INVESTIGATING THE IMPACT OF MAGNESIUM ON *MOEHRINGIA MACROPHYLLA*, A SERPENTINE-TOLERANT SPECIES

by

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for eight credits.

This work is dedicated to my mom, who raised me to think both scientifically and creatively, I love you!

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ABSTRACT

Moehringia macrophylla Hook. (Fenzl) (large-leaved sandwort) is an understudied species of the Caryophyllaceae family that shows tolerance to serpentine soil and is rare in New England. Serpentine soils are nutrient-poor and metal-rich, and produce "serpentine syndrome," or decreased growth and tissue necrosis, in plants growing on it. As the specific mechanisms of *M. macrophylla*'s response to serpentine conditions are largely unknown, I tested how its growth and cellular accumulation of magnesium were altered by elevated magnesium exposure, a key stressful feature of serpentine environments. I optimized a germination procedure, grew the plant on substrates containing magnesium, and performed confocal microscopy using a magnesium-binding fluorescent dye to determine if and where magnesium localization occurs in the roots, stems, and leaves of this species. Examining the effects of magnesium in *M. macrophylla* is essential to understanding the mechanisms of tolerance this species employs, and if it follows expected morphological and physiological characteristics for serpentine tolerators. My study establishes a basic understanding of serpentine response, setting the stage for further research on the genetic mechanisms of serpentine tolerance and the impact of the serpentine habitat on the rarity of this species, as well as the possible role of ecotypic differentiation in *M. macrophylla.*

INTRODUCTION

Overview of the study

In this study, I examined the physiological and morphological effects of magnesium on *Moehringia macrophylla* (Hook.) Fenzl, an understudied and rare plant species known to grow on ultramafic, serpentine soils. The response of *M. macrophylla* to serpentine conditions may contribute to its geographic distribution and rarity but is largely unknown; therefore, this study aims to determine how its growth and cellular accumulation of magnesium are altered under elevated magnesium exposure, simulating a key abiotic stressor characteristic of serpentine environments.

Soil endemism and ultramafic rock

The evolutionary processes that result in plants becoming tolerant of, or even endemic (restricted) to, specific or challenging environments are not fully understood. This type of work, on why certain species or genera of plants adapt to live in environments that are inhibitory or toxic to other species, provides valuable insight into processes of adaptation, speciation, and community ecology (Armbruster, 2014). One particular type of challenging environment that plants can become endemic to is ultramafic outcrops, where ultramafic rock, a type of igneous rock, is exposed (McTaggart, 1971). The weathering of these rocks, which occurs across diverse geographic regions and ecosystems, leads to the

formation of ultramafic soils, which retain the rich minerality and heavy metal content of the rocks they are derived from (*Ultramafic Lands*, 2024). Depending on their mineral composition, ultramafic rocks can be broadly classified as either serpentinite or peridotite, with the soil derived from both types commonly referred to as "serpentine" (*Soils and Plant Adaptations*, n.d.). Serpentine soil tends to be nutrient-poor and metal-rich, creating harsh conditions that are inhospitable to many plant species (Gordon & Lipman, 1926). As a result of their unique characteristics, serpentine soil tends to form distinct ecological zones, known as "edaphic islands," where plants must evolve specialized adaptations to cope with the edaphic stressors present (O'Dell & Rajakaruna, 2011).

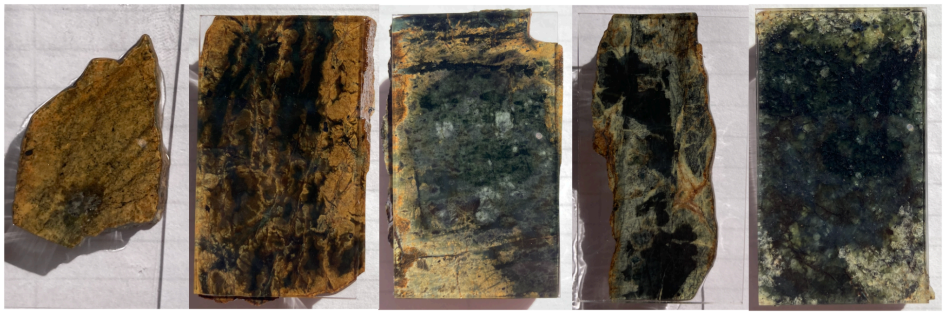

Figure 1. Cross sections of serpentine outcrop billets obtained from *M. macrophylla* population sites in Vermont and Massachusetts. Samples obtained by Dr. Chloe P. Drummond, billets prepared by Claribel Connors (Connors, 2026).

The Caryophyllaceae family

Caryophyllaceae, or the carnation family, is among the many plant families that display soil endemism/tolerance to the unique conditions of

ultramafic soils, and has been found on North American ultramafic soils (Reeves & Kruckeberg, 2018). This family comprises approximately 100 genera and 3,000 species distributed across North America, South America, Africa, and Asia (Greenberg & Donoghue, 2011; Yang et al., 2024). While many Caryophyllaceae species inhabit dry, exposed environments, others thrive in more moist habitats such as temperate forests and meadows (Greenberg & Donoghue, 2011). Several members of this family have been documented on North American ultramafic soils, where some exhibit the ability to hyperaccumulate, or accumulate high concentrations of metals, namely nickel, in their tissue (Reeves & Kruckeberg, 2018). Brooks (1987) identified 128 species of Caryophyllaceae occurring in ultramafic environments (Reeves & Kruckeberg, 2018), meaning that some members of the Caryophyllaceae likely have specialized adaptations to cope with these edaphic environments.

Moehringia macrophylla

Within the Caryophyllaceae family, one species of particular interest for its edaphic specialization is *M. macrophylla* (Hook.) Fenzl, which is known to grow on ultramafic, specifically serpentine, soils. Additionally, the *Moehringia* genus is paraphyletic with the *Arenaria* genus, which has been shown to explicitly display ultramafic endemism and hyperaccumulation (Reeves & Kruckeberg, 2018). *Moehringia macrophylla*, commonly known as large-leaved sandwort, is a

small, delicate, perennial, vascular plant (*Moehringia macrophylla (Large-Leaved Grove-Sandwort): Go Botany*, n.d.) (Fig. 2, Fig. 3).

Figure 2. *M. macrophylla* growing on serpentine soil in Connecticut. (Drummond, 2025).

This species is characterized by having simple, entire leaves, arranged oppositely on the stem, with two leaves per node (*Moehringia macrophylla* (Large-Leaved Grove-Sandwort): Go Botany, n.d.). The flowers are radially symmetrical, with 5 sepals, 5 white petals, 10 stamens, and 3 styles (*Moehringia Macrophylla (Large-Leaved Grove-Sandwort): Go Botany*, n.d.). The fruit of *M. macrophylla* is dry and splits open when ripe, and is up to 5 mm in length

(*Moehringia macrophylla* (Large-Leaved Grove-Sandwort): Go Botany, n.d.). *M. macrophylla* tends to grow between 2-18 centimeters in height, but is rhizomatous, so one individual spreads through branching underground rhizomes (*Moehringia macrophylla (Large-Leaved Sandwort)*, n.d.). Ant dispersal of the seeds of some members of the *Moehringia* genus has been observed (Mehrhoff, 1989). It has been suggested that the physical removal of the eliasome by ants may promote germination, but this has not been studied for *M. macrophylla* (Casazza et al., 2008).

Figure 3. Flowering *M. macrophylla* specimen. (Carr, n.d.).

Although the species is found more abundantly in western North America, it is rare in New England, where disjunct and small populations persist in Connecticut, Massachusetts, and Vermont (*Moehringia macrophylla (Large-Leaved Grove-Sandwort): Go Botany*, n.d.). In New England, *M. macrophylla* occurs on cliffs and talus slopes, frequently those of serpentine outcrops. These small populations, ranging from a few plants to over 200, are

restricted to fragile habitats such as rock crevices, where organic debris accumulates to form a substrate for plants to take root in (*Moehringia macrophylla*, n.d.). Despite its rarity and intriguing association with serpentine environments, little is known about the mechanism of *M. macrophylla*'s tolerance to such chemically stressful substrates, and whether its persistence on those substrates represents physiological specialization or environmental plasticity and competition avoidance.

The properties of serpentine substrates

M. macrophylla has a reported ability to withstand the harsh conditions that define serpentine substrates (*Moehringia macrophylla (Large-Leaved Grove-Sandwort): Go Botany*, n.d.). Serpentine soils are typically shallow and rocky, have low water-holding capacity, and lack essential elements such as nitrogen, phosphorus, potassium, and calcium. These deficiencies are coupled with toxically high levels of bioavailable magnesium, as well as heavy metals such as nickel, chromium, and cobalt (Jenny, 2012). These properties result in a very low calcium-to-magnesium (Ca:Mg) molar ratio, which is detrimental to plant growth (O'Dell & Rajakaruna, 2011). Plants inhabiting serpentine landscapes face complex challenges, including drought stress, macronutrient deficiency, macronutrient toxicity, and heavy metal toxicity (O'Dell & Rajakaruna, 2011). These combined stressors are referred to as "serpentine syndrome," which is largely attributed to the low Ca:Mg ratio. Although

serpentine soils contain little calcium to begin with, this limitation is intensified by the high concentration of magnesium. This further reduces calcium availability through magnesium toxicity, where excessive levels of magnesium competitively inhibits calcium uptake, resulting in magnesium poisoning in plants (Brady et al., 2005). Plants that are able to negotiate serpentine syndrome do so through physiological mechanisms that ensure an adequate internal concentration of calcium (O'Dell & Rajakaruna, 2011).

Calcium and magnesium: two essential macronutrients

To understand the importance of the Mg:Ca ratio in *M. macrophylla,* it is necessary to look at the roles of Ca and Mg in plants. Calcium is a core macronutrient that is required extracellularly, in the vacuole, and cytoplasmically (White, 1998). It is required in its ionic form (Ca^{2+}), functioning both structurally and as a signaling molecule (Broadley et al., 2003). Extracellularly, Ca^{2+} maintains cell wall structure by crosslinking pectin chains to form a framework known as calcium pectate that retains cell shape (Broadley et al., 2003). Within the vacuole, Ca^{2+} acts as a counter-cation, neutralizing inorganic and organic anions to maintain osmotic and ionic balance (White & Broadley, 2003). Within the cytoplasm, Ca^{2+} serves as a critical second messenger in signal transduction pathways (Broadley et al., 2003). The cytoplasmic concentration of Ca^{2+} is essential for cellular metabolism as well as responses to abiotic stress (Broadley et al., 2003).

Calcium enters the root system from the substrate and is transported through the xylem to the shoot (White, 2001). The cation moves through the root via both symplastic and apoplastic pathways (White & Broadley, 2003). These pathways are highly regulated, as cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) are crucial for root cell signaling, while elevated $[Ca^{2+}]_{cyt}$ levels are cytotoxic (White & Broadley, 2003). From the xylem, regulated quantities of Ca^{2+} enter cells through Ca^{2+}-permeable ion channels located within the plasma membrane (White & Broadley, 2003). This process is further regulated by Ca^{2+}-ATPase enzymes and antiporter membrane transport proteins, which translocate Ca^{2+} to both the apoplast and the lumen of intracellular organelles, including the vacuole (White & Broadley, 2003). The process of Ca^{2+} moving through these ion channels triggers changes in $[Ca^{2+}]_{cyt}$ that initiate signal transduction and cellular responses to environmental and physiological stimuli, including stressors such as elevated magnesium (White, 2001).

Magnesium is another essential macronutrient, imperative for organismal development and function (Guo et al., 2015). It is structurally essential to chlorophyll molecules and ribosomal complexes (Shaul, 2002). Chlorophyll molecules are required for photosynthesis and ribosomal complexes are required to make proteins. Magnesium plays key roles in photosynthesis, enzymatic activity, and carbon metabolism, making it one of the most important plant nutrients (Guo et al., 2015). However, both magnesium deficiency (MgD) and

magnesium toxicity (MgT), collectively referred to as magnesium stresses (MgSs), can disrupt plant development (Guo et al., 2015).

Magnesium stresses

Currently, the physiological and molecular mechanisms underlying MgSs are not well understood (Guo et al., 2015). This knowledge gap is a result of magnesium's commonality, its high concentration in soil, as well as its solubility in water, which facilitates rapid uptake by plants (Guo et al., 2015). However, the discovery of magnesium transporters over the last 25 years has reignited interest in understanding its role in cellular homeostasis and stress responses (Guo et al., 2015). It is unclear how transcriptional levels of magnesium transporters change under MgD or MgT conditions; it has been resported that MgSs activate signaling pathways involving other transport systems (Guo et al., 2015). Although many magnesium-dependent cellular processes are well characterized, less is known about the signaling pathways that mediate responses to magnesium stress (Guo et al., 2015). Consequently, it remains unclear how plants respond to environments with abnormal magnesium levels or how prolonged exposure to MgSs may influence genetic adaptation across generations.

Morphologically, it has been indicated that MgSs, specifically MgT conditions, reduce overall plant growth, including shorter roots, smaller shoots, reduced leaf size, decreased growth rate, and the formation of localized areas of necrotic tissue on leaves (Guo et al., 2015).

MgT occurs when excess magnesium accumulates in plant tissues, disrupting normal growth and physiological processes (Guo et al., 2015). MgT alters carbohydrate balance by promoting starch and sugar breakdown rather than accumulation, as well as the uptake of other ions, specifically calcium, inducing broader nutrient imbalance (Guo et al., 2015). Calcium deficiency resulting from magnesium toxicity occurs because both ions are divalent cations, meaning they competitively inhibit one another (Jeong et al., 2020). This means that in high Mg:low Ca environments, Mg^{2+} competitively binds Ca^{2+}-binding sites, inhibiting Ca^{2+} uptake, resulting in calcium deficiency and magnesium poisoning (Jeong et al., 2020). Calcium deficiency is especially detrimental in dicotyledonous angiosperms, including *M. macrophylla*, because a large proportion of cellular calcium is bound to pectins in the middle lamella of the cell wall, where it stabilizes tissue structure and mediates cell-to-cell adhesion (O'Dell & Rajakaruna, 2011). When calcium becomes deficient, plants can no longer expand their cell walls, their pectin breaks down, and their tissue goes through necrosis, especially in actively growing tissues where calcium demand is high and cannot be remobilized due to its phloem immobility (O'Dell & Rajakaruna, 2011).

At the molecular level, MgT strongly activates stress signaling pathways, particularly those involving the stress hormone abscisic acid (ABA), whose levels increase significantly (Guo et al., 2015). This increase triggers downstream signaling through proteins such as DELLA, which act as growth regulators and ultimately inhibit plant development (Guo et al., 2015). Additionally, MgT is

associated with increased production of reactive oxygen species (ROS), which are byproducts of cellular metabolism that contribute to cellular stress in excess quantities (Guo et al., 2015; Mhamdi & Van Breusegem, 2018). Although certain magnesium transporters are upregulated under MgT and may help regulate excess Mg, the exact mechanisms remain unclear (Bradshaw, 2005)

In one study, exposure to MgT disrupted ionic balance in *Arabidopsis thaliana*, reducing Ca^{2+} uptake, and inhibiting overall plant growth, particularly root elongation, while paradoxically stimulating root hair development as an adaptive morphological response (Guo et al., 2014). A central finding was that MgT induces accumulation of ABA, stabilizing DELLA proteins, which in turn negatively regulate gibberellin signaling (Guo et al., 2014). Gibberellin is a major hormone that regulates growth, essential for stem elongation, leaf expansion, and overall development (Davière & Achard, 2013). This ABA-DELLA interaction forms a regulatory module that suppresses growth while promoting stress adaptation (Guo et al., 2014). Genetic evidence from DELLA-deficient mutants demonstrates that reduced DELLA activity alleviates MgT-induced growth inhibition, lowers Mg accumulation, and improves ion balance, indicating that DELLAs actively regulate Mg transporter gene expression and ion uptake (Guo et al., 2014).

Additionally, MgT alters carbon metabolism by enhancing starch degradation and reducing sucrose levels; these effects are partially mitigated in DELLA mutants, suggesting that DELLAs also regulate metabolic responses

under stress (Guo et al., 2014). Mg toxicity responses are coordinated through a complex signaling network in which ABA-induced DELLA accumulation integrates environmental stress signals to reshape ion transport, metabolism, and development, thereby balancing survival and growth under high-magnesium conditions (Guo et al., 2014). Furthermore, there is evidence for active regulation of Mg transporter genes rather than passive accumulation (Guo et al., 2014). In wild-type plants, many Mg transporter genes are upregulated under MgT, contributing to increased Mg uptake (Guo et al., 2014). In contrast, DELLA mutants suppress several of these transporters, correlating with reduced Mg accumulation (Guo et al., 2014). This suggests that serpentine-adapted plants may regulate or downregulate Mg transport systems to prevent excessive intracellular Mg accumulation (Guo et al., 2014).

Mechanisms for serpentine tolerance

Both the cellular mechanisms underlying magnesium and calcium homeostasis and the physiological and evolutionary strategies enabling survival under extreme edaphic stress remain incompletely understood and are active areas of ongoing study (O'Dell & Rajakaruna, 2011).

Serpentine endemics therefore require specialized physiological mechanisms to maintain adequate internal calcium levels, including selective calcium uptake and translocation, magnesium exclusion, and vacuolar sequestration of excess magnesium (Inoue et al., 2022). One central molecular

mechanism involves the vacuolar cation exchanger CAX1, a high-capacity Ca^{2+} antiporter (O'Dell & Rajakaruna, 2011). Loss-of-function mutations in CAX1 have been shown to reduce traits of serpentine tolerance, meaning that functional CAX1 increases resistance to low Ca^{2+} and high Mg^{2+} conditions, reduced Mg^{2+} uptake, and exclusion of Mg^{2+} from leaf tissues, thereby maintaining cytoplasmic calcium homeostasis under low Ca:Mg conditions (Bradshaw, 2005).

Beyond this cellular processes, serpentine-adapted species employ a range of stress-tolerance strategies to cope with magnesium (Brady et al., 2005). Some plants avoid toxicity by limiting Mg uptake at the root level while maintaining sufficient calcium acquisition, demonstrating selective ion uptake mechanisms, or magnesium exclusion (Brady et al., 2005). For example, serpentine-tolerant strains of *Agrostis stolonifera* of the Poaceae family similarly resist Mg uptake (Brady et al., 2005).

In contrast, other species tolerate high internal Mg concentrations through physiological mechanisms such as compartmentalization or biochemical buffering (Proctor, 1970). For instance, *Agrostis canina*, a serpentine-tolerant member of the Poaceae family, exhibits increased tolerance to elevated Mg relative to non-serpentine populations (Proctor, 1970). *Agropyron spicatum,* another member of the Poaceae family, shows genetically based tolerance with intermediate responses in hybrids, and *Lasthenia californica* of the Asteraceae family accumulates high Mg concentrations in shoots while maintaining normal function (Rajakaruna et al., 2003).

As Mg toxicity often interferes with calcium uptake, many serpentine-adapted plants maintain Ca:Mg balance by enhancing calcium uptake or retention despite elevated Mg levels (Brady et al., 2005). For example, *Helianthus bolanderi ssp. exilis* of the Asteraceae family tolerates high Mg partly by sustaining Ca uptake, while *Streptanthus glandulosus ssp. pulchellus* of the Brassicaceae family continues to efficiently absorb Ca even in low Ca:Mg soils (Walker, 1948).

Additionally, some serpentine species exhibit a higher physiological requirement for magnesium, growing optimally under elevated Mg conditions (Brady et al., 2005). This is observed in species such as *Poa curtifolia*, where growth requires higher external Mg to maintain internal balance (Main, 1981). Growth in *Helianthus bolanderi ssp. exilis* is also positively correlated with soil Mg levels (Madhok & Walker, 1969).

Another important mechanism involved is ion sequestration and detoxification, in which plants reduce Mg toxicity by binding magnesium through chelation with organic acids such as citrate or malate, followed by compartmentalizing the bound magnesium in cellular structures like vacuoles (Brady et al., 2005). This reduces the concentration of free toxic ions (Brady et al., 2005). For example, *Sedum anglicum* utilizes vacuolar chelation to store both Mg and Ca (Brady et al., 2005).

Another strategy is metal hyperaccumulation, in which plants concentrate metals such as nickel in leaf tissue, potentially providing defense against

herbivory, allelopathy, or pathogens (Anderson et al., 1999). However, only a minority of serpentine species are true hyperaccumulators, with most relying on exclusion or sequestration mechanisms (Brady et al., 2005).

In addition to these mechanisms, serpentine-adapted species exhibit broader ecological strategies, including drought resistance, osmotic adjustment through elevated cation concentrations, and slow, resource-conservative growth (O'Dell & Rajakaruna, 2011).

Additionally, preadaptation likely contributes to serpentine tolerance. Species already adapted to high magnesium or nutrient-poor conditions in other harsh habitats, such as coastal saline environments or mine tailings, may more readily establish in serpentine ecosystems (Brady et al., 2005). This suggests that some species exhibit genetic adaptations for Mg tolerance, indicating that these traits are heritable and subject to natural selection under strong environmental pressures (Brady et al., 2005)

Together, these interacting cellular, physiological, and evolutionary mechanisms demonstrate how complex serpentine tolerance is, as well as the adaptability of plant species such as *M. macrophylla* to persist on these challenging soils.

The goals and hypotheses of this study

Excess magnesium is a key component of serpentine soils that contributes to serpentine syndrome in plants (O'Dell & Rajakaruna, 2011). At present, it

remains unclear which mechanisms of tolerance (exclusion, sequestration, and/or hyperaccumulation) are employed by *M. macrophylla* and how its growth characteristics respond to magnesium-enriched substrates. Therefore, it is necessary to ask fundamental questions about how this rare serpentine-tolerant plant responds to varying magnesium conditions. To address this, I germinated and grew *M. macrophylla* on substrates containing differing concentrations of magnesium, and subsequently tested if and where excess magnesium accumulates within the plant.

There are three overarching goals of this research: (1) optimize a germination protocol for *M. macrophylla*, as there is no available information on its germination in the literature or from the New England Nasami seed bank, (2) to measure growth characteristics of *M. macrophylla* under varying magnesium concentrations in order to simulate serpentine syndrome and assess whether the species exhibits expected morphological and physiological characteristics of serpentine endemics; and (3) to use a magnesium-binding fluorescent dye to visually determine whether and where magnesium localization occurs within root, stem, and leaf tissues.

Based on these objectives, I hypothesize that *M. macrophylla* will exhibit morphological and physiological characteristics consistent with serpentine tolerators when exposed to elevated magnesium levels, including decreased growth rate and overall growth, reduced leaf size and quantity, and increased necrosis of leaf tissue. I further hypothesize that *M. macrophylla* will exhibit Mg^{2+}

accumulation in root, stem, and leaf tissues when grown in substrates containing heightened levels of magnesium. Additionally, I am testing between hypotheses of leaf and root sequestration: I hypothesize that if *M. macrophylla* shows higher levels of magnesium in its roots, there will be a less drastic change in the magnesium levels in its stems and leaves under varying magnesium conditions, thereby supporting the root sequestration hypothesis. If *M. macrophylla* shows the highest levels of magnesium in its leaves under high magnesium conditions compared to low magnesium conditions, then I hypothesize its roots and stems will show a less drastic change in magnesium levels under varying magnesium conditions, thereby supporting the hypothesis of leaf sequestration.

METHODS

Obtaining *M. macrophylla*

Seeds of *M. macrophylla* were obtained from 7 different sites across Massachusetts and Vermont, totaling to 67 seeds. Three of these sites were in Massachusetts, with 10, 5, and 11 seeds each, collected by Chloe P. Drummond in 2025. The other 4 sites were in Vermont, with 7, 9, 22, and 9 seeds each, and were collected by volunteers from the New England Nasami seed bank in Whately, Massachusetts. As *M. macrophylla* is a protected rare species, specific locations are withheld.

In addition to seeds, 60 mature *M. macrophylla* plants were obtained from Echo Valley Natives, a nursery in Sandy, Oregon.

Germination protocol of *M. macrophylla* seeds: seed stratification and surface sterilization

A germination experiment was completed to establish the following *M. macrophylla* germination protocol. Seeds were stratified in dry paper packets in a growth chamber kept at 20 °C in 24 hour darkness for 7 days (Vandelook et al., 2008).

Seeds were surface sterilized in 2 separate 1.5 mL microcentrifuge Eppendorf tubes based on collection source: one for the seeds from the seed bank and one for the seeds collected by Dr. Drummond. 1 mL of 70% EtOH was added to each tube, and tubes were inverted four times to rinse the seeds. EtOH was then

removed by pitpetting. Seeds were then transferred to a 10% bleach and 0.1% Tween-20 solution, and stirred four times over a 10 minute period. The bleach solution was then decanted, and seeds were rinsed four times with sterile, autoclaved Milli-Q water.

An attempt was made to remove the eliasome of each seed, as Casazza et al. (2008) suggested it may enhance germination results. However, removal was unsuccessful and all seeds were sown with their eliasomes intact.

Agar media preparation, seed sowing, and growth chamber conditions

Magenta boxes were poured containing half strength Murashige and Skoog (MS) medium. The control medium consisted of 2.165 g/L MS and 20 g/L sucrose. The pH was adjusted to 5.8 using KOH and HCl until pH 5.8 was reached. 1L of solution was then combined with 0.5 g active charcoal and 10 g agar, then autoclaved at 121 ºC for 45 min (liquid cycle), and poured into Magenta boxes to a depth of 2 cm. Boxes were sealed with parafilm, and stored at 4 °C until use.

The baseline magnesium concentration of ½ MS medium (0.75mM of $MgSO_4$) served as the control condition. Experimental treatments were prepared by supplementing the media with $MgSO_4$ to achieve final concentrations of 20 mM, 24 mM, and 28 mM $MgSO_4$ (Table 1).

Following sterilization, one seed was sown into each magenta box within a laminar flow hood. The 67 available seeds were distributed evenly across four

magnesium treatments. Seeds from each collection site were divided by four, and when the site totals were not divisible by four, assignments were randomized. Each treatment group consisted of 16 seeds, each grown individually in separate Magenta boxes. Boxes were labelled according to seed source (collected from sites across New England or received from the seed bank) and treatment, sealed, and placed in a growth chamber maintained at 20 °C during a 12 hour photoperiod and 10 °C during a 12 hour dark period. Boxes were originally sealed with parafilm, but then resealed with micropore tape to allow for gas exchange. Germination was monitored weekly, and contaminated seeds were removed and documented.

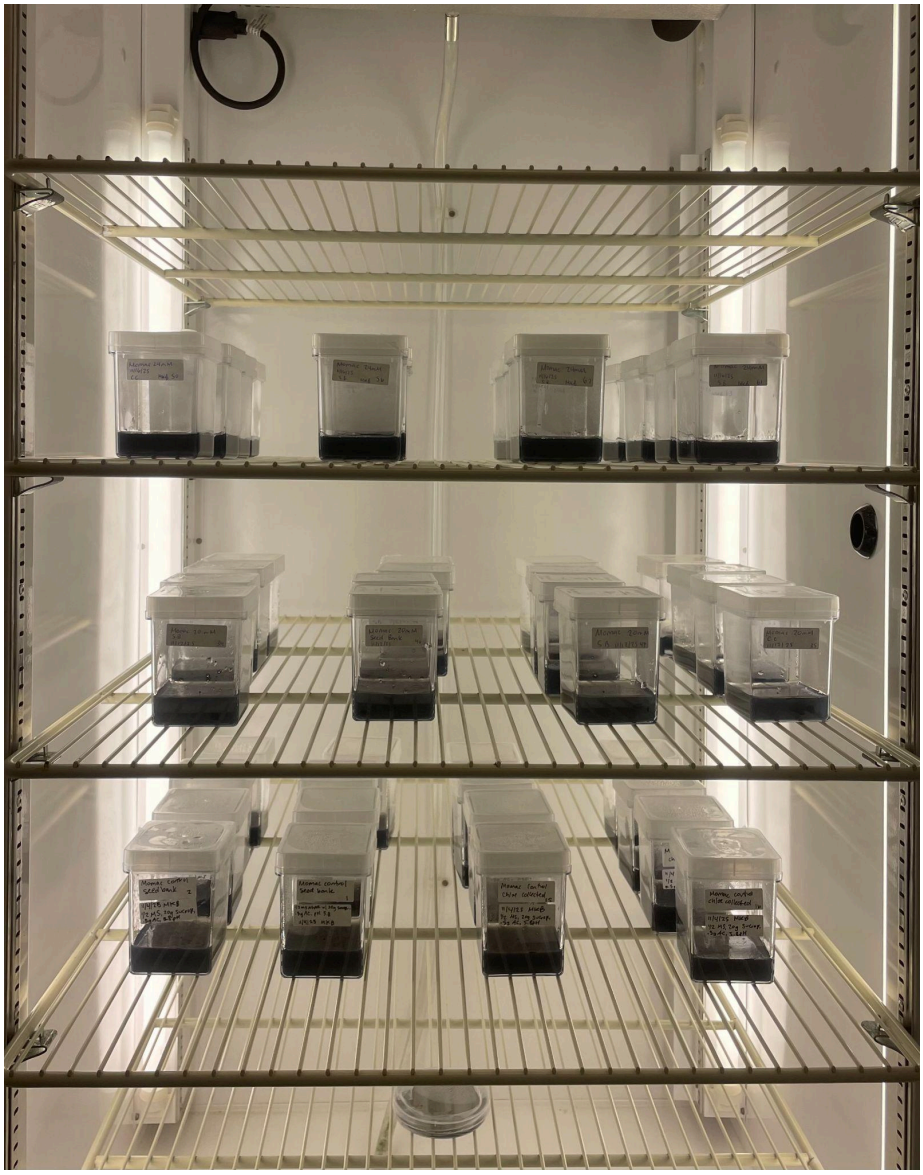

Figure 4. Magenta boxes in the growth chamber, containing 1/2MS media with varying concentrations of $MgSO_4$, and 1 seed of *M. macrophylla* sown per box.

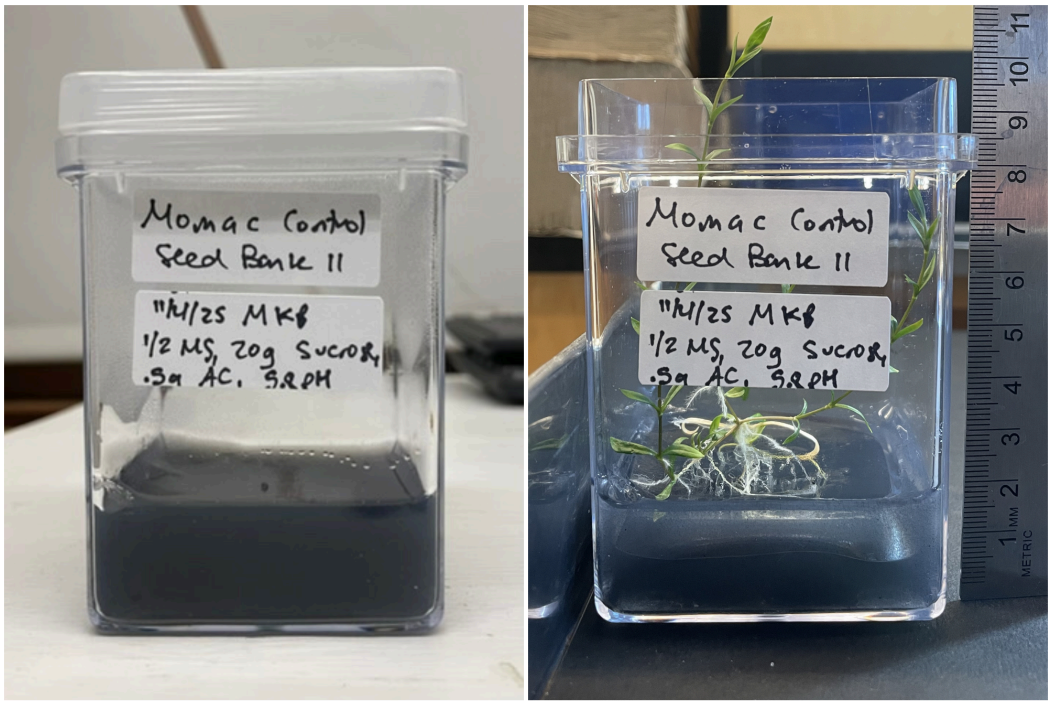

Figure 5. Labelled magenta box containing control group 1/2MS media and 1 inoculated *M. macrophylla* seed.

Table 1. $MgSO_4$ concentrations added to agar for each experimental group (½ MS medium has a baseline level of 0.75mM $MgSO_4$)

Group	Amount of $MgSO_4$ in agar
Control group	0.75mM $MgSO_4$
Group 2	20mM $MgSO_4$
Group 3	24mM $MgSO_4$
Group 4	28mM $MgSO_4$

Greenhouse acclimation

60 mature *M. macrophylla* plants obtained from a forest patch at Echo Valley Natives in Sandy, OR were transplanted into pots containing a 1:1 mixture of ProMix and perlite and maintained in the research house of the Talcott Greenhouse on Mount Holyoke College campus. The research house remained between 60 and 70 °F during a 14-hour photoperiod and between 68 and 74 °F during a 10-hour dark period. Plants were acclimated to greenhouse conditions without exposure to experimental treatments for 8 weeks.

Magnesium treatments in greenhouse study

Magnesium was supplied as Epsom salt (magnesium sulfate heptahydrate, MgSO4 · 7H2O), which contains approximately 10% elemental magnesium. Epsom salt was used as the source of experimental magnesium for this study because previous literature determined that the direct application of epsom salt onto soil results in a similar tissue magnesium concentration as ground serpentine rock when used as a fertilizer (Hanly et al., 2005). Solutions were mixed at 0 ppm, 40 ppm, 80 ppm, and 160 ppm (Table 2) and stored in labeled bins and sealed to prevent evaporation and salt concentration changes. Although ProMix contained a low level of fertilizer, including magnesium, its magnesium contribution was considered experimentally negligible relative to the experimental concentrations and was not included in calculations.

Table 2. Epsom salt solution concentrations for each experimental group.

Treatment Group	Epsom salt per 5 gal	Final Mg (ppm)
Control	0 g	0 ppm
Group 2	7.56 g	40 ppm
Group 3	15.12 g	80 ppm
Group 4	30.24 g	160 ppm

Figure 6. *M. macrophylla* growing in greenhouse conditions, separated into trays based on magnesium treatment.

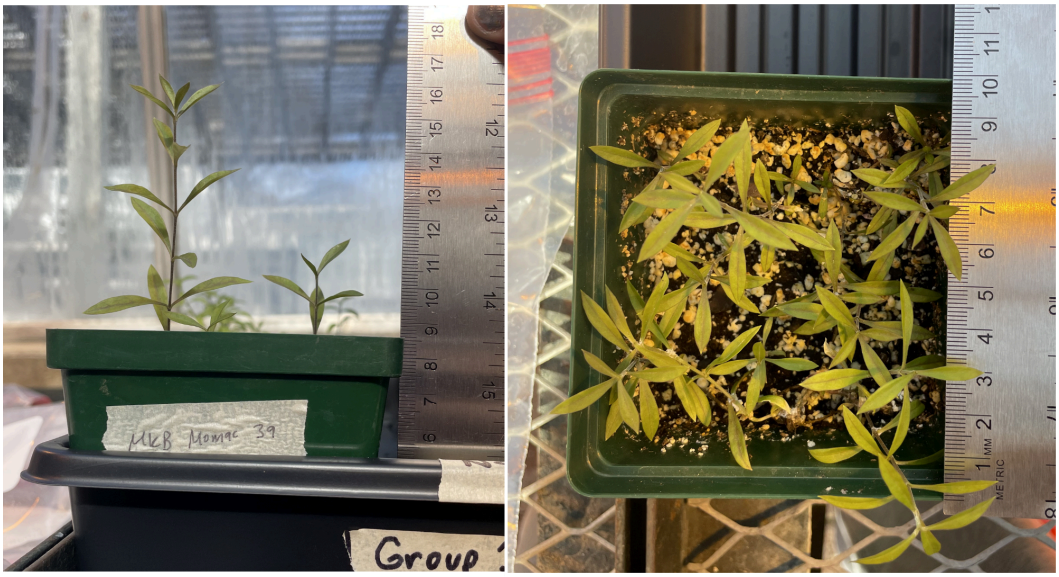

Figure 7. Side and aerial views of *M. macrophylla* in the greenhouse at the commencement of experimentation.

Figure 8. Side and aerial views of *M. macrophylla* in the greenhouse at the conclusion of experimentation.

Experimental conditions and watering of greenhouse plants

After the acclimation period, plants were randomly assigned to 4 treatment groups, totaling to 14 plants per group, and 7 individuals per tray. Four individuals were excluded from the experiment and used for staining protocol optimization.

The experiment duration (5 weeks) was determined based on observed growth rates during the 8 week acclimation period (mean growth of approximately 0.5 cm/week), The experiment duration of 5 weeks allowed for at least 2 cm of new growth including one mature node. Plants were bottom-watered using trays to promote capillary action and minimize nutrient leaching. Magnesium ions are less bound to soil charges than other ions, and therefore highly mobile in soil, meaning that they are extremely susceptible to leaching (Senbayram et al., 2015). As magnesium leaching occurs very quickly, including from the soilless media used in this experiment, magnesium treatments were applied with each major watering rather than as a single application (Senbayram et al., 2015). 1 L of solution per tray was applied every 5-6 days, depending on the time required for soil drying, as it was suggested by Echo Valley Native staff that *M. macrophylla* does best when its substrate is allowed to dry between waterings. After several waterings, it was decided that trays should be removed after 24 hours, after the solution had been absorbed, in order to allow for better drainage and faster drying of the soil.

Magnesium concentration quantification

Soil and tissue samples from each treatment group were collected and sent to the University and Massachusetts Amherst Soil and Plant Nutrient Testing Laboratory for magnesium quantification to verify treatment differentiation (Soil and Plant Nutrient Testing Laboratory, n.d.). 300 mg of pooled leaf tissue from each experimental condition, as well as 1 cup of pooled soil from each experimental condition were delivered to the laboratory after experimentation concluded.

Growth analysis for growth chamber plants

Individuals were destructively sampled for morphological trait analysis after 20 weeks of growth. Plants were carefully removed from agar, in order to not destroy the root tissue. Morphological measurements included total plant length (measured from the root apical meristem to the central shoot apical meristem), whole-plant wet biomass, and quantity of stems. Total leaf quantity was recorded, as well as categorized into tissue quality (yellow or green) and size (juvenile or mature). Leaves were categorized as juvenile or mature depending on their size at the time of sampling. As leaf growth inhibition is a trait associated with serpentine syndrome, categories of juvenile and mature were used to determine if leaf growth suppression occurred across experimental groups. In preparation for microscopic tissue analysis, each type of tissue was sampled in a consistent manner across replicates. Leaf samples were taken from the 2nd node

down from the shoot apical meristem of the tallest stem. Stem samples were taken from the internode length between the 2nd and 3rd nodes down from the SAM of the tallest stem. Root samples were taken from just under the base of the stem.

Growth analysis for greenhouse plants

For the greenhouse-grown *M. macrophylla*, plants were already at a mature stage. The length of a random stem of each plant was measured at the beginning of the experimental period, and the length of the tallest stem of each plant was measured at the end of the experimental period, to assess growth. One leaf was collected from the 2nd node of the tallest stem of each plant, which ensured that analyzed tissue was growing in experimental conditions. Each leaf was photographed for surface area analysis.

Statistical analysis of morphological traits

One-way and two-way analyses of variance (ANOVA) were performed to determine whether differences obtained from data analysis were statistically significant. A significance threshold of $p < 0.05$ was applied. If significance was found, a post-hoc test, the Tukey's Honestly-Significant-Difference test (TukeyHSD), was used to determine pairwise differences among groups.

Magnesium quantification in plant tissue

Magnesium accumulation in plant tissues was visualized using magnesium-binding fluorescent dye. Ion-specific fluorescent indicators used for the measurement of intracellular ions typically rely on a chelating structure to bind the target ion and result in a visual change in fluorescence (Patel et al., 2020). However, most commercially available indicators used for the intracellular measurement of Mg^{2+} are derived from the tetracarboxylate BAPTA Ca^{2+} chelator (Patel et al., 2020). This results in these fluorescent indicators having a higher affinity to Ca^{2+} than Mg^{2+}, resulting in unrepresentative measurement of magnesium ion quantity (Pesco et al., 2001). This challenge is increased by the intertwined nature of magnesium and calcium signaling pathways (Patel et al., 2020). Thus, Mg^{2+} measurement requires a very selective Mg^{2+} indicator that is sensitive to the subtle change in Mg^{2+} quantity while avoiding the subsequent and often greater change in Ca^{2+} concentration (Patel et al., 2020). Mag-520 AM dye was used because it has a ten-fold greater affinity for Mg^{2+} than Ca^{2+}, and is capable of monitoring cellular Mg^{2+} influx without Ca^{2+} interference (Patel et al., 2020).

Buffers and solutions for tissue staining

A wash consisting of Dulbecco's phosphate-buffered saline (DPBS) supplemented with HEPES was prepared for tissue washing and staining procedures. The DPBS solution was prepared by dissolving 0.8 g sodium chloride

(NaCl), 0.02 g potassium chloride (KCl), 0.02 g monopotassium phospate (KH_2PO_4), and 0.115 g disodium phosphate (Na2HPO4) in distilled water. HEPES buffer was added to achieve a final buffer concentration of approximately 20 mM. Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were used to adjust the pH to approximately 7.4.

The Mag-520 AM dye was prepared as a stock solution by dissolving lyophilized dye in anhydrous dimethyl sulfoxide (DMSO) to obtain a final concentration of 5 mM. The dye vial was briefly centrifuged prior to dissolution to ensure the reagent was collected at the bottom of the tube. The solution was mixed by pipetting and vortexed until the dye was fully dissolved in DMSO. Stock solutions were used immediately or stored in aliquots at -20 °C and protected from light to prevent degradation. Repeated freeze-thaw cycles were avoided.

Working solutions of Mag-520 were prepared by diluting the stock solution with the DPBS HEPES buffer to achieve a final concentration of 10 μM. These working solutions were prepared immediately prior to use and were protected from light throughout the preparation and staining process.

Tissue preparation and staining

Leaf, stem, and root tissues were collected from *M. macrophylla* individuals grown in the control and highest magnesium conditions (28 mM) in the growth chamber experiment. Only leaf tissue was collected from *M.*

macrophylla individuals grown in the control and highest magnesium conditions (160 ppm) in the greenhouse experiment.

Tissue was collected from one plant from each experimental condition, both from the greenhouse and the growth chamber populations. For growth chamber plants, leaf samples were collected from the 2nd node down from the shoot apical meristem of the tallest stem, stem samples were collected from the internode length between the 2nd and 3rd nodes down from the SAM of the tallest stem, and root samples were collected from just under the base of the stem. For greenhouse plants, leaf samples were collected from the 2nd node of the tallest stem of each imaged plant, ensuring the analyzed tissue grew in experimental conditions.

Tissues were prepared for staining by gently rinsing samples in the buffer. Tissue was placed into microcentrifuge tubes containing approximately 2 mL of buffer and allowed to incubate for 10 minutes. After this initial wash step, the buffer was removed by decanting.

16 µL of stock solution was diluted into 2 mL of HEPES-DPBS to produce a final dye concentration of 40 µM. Tissue was transferred into Eppendorf tubes containing 2 mL of the working solution and incubated for approximately 30 minutes at 37 °C. During staining and incubation, tissue was protected from light using aluminum foil to prevent photodegradation of the dye.

Following incubation, dye solution was removed and tissue samples were washed two to three times with HEPES-DPBS buffer to remove extracellular dye.

During each wash step, tissue samples were transferred into a fresh Eppendorf tube containing 1 mL of buffer and allowed to sit for 10 minutes before the buffer was pipetted out. During all steps of the staining process, samples were protected from light using aluminum foil to prevent photodegradation of the dye.

Slide preparation and fluorescence microscopy

Following staining and washing, tissue samples were mounted on microscope slides for fluorescence imaging. Slides were cleaned with ethanol and KimWipes prior to mounting. Stained tissue samples were placed on the slide surface and mounted using a mounting solution composed of glycerol and HEPES-DPBS in a 9:1 ratio. Approximately 50 µL of mountant was applied to the sample before a coverslip was placed over the tissue.

Prepared slides were protected from light and imaged as soon as possible following mounting. Fluorescence imaging was performed using confocal microscopy to visualize the intracellular distribution of magnesium within plant tissues stained with the Mag-520 AM dye. Mag-520 AM is excited at 488 nm with an emission peak around 520–525 nm. The dye's peak excitation and emission wavelengths match with Cy5 and GFP filter sets (Mag-520TM AM | AAT Bioquest, n.d.). For each sample, a Z-stacked image was taken to create three-dimensional views of the internal tissue structures, with each step in the Z-stack taken 2 µm apart. Z-stacks using both the Cy5 (pink) and GFP (green) filter sets were taken and overlaid. From these scans, maximum intensity

projections were taken, in order to visualize a two-dimensional image that displays the brightest pixel in each layer. For each tissue type, four images were taken: stained experimental tissue, unstained experimental tissue, stained control tissue, and unstained control tissue. This process allowed for the verification of the staining process, in addition to a comparison across experimental groups. In order to make all images consistent across tissue preparation, experimental group, and tissue type, the stained experimental tissue was always imaged first and microscope settings were adjusted in regards to its fluorescence. This is because the stained experimental tissue was hypothesized to fluoresce with the highest intensity. Additionally, each leaf sample image was taken with the upper epidermis closest to the camera, with the frame of view focused on the basal half of the leaf. Each root sample image was taken of the primary root, with the frame of view focused just below the base of the stem. Each stem sample image was taken with the frame of view focused just below the 2nd node down from the SAM.

Within these images, magnesium, as well as some other plant elements, produce fluorescence signal. These other elements (such as chlorophyll and lignon) produce fluorescence signal as a result of autofluorescence. Although autofluorescence is included, fluorescence intensity across unstained and stained tissues as well as control and experimental tissues can be compared to pinpoint magnesium accumulation in specific.

RESULTS

Magnesium fluorescence in growth chamber samples

Confocal fluorescence microscopy revealed clear differences in magnesium-associated fluorescence between control and high-magnesium treatments. In growth chamber leaf tissue (Fig. 9), the stained experimental sample exhibited significantly higher GFP fluorescence relative to all controls. Visually, this fluorescence was concentrated along the midvein, extending throughout the lamina, and peripherally localized along the edges of the cell walls.

Root tissue from growth chamber plants (Fig. 10) also showed some elevated fluorescence in experimental samples, particularly in the outer cell layers. This can be identified by the overexposed area of GFP (green) fluorescence on the right side of the stained experimental tissue (Fig. 10). Visually, this increase in GFP fluorescent intensity in the root epidermal cells may indicate magnesium accumulation.

In contrast, stem tissue (Fig. 11) did not show differences in fluorescent intensity between control and high-magnesium treatments. Observed Cy5 (pink) fluorescence in stems was largely attributed to autofluorescence, as similar levels of intensity in control samples were present whether stained or unstained. There is some bright GFP (green) fluorescence on the lateral shoot of the stained experimental tissue sample, however this structure is not visible in the unstained tissue samples, and thus cannot be evaluated. Additionally, there is some GFP

fluorescence in the epidermis of the stained experimental tissue, but this is difficult to evaluate as magnesium accumulation due to the GFP fluorescence occurring similarly in the stained control tissue. Overall, there is minimal, but potentially some, detectable accumulation of magnesium in stem tissue under the conditions tested.

Magnesium fluorescence in greenhouse samples

Fluorescence imaging of greenhouse-grown plants (Fig. 12) showed similar results to the growth chamber plants. The stained experimental leaf tissue exhibited increased fluorescence intensity relative to controls, particularly in cells surrounding the midvein. Some additional GFP fluorescence within the midvein may be visible, but this is difficult to evaluate as magnesium accumulation due to the GFP fluorescence occurring similarly in the stained control tissue.

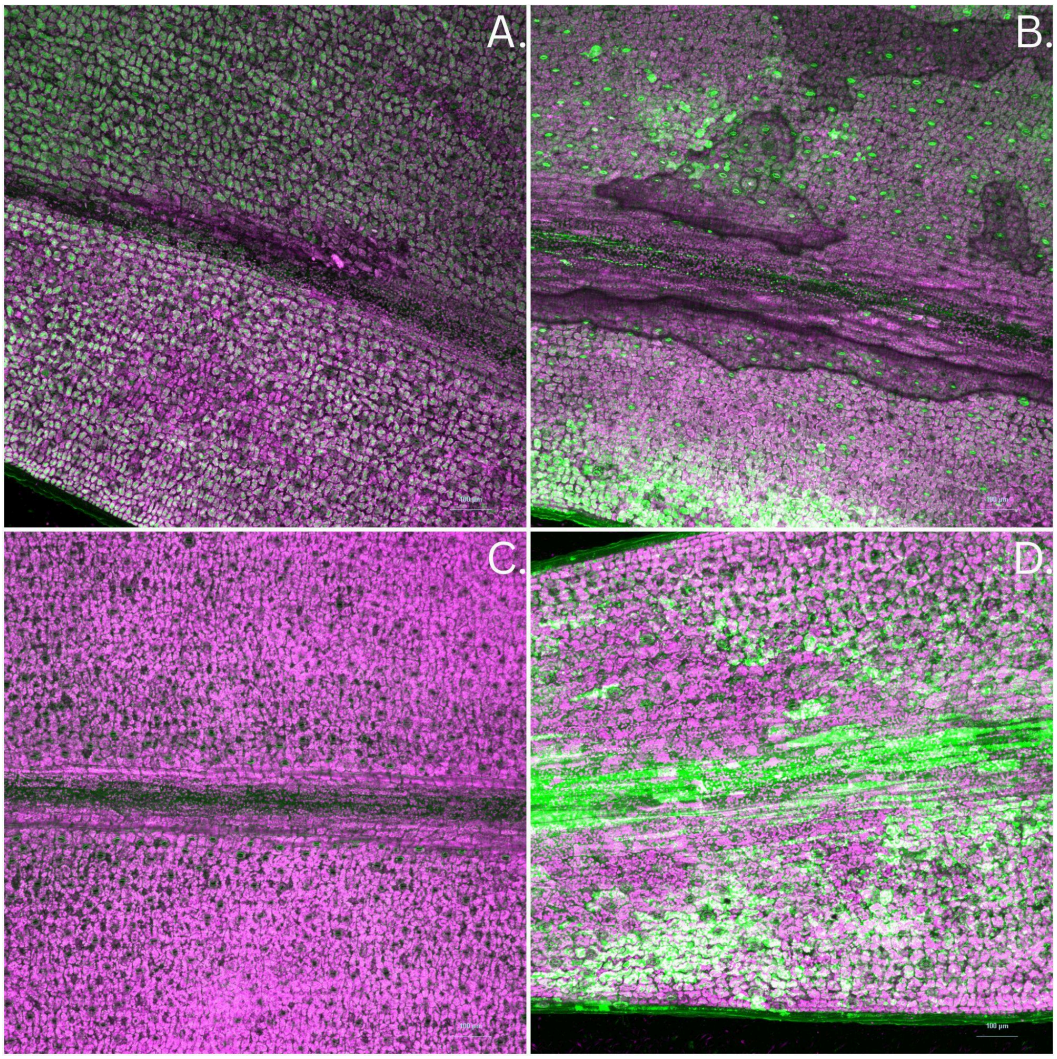

Figure 9. *M. macrophylla* growth chamber leaf tissue observed with confocal fluorescence microscopy. Nikon confocal microscope with 4x objective lens used to take Maximum Intensity Projections using the Cy5 and GFP filter sets. Scale bar is 100 µm. A) unstained control tissue, B) stained control tissue, C) experimental unstained tissue, and D) stained experimental tissue.

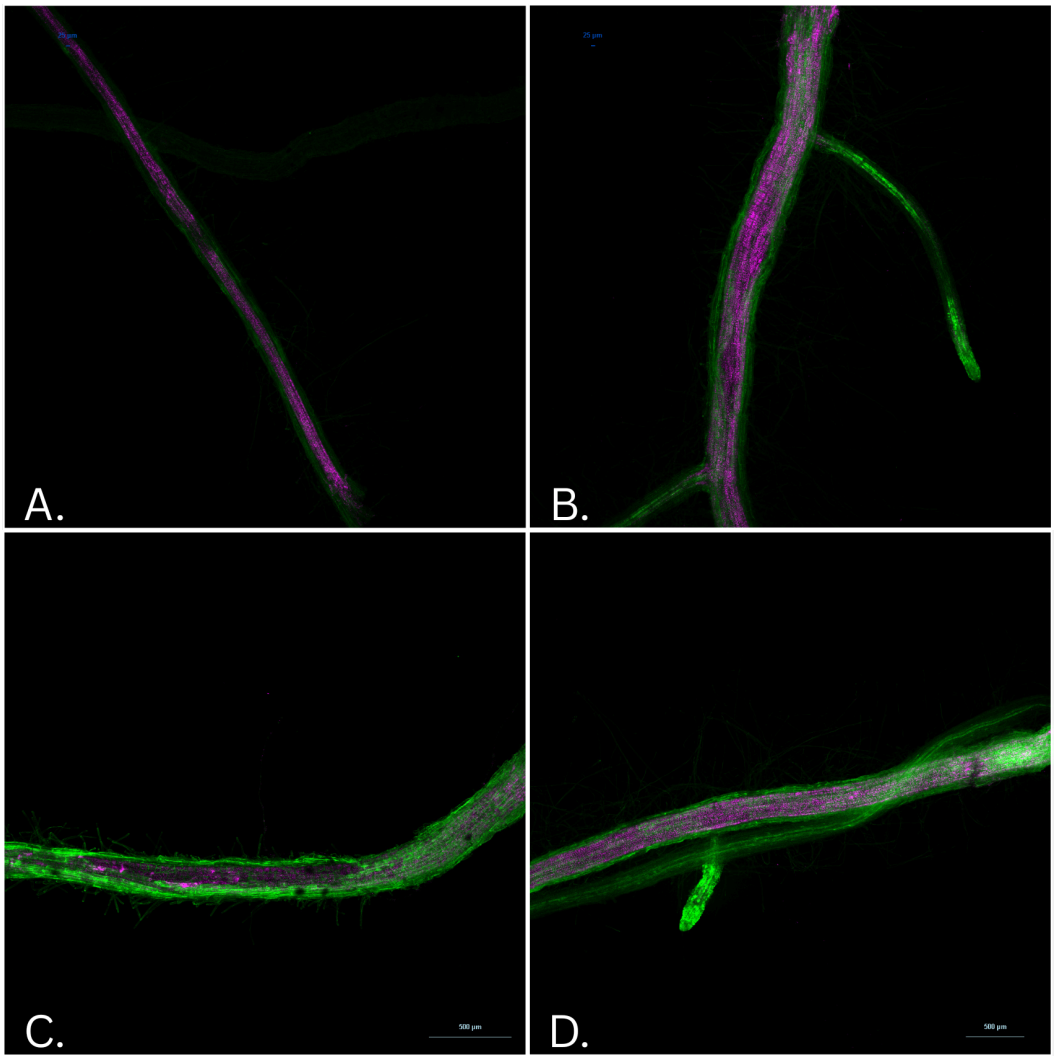

Figure 10. *M. macrophylla* growth chamber root tissue observed with confocal fluorescence microscopy. Nikon confocal microscope with 10x objective lens used to take Maximum Intensity Projections using the Cy5 and GFP filter sets. Scale bar is 500 µm. A) unstained control tissue, B) stained control tissue, C) experimental unstained tissue, and D) stained experimental tissue.

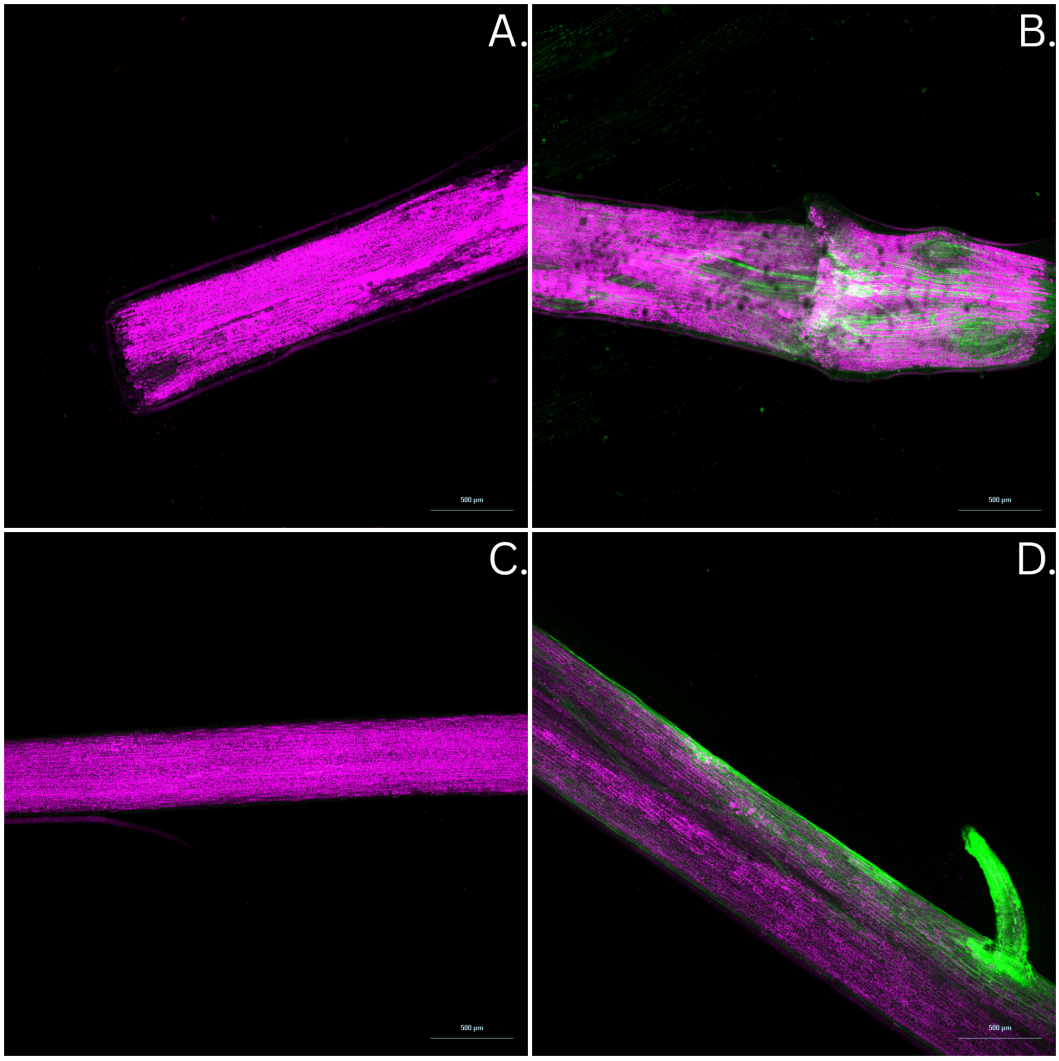

Figure 11. *M. macrophylla* growth chamber stem tissue observed with confocal fluorescence microscopy. Nikon confocal microscope with 4x objective lens used to take Maximum Intensity Projections using the Cy5 and GFP filter sets. Scale bar is 500 µm. A) unstained control tissue, B) stained control tissue, C) experimental unstained tissue, D) stained experimental tissue.

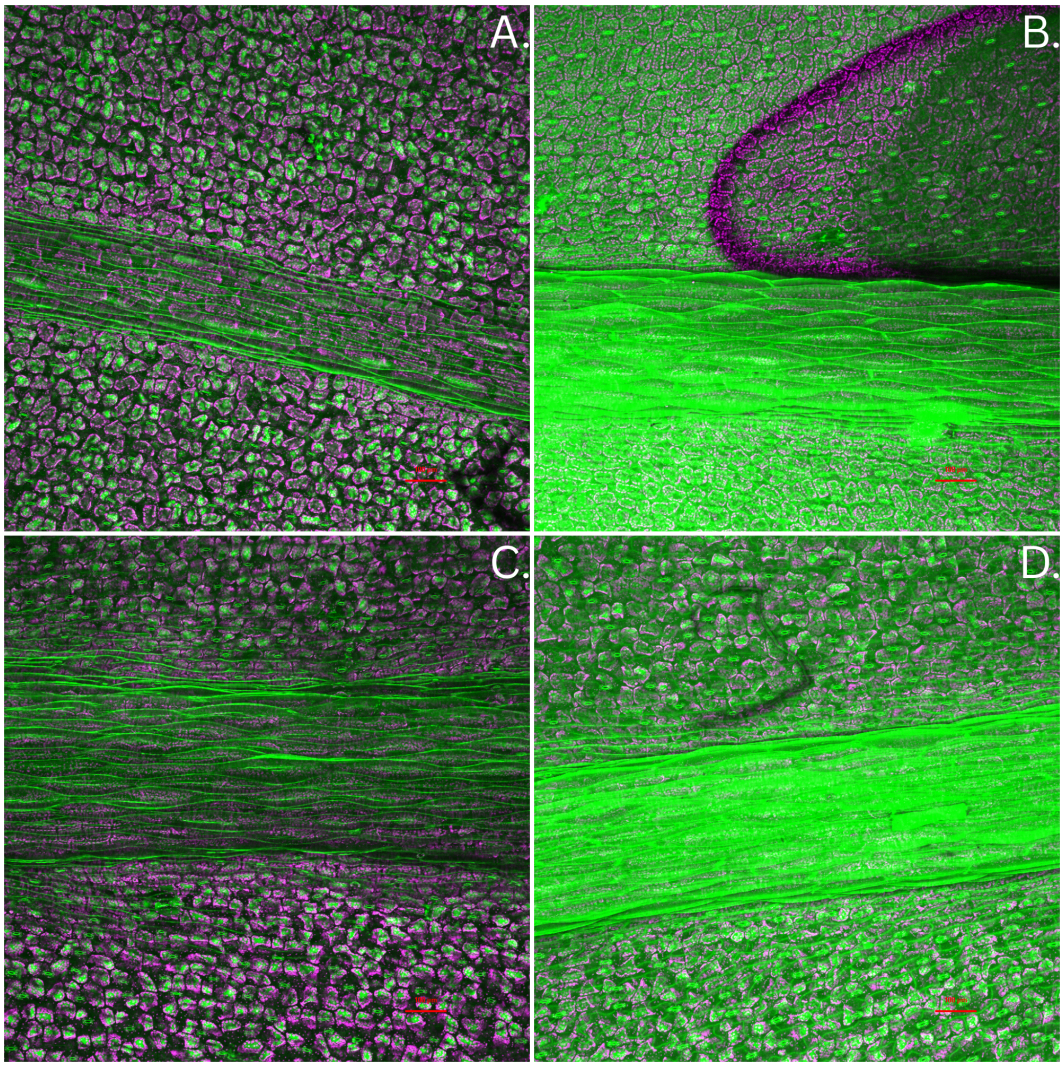

Figure 12. *M. macrophyll*a green house leaf tissue observed with confocal fluorescence microscopy. Nikon confocal microscope with 10x objective lens used to take Maximum Intensity Projections using the Cy5 and GFP filter sets. Scale bar is 100 µm. A) unstained control tissue, B) stained control tissue, C) experimental unstained tissue, and D) stained experimental tissue.

Growth trait analysis in growth chamber samples

Across both growth chamber and greenhouse experiments, morphological traits showed little responsiveness to increasing magnesium conditions.

In growth chamber-grown plants, the two-way ANOVA analysis showed no statistically significant difference among treatments for the number of juvenile vs. mature leaves ($F(3,34)=0.423$, $p = 0.737988$) (Fig. 13; Table 3). In growth chamber-grown plants, one-way ANOVA analyses showed no statistically significant differences among treatments for whole plant wet biomass ($F(1,19)=1.982$, $p = 0.175$), percentage of yellow leaves per plant ($F(3,17)=0.58$, $p = 0.636$), total number of leaves per plant ($F(3,17)=0.869$, $p = 0.476$), total number of mature leaves per plant ($F(3,17)=1.176$, $p = 0.348$), total stem length ($F(3,17)=0.233$, $p = 0.872$), and root length ($F(3,17)=1.034$, $p = 0.402$) (Figs. 14-19; Tables 4-9).

Similarly, for greenhouse-grown plants, the two-way ANOVA analysis showed showed no statistically significant difference among treatments for stem growth over the experiment duration ($F(3,84)=0.038$, $p = 0.9901$) (Fig. 20; Table 10). The one-way ANOVA analysis also did not show statistically significant difference in leaf surface area among treatments ($F(3,42)=0.171$, $p=0.916$) (Fig. 21; Table 11).

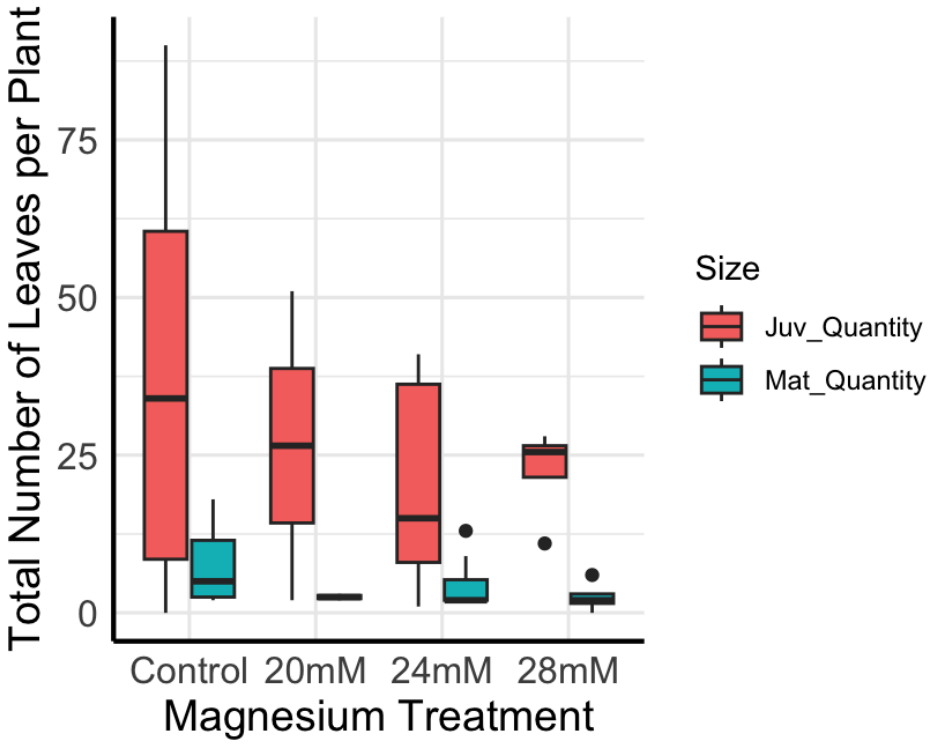

Figure 13. Total number of juvenile and mature leaves per plant compared across magnesium treatments of growth chamber grown plants. Control (n=7), Mg 40 ppm (n= 2), Mg 80 ppm (n=8), Mg 160 ppm (n=4).

Table 3. A 2x4 ANOVA summary table for the effects of treatment, leaf maturity, and their interaction on the number of leaves.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Treatment	3	946	315	0.959	0.423243	
Size	1	4994	4994	15.194	0.000433	***
Treatment:Size	3	417	139	0.423	0.737988	
Residuals	34	11176	329			

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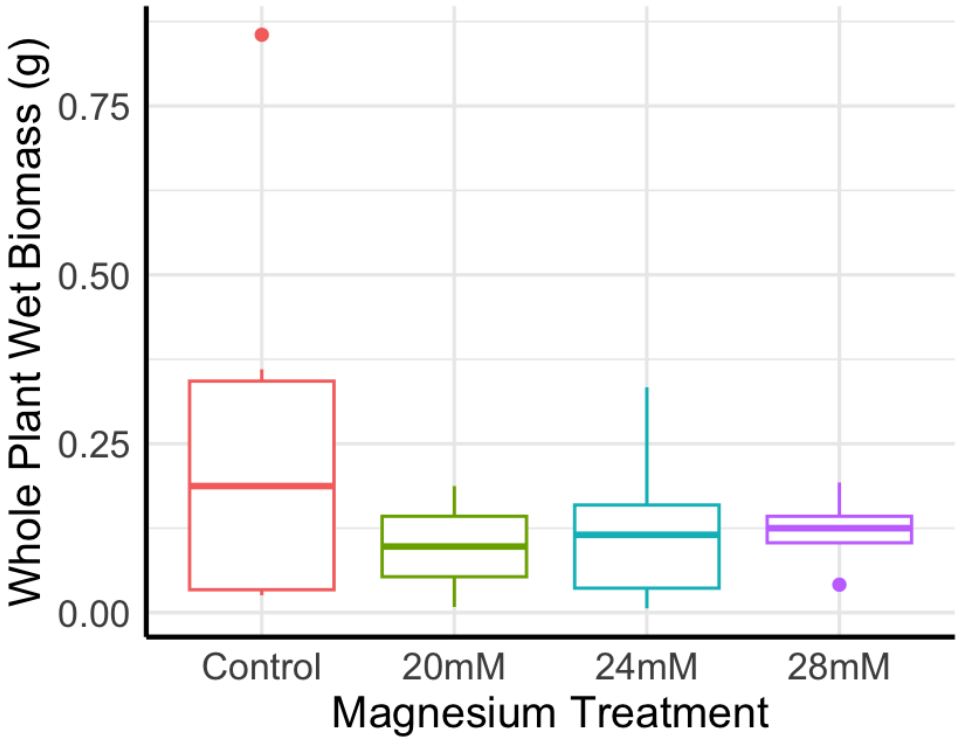

Figure 14. Whole plant wet biomass (g) compared across magnesium treatments in growth chamber grown plants. Control (n=7), Mg 40 ppm (n= 2), Mg 80 ppm (n=8), Mg 160 ppm (n=4).

Table 4. A one-way ANOVA summary table for the effects of treatment on whole plant wet biomass (g).

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	1	0.0696	0.06962	1.982	0.175
Residuals	19	0.6674	0.03513		

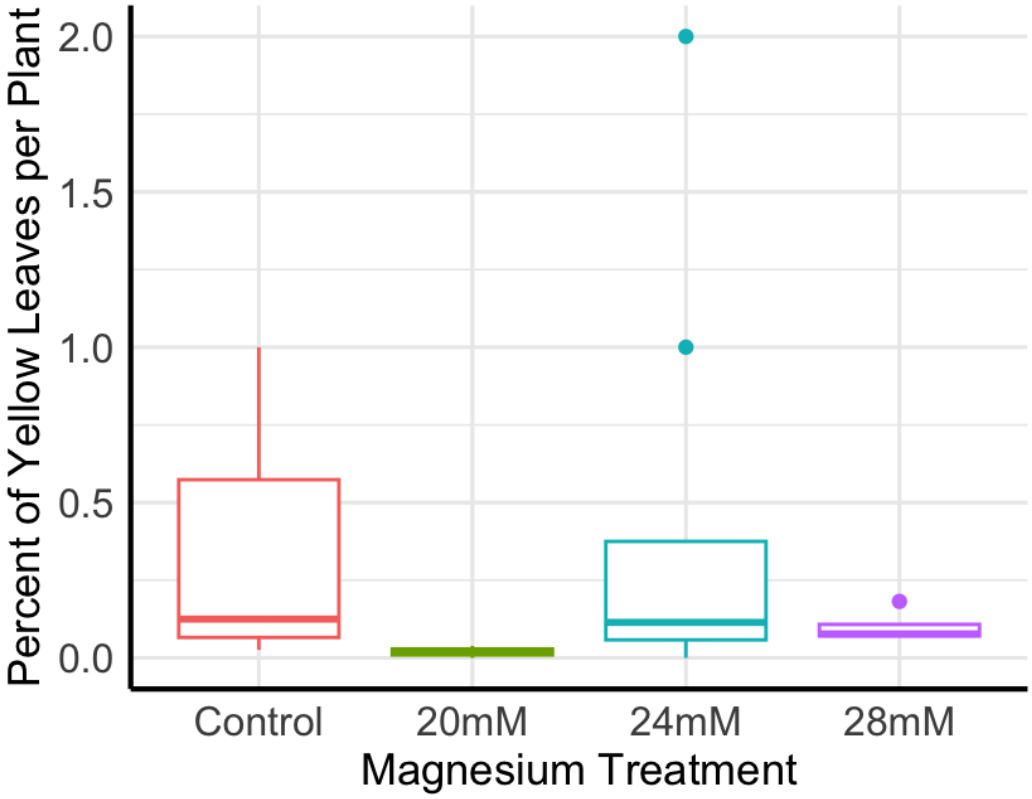

Figure 15. Percent of yellow leaves per plant compared across magnesium treatments in growth chamber grown plants. Control (n=7), Mg 40 ppm (n= 2), Mg 80 ppm (n=8), Mg 160 ppm (n=4).

Table 5. A one-way ANOVA summary table for the effects of treatment on percent of yellow leaves per plant.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	0.485	0.1615	0.58	0.636
Residuals	17	4.735	0.2785		

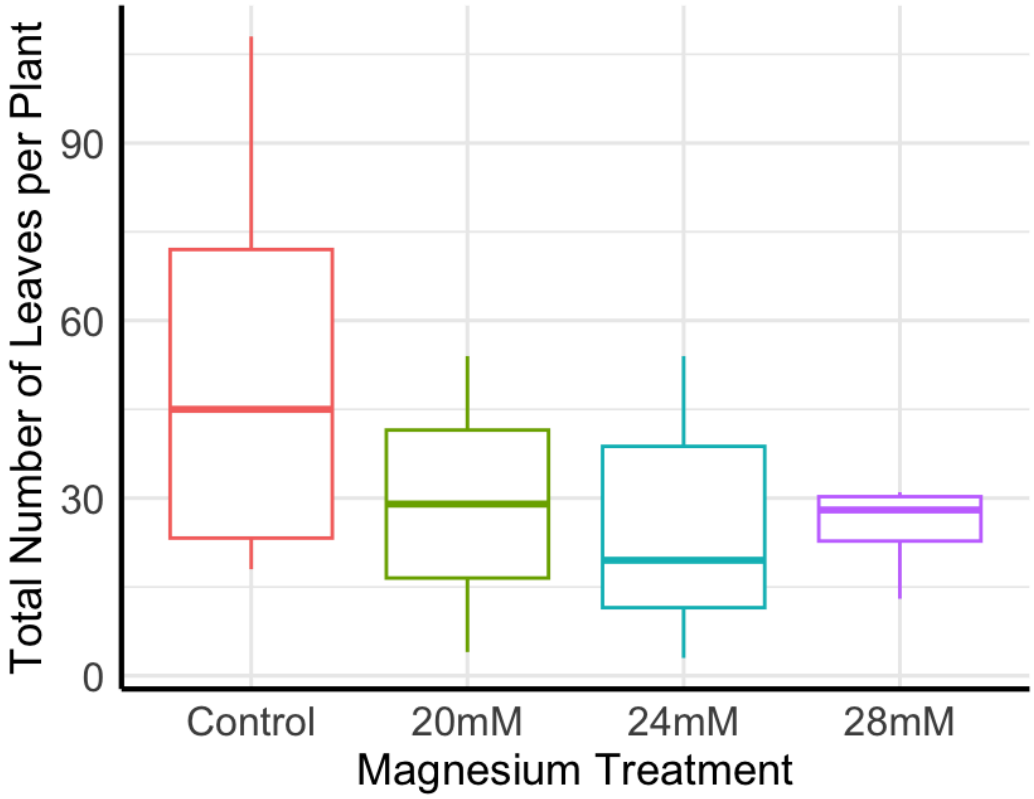

Figure 16. Total number of leaves per plant compared across magnesium treatments in growth chamber grown plants. Control (n=7), Mg 40 ppm (n= 2), Mg 80 ppm (n=8), Mg 160 ppm (n=4).

Table 6. A one-way ANOVA summary table for the effects of treatment on total number of leaves per plant.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	1891	630.5	0.869	0.476
Residuals	17	12327	725.1		

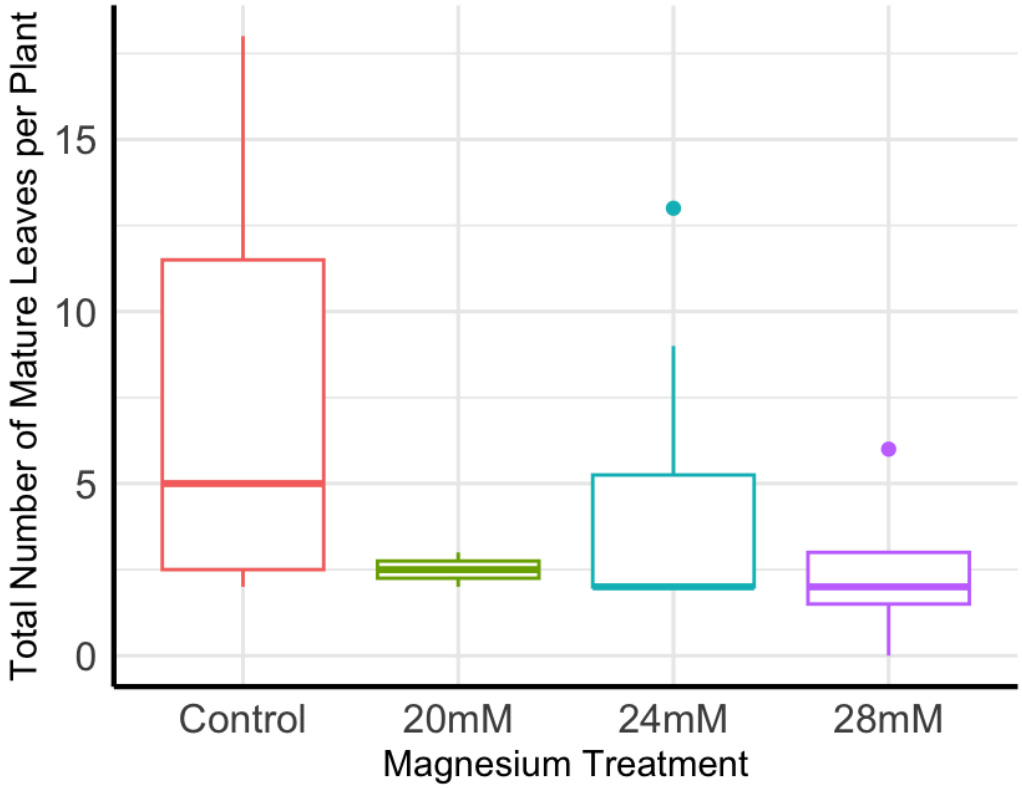

Figure 17. Total number of mature leaves per plant compared across magnesium treatments in growth chamber grown plants. Control (n=7), Mg 40 ppm (n= 2), Mg 80 ppm (n=8), Mg 160 ppm (n=4).

Table 7. A one-way ANOVA summary table for the effects of treatment on total number of mature leaves per plant.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	85.7	28.58	1.176	0.348
Residuals	17	413.2	24.31		

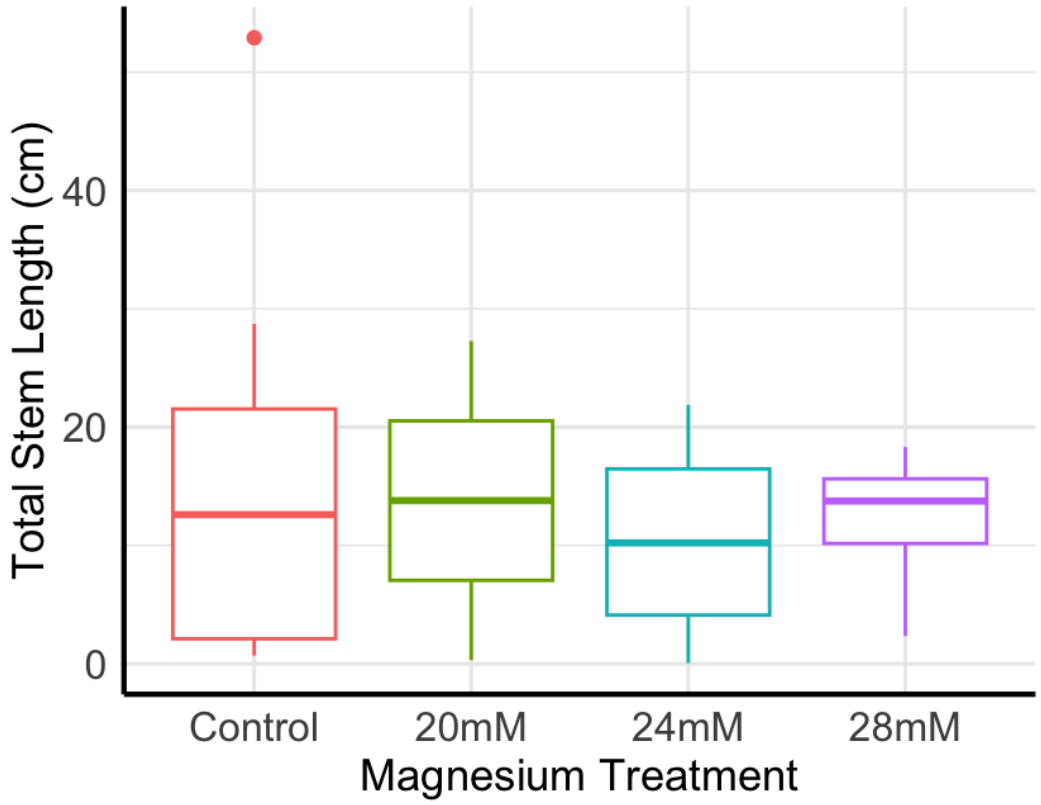

Figure 18. Total stem length (cm) per plant compared across magnesium treatments in growth chamber grown plants. Control (n=7), Mg 40 ppm (n= 2), Mg 80 ppm (n=8), Mg 160 ppm (n=4). The length (cm) of each major branching stem was measured and totaled to find the "total stem length" of all the stems on each individual.

Table 8. A one-way ANOVA summary table for the effects of treatment on total stem length (cm).

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
treatment	3	130	43.32	0.233	0.872
Residuals	17	3157	185.69		

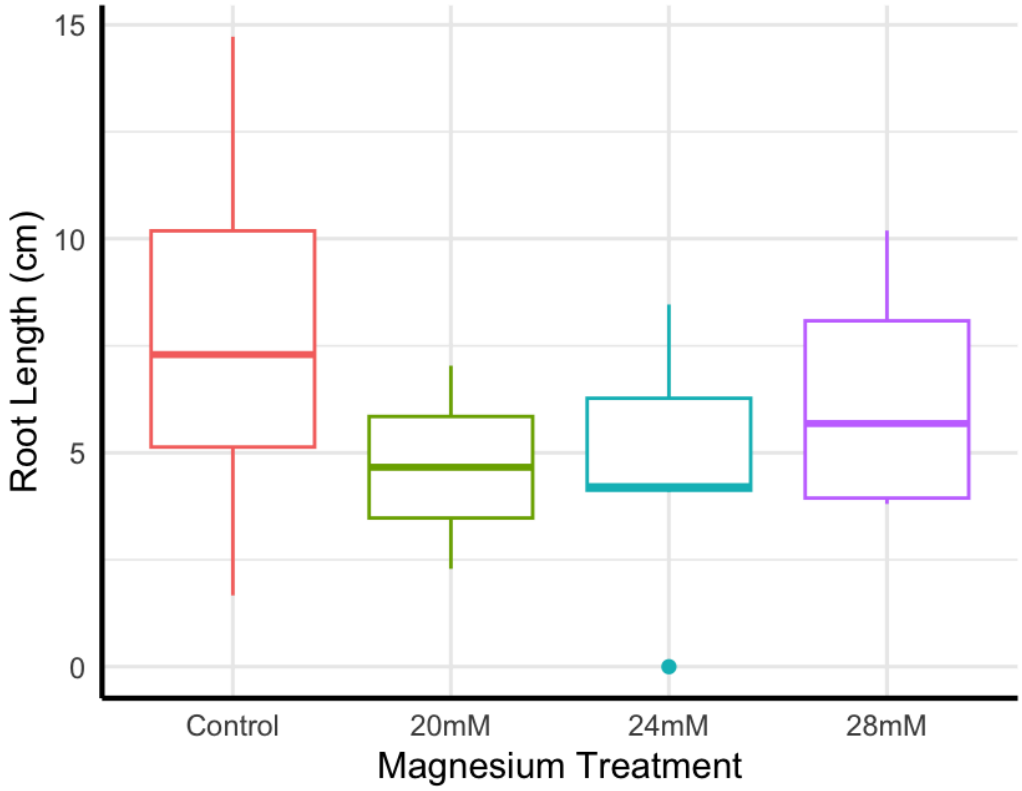

Figure 19. Total root length (cm) per plant compared across magnesium treatments in growth chamber grown plants. Control (n=7), Mg 40 ppm (n= 2), Mg 80 ppm (n=8), Mg 160 ppm (n=4). The length (cm) was measured from the base of the stem to the longest root apical meristem of each individual.

Table 9. A one-way ANOVA summary table for the effects of treatment on root length (cm).

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	36.55	12.18	1.034	0.402
Residuals	17	200.25	11.78		

Growth trait analysis in greenhouse samples

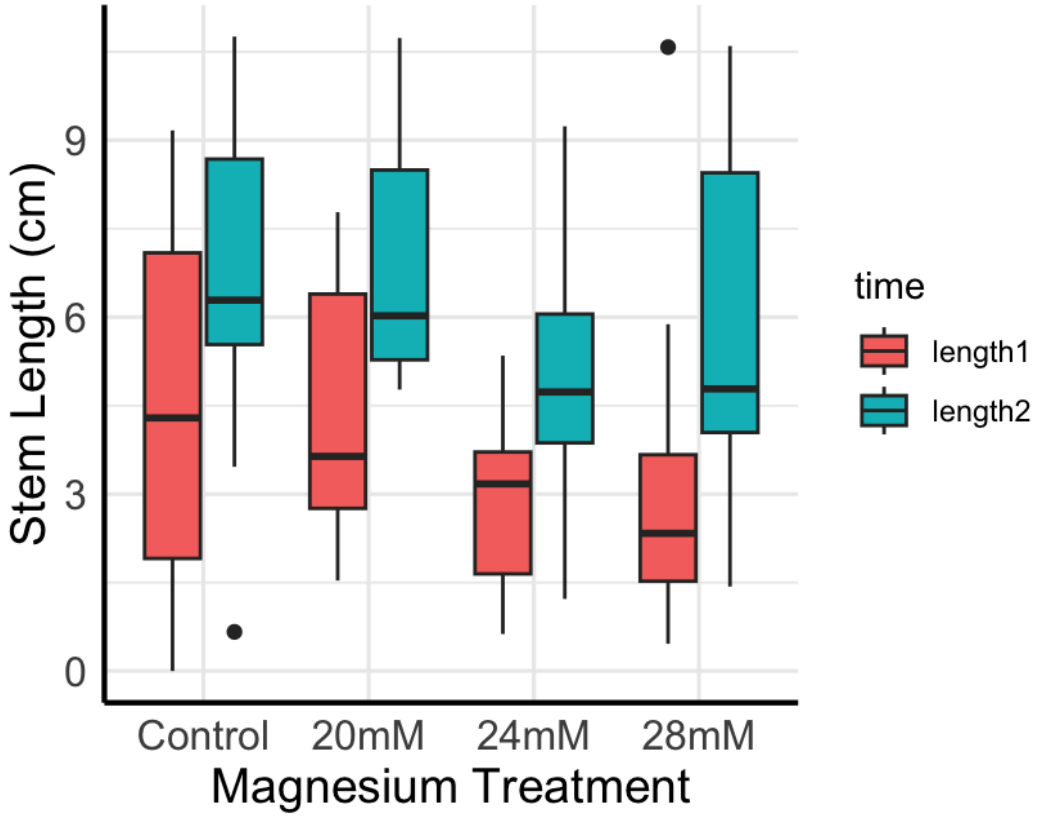

Figure 20. Stem length (cm) of greenhouse individuals per magnesium treatment compared between commencement and conclusion of experimentation. Control (n=11), Mg 40 ppm (n= 11), Mg 80 ppm (n=11), Mg 160 ppm (n=13). Length1 was taken from one random stem of each individual. Length2 was taken at the end of experimentation from the longest stem of each individual.

Table 10. A 2x4 ANOVA summary table for the effects of treatment, stem length, and their interaction on stem length over time.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
treatment	3	45.0	15.00	2.334	0.0797	.
time	1	131.1	131.12	20.410	2.03e-05	***
treatment:time	3	0.7	0.24	0.038	0.9901	
Residuals	84	539.6	6.42			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

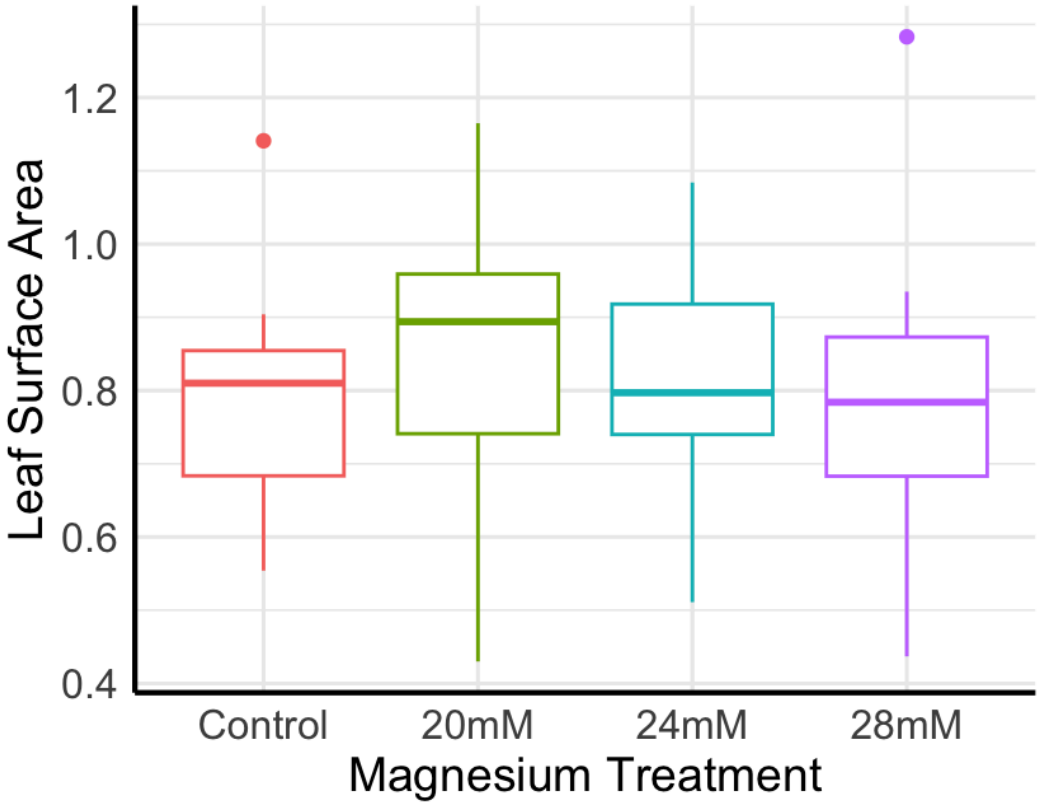

Figure 21. Leaf surface area (cm^2) compared across magnesium treatments in greenhouse grown plants. Control (n=11), Mg 40 ppm (n= 11), Mg 80 ppm (n=11), Mg 160 ppm (n=13).

Table 11. A one-way ANOVA summary table for the effects of treatment on leaf surface area.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
treatment	3	0.0182	0.00608	0.171	0.916
Residuals	42	1.4969	0.03564		

Soil and tissue nutrient analyses from greenhouse experiment

Soil nutrient analysis was performed for the greenhouse experiment, because, due to the mobility of Mg^{2+}, controling magnesium concentration in soil is more difficult than in agar.

Tissue nutrient analysis was performed for the greenhouse experiment, to quantify any increase in Mg. This process could not be replicated in growth chamber tissue due to lack of tissue.

Soil nutrient analysis confirmed that magnesium concentrations increased across treatments, validating the experimental maipulation (Fig. 22; Table 12). Magnesium levels in soil increased from 2035 ppm in the pooled control soil sample to 5740 ppm in the pooled high magnesium concentration soil sample, but calcium levels also remained relatively high across all treatments (Fig. 22).

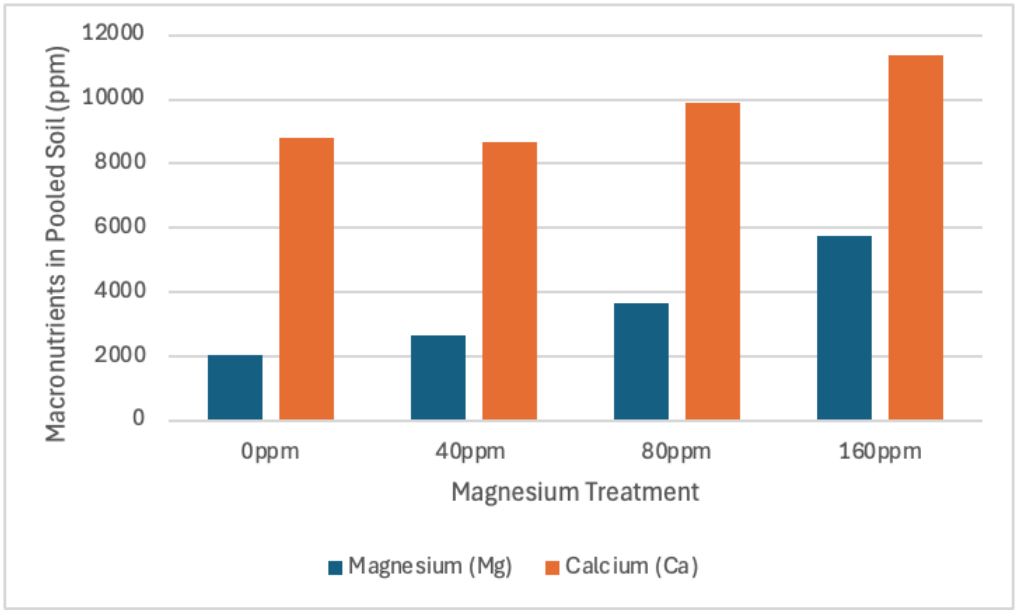

Figure 22. Quantity of total magnesium (Mg) and calcium (Ca) (ppm) found in soil nutrient analysis report from pooled greenhouse soil samples, compared across magnesium treatments. Control (n=11), Mg 40 ppm (n= 11), Mg 80 ppm (n=11), Mg 160 ppm (n=13).

Table 12. Quantity of total macronutrients (ppm) found in soil nutrient analysis report from pooled greenhouse soil samples, compared across magnesium treatments. Nutrient analysis report performed by the University of Massachusetts Amherst Extension Soil & Plant Nutrient Testing Laboratory using the Modified Morgan Extractable Nutrients procedure.

Macronutrients in pooled soil (ppm)					
Mg Treatment	**Magnesium (Mg)**	**Phosphorous (P)**	**Potassium (K)**	**Calcium (Ca)**	**Sulfur (S)**
0ppm	2035	25.8	328	8787	377
40ppm	2650	29.2	414	8677	1222.3
80ppm	3639	30.7	387	9914	2206.2
160ppm	5740	38.4	507	11382	5126

Leaf tissue analysis revealed relatively stable internal magnesium concentrations across treatments, ranging from 1.45% to 1.72% (Fig. 23; Table 13). Additionally, the low magnesium group (40 ppm) showed lower levels of magnesium than the control (0 ppm) group, while the medium magnesium group (80 ppm) showed higher levels of magnesium than the highest magnesium group (160 ppm) (Fig. 23). Calcium concentrations also remained relatively consistent across groups, ranging from 3.20% to 3.67%, and no strong inverse relationship between magnesium and calcium was observed (Fig. 23).

Other macronutrients, such as potassium and phosphorous, showed some variability across treatments, but there does not appear to be a clear trend between these nutrients and experimental treatment (Table 13). Micronutrient concentrations also varied between groups but did not indicate clear trends or imbalances (Table 14).

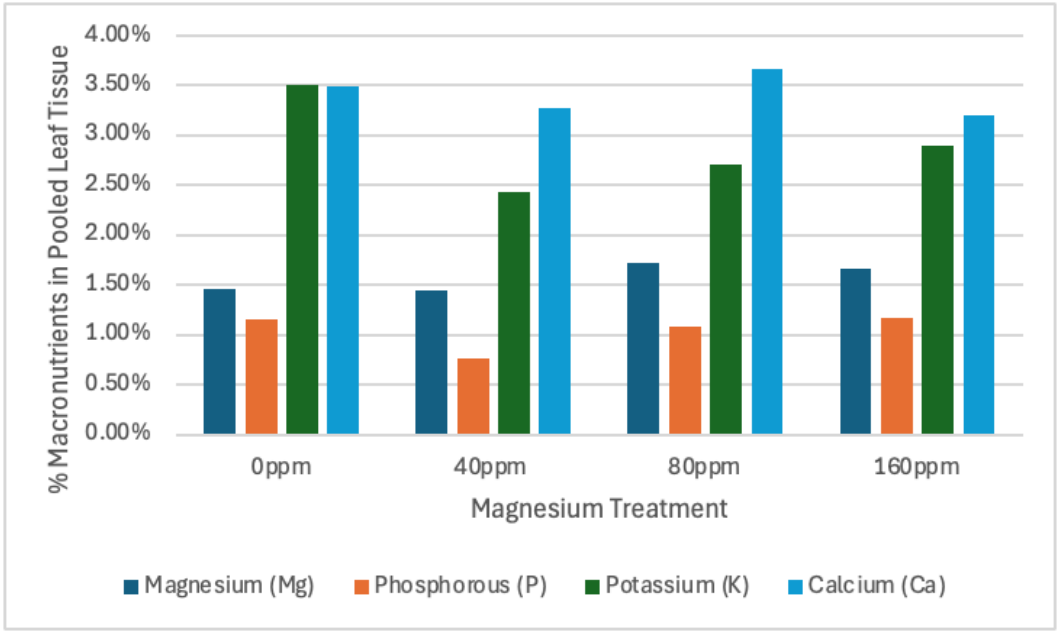

Figure 23. Quantity of total magnesium (Mg), phosphorus (P), potassium (K), and calcium (Ca) (%) found in plant nutrient analysis report from pooled greenhouse leaf tissue samples, compared across magnesium treatments.

Table 13. Quantity of total macronutrients (%) found in plant nutrient analysis report (Acid Wet Digestion in HCl, HNO3, H2O2) in greenhouse leaf tissue, compared across magnesium treatments. Nutrient analysis report performed by the University of Massachusetts Amherst Extension Soil & Plant Nutrient Testing Laboratory.

Macronutrients in pooled leaf tissue (%)				
Mg Treatment	**Magnesium (Mg)**	**Phosphorous (P)**	**Potassium (K)**	**Calcium (Ca)**
0 ppm	1.46%	1.15%	3.50%	3.49%
40 ppm	1.45%	0.77%	2.43%	3.27%
80 ppm	1.72%	1.08%	2.71%	3.67%
160 ppm	1.66%	1.17%	2.89%	3.20%

Table 14. Quantity of total micronutrients (ppm) found in plant nutrient analysis report (Acid Wet Digestion in HCl, HNO3, H2O2) in greenhouse leaf tissue, compared across magnesium treatments. Nutrient analysis report performed by the University of Massachusetts Amherst Extension Soil & Plant Nutrient Testing Laboratory.

Micronutrients in pooled leaf tissue (ppm)					
Mg Treatment	**Zinc (Zn)**	**Copper (Cu)**	**Manganese (Mn)**	**Iron (Fe)**	**Boron (B)**
0 ppm	58.1	6.5	132.3	39	77.4
40 ppm	54.3	4.2	135.7	27	73.1
80 ppm	70.7	7.1	150.8	33	73
160 ppm	64.4	4.6	133.3	32	69

DISCUSSION

This study established that *M. macrophylla* maintains consistent growth across a range of elevated magnesium conditions, indicating a high level of magnesium tolerance. If magnesium was majorly inhibitory to *M. macrophylla*, we would expect to observe typical symptoms associated with serpentine syndrome, specifically clear stunting, necrosis, or major decreases in biomass. However, none of these patterns were statistically supported in either the growth chamber or greenhouse experiments. These findings therefore contradict the first hypothesis that elevated magnesium would induce serpentine stress phenotypes.

Instead, the absence of significant morphological responses suggests that the studied populations of *M. macrophylla* are are either well adapted to or have the ability to plastically respond to elevated magnesium levels within the tested range. This tolerance is notable given that magnesium is considered a key causative agent of serpentine syndrome through its disruption of calcium uptake and cellular homeostasis. The ability of *M. macrophylla* to maintain normal growth under high magnesium conditions while actively accumulating magnesium within its tissues suggests that it possesses effective physiological mechanisms to mitigate magnesium toxicity, rather than solely avoiding magnesium uptake.

Importantly, these results also suggest that the tested populations of *M. macrophylla* did just as well on and off magnesium-containing substrates, demonstrating that this species does not exhibit an obligate dependence on

high-magnesium substrates. This suggests that this species is capable of showing plasticity to serpentine and non-serpentine substrates, rather than exhibiting an increased requirement for magnesium. This supports the idea that the species' occurrence on serpentine habitats may be influenced by ecological factors such as competition, rather than physiological specialization.

Tissue-specific sequestration of magnesium

Fluorescence imaging provides further insight into the mechanisms underlying this tolerance. Magnesium accumulation was not uniform across all tested tissue types but instead localized primarily to leaves and roots, with little evidence of accumulation in stems. This pattern partially supports the hypothesis that magnesium accumulation in root, stem, and leaf tissues will increase in experimental tissue, and supports the hypothesis that magnesium sequestration might occur at a increased level specifically in root and leaf tissue. This supports the idea that *M. macrophylla* employs tissue-specific sequestration as a tolerance strategy.

In both growth chamber and greenhouse-grown plants, leaf fluorescence was strongest along the midvein. The midvein contains vascular tissue (xylem and phloem) as well as ground tissue such as collenchyma. Xylem transports water and nutrients (including Mg^{2+}), and phloem transports the products of photosynthesis, suggesting an active transport of magnesium. Ground tissue contains large vacuoles, suggesting that magnesium may be compartmentalized

within vacuoles to reduce cytoplasmic toxicity. Additionally, increased fluorescence can be seen in the cells across the lamina, as well as some peripheral localization of fluorescence along cell edges. This may point to magnesium accumulation occurring in the cytoplasm (as vacuolar expansion can displace cytoplasmic contents outward) as well as potentially within the cell wall. This suggests that magnesium accumulates, potentially through sequestration, within both vascular tissues as well as in epidermal tissue. These findings support the hypothesis of leaf sequestration as a primary site of magnesium accumulation.

Root tissues, particularly epidermal cells, also showed elevated fluorescence. This suggests that roots may function as an initial regulatory barrier, limiting the translocation of excess magnesium into the shoots. Since the epidermis is the contact zone, a concentration gradient of fluorescence intensity would occur, making the epidermis brighter than other parts of the root. Such a mechanism is consistent with known strategies in other serpentine-tolerant species, where ion regulation occurs at the point of uptake. Increased signal in epidermal cells indicates that roots may serve as an initial site of magnesium sequestration, compartmentalization, or regulation. However, variability in root sample structure due to limited tissue restricted higher analysis of specific tissue-level localization, specifically within lateral roots. These findings support the hypothesis of root sequestration.

In contrast, stems showed minimal fluorescence difference between treatments, indicating that they are unlikely to serve as primary storage sites for

magnesium. This suggests that it is likely that stems function primarily in transport. However, this does not exclude the possibility of localized sequestration in specific stem tissue (specifically, the cortex or lateral shoots), which were not resolved in this study. Additionally, the minor GFP fluorescence on the epidermis in the stained experimental tissue may indicate that epidermal stem tissue accumulates magnesium, but this association would need to be more specifically measured, as similar fluorescence was visible in the stained control tissue. Future work should examine stem cross sections with increased specificity to test whether these tissues serve as additional sequestration sites.

Together, these findings support the idea that *M. macrophylla* actively regulates internal magnesium through compartmentalization, rather than passively accumulating it. This aligns with broader literature suggesting that serpentine-tolerant plants maintain homeostasis through sequestration, exclusion, or selective transport mechanisms.

Soil and tissue nutrient analysis

Additionally, the results from the leaf tissue analysis showed relatively stable internal magnesium concentrations across treatments, even though the results from the soil analysis showed increasing magnesium concentrations across treatments. This may suggest that *M. macrophylla* is capable of maintaining internal nutrient homeostasis despite high differences in magnesium availability in soil.

Experimental considerations and limitations

Several limitations likely influenced the results of this study. First, sample sizes, particularly in the growth chamber, were small due to limited seed availability, which may have reduced statistical power and obscured subtle treatment effects.

Second, the duration of the green house experiment (five weeks) may not have been sufficient to capture long-term physiological or cumulative stress responses to elevated magnesium exposure. It is unclear how long it takes for magnesium toxicity to manifest.

Third, the experimental design did not fully replicate natural serpentine conditions, and only focused on one causative agent of serpentine syndrome: magnesium. The use of liquid magnesium sulfate (growth chamber) and dissolved Epsom salt (greenhouse) to replicate serpentine conditions excluded the additional stressors present in natural serpentine soils, such as heavy metals and drought.

Although this is a limitation, previous literature examining magnesium stress on plants set the stage in prioritizing elevated magnesium, and even utilizing Epsom salt as a substitute for serpentine soil, to experiment on the effect of serpentine substrates (Hanly et al., 2005). As magnesium stress, including both magnesium toxicity and deficiency, is a factor of concern in agriculture, studies have been conducted determining that the direct application of Epsom salt onto soil results in a similar tissue magnesium concentration as ground serpentine rock

when used as a fertilizer (Hanly et al., 2005). However, the application of Epsom salt in this study was dissolved, not direct, which could have affected the results. There was a notable increase in magnesium concentration in the results of the soil tests, but not a major accumulation in the tissue that was tested. This remains an unresolved area of the study, as that discrepancy in magnesium concentration was likely not due to faulty magnesium application (as indicated by the soil tests) and yet the tissue test and microscopy analyses results were not congruent. More thorough testing of other tissues with additional replicates might help to resolve this discrepancy between microscopy images and leaf magnesium content. Microscopy looking at magnesium content within the whole leaf rather than one section, as well as not pooling leaf samples for tissue analysis, could have led to different results as well.

Additionally, specifically in the greenhouse experiment, calcium concentrations remained relatively high in addition to magnesium levels (Fig. 23). This resulted in Ca:Mg molar ratios that were not as extreme as those found in serpentine soils. As many of the inhibitory effects of magnesium are mediated through calcium deficiency, the absence of low-calcium conditions may have impacted the lack of strong stress response in greenhouse tissue.

Finally, while Mag-520 AM was formulated to have a higher affinity for Mg^{2+} than Ca^{2+}, it is difficult to completely outrule signal overlap with calcium ions present in leaf tissue. However, if there was signal overlap, it is likely that it was quite minimal. Using the greenhouse leaf tissue analysis, we know that there

was an increase in Ca^{2+} content across experimental groups in the greenhouse experiment. Future studies incorporating quantitative methods, such as ion quantification, would strengthen my confidence in the results.

Broader implications and applications

These findings contribute to a growing understanding of how plants maintain ion homeostasis under chemically stressful conditions. The ability of *M. macrophylla* to tolerate elevated magnesium without major growth consequences suggests a plethora of possible species-specific factors that influence magnesium toxicity.

From an ecological perspective, studying serpentine endemics helps address broader ecological and evolutionary questions regarding community composition, species distribution, and soil adaptation: are these plants confined to serpentine environments because they are competitively excluded from more fertile habitats, or because they have evolved unique specializations that restrict them to this narrow niche (O'Dell & Rajakaruna, 2011)? These questions contribute to a broader understanding of local adaptation, edaphic island systems, and modes of speciation (O'Dell & Rajakaruna, 2011). Examining the effects of magnesium on plant growth in serpentine-tolerant plants is essential to understanding whether and how serpentine tolerance is playing a role in ecotypic differentiation, speciation, and competition. The ability to tolerate elevated magnesium without major growth consequences raises interesting questions about

competition ecology. If *M. macrophylla* is not strongly limited by magnesium toxicity, its restriction to serpentine habitats may reflect other factors, such as competition. In more fertile soils, faster-growing or larger species may outcompete it, whereas serpentine conditions reduce competition and allow it to persist.

Future directions

My preliminary assessment establishes a basic understanding of *M. macrophylla*'s serpentine response, setting the stage for further research. A key next step is to directly compare serpentine and non-serpentine populations of *M. macrophylla* to test for ecotypic differentiation. If serpentine-orgin populations perform equally well across conditions while non-serpentine populations show reduced performance under high magnesium, this would provide strong evidence for ecotypic differentiation.

Comparative studies with closely related non-serpentine species, such as *Moerhingia lateriflora*, could further test how genetically engrained the ability to withstand elevated magnesium levels is in this lineage. Measuring the expression of calcium and magnesium transporter genes would provide insight into the molecular basis of tolerance. Perhaps there are pre-adaptations to serpentine tolerance in this lineage that are shared even among non-serpentine relatives.

Future experimentation should also more accurately simulate serpentine soils by manipulating calcium availability alongside magnesium to achieve consistent and low Ca:Mg ratios.

Additionally, despite the lack of statistical significance in the measurement of morphological traits, some non-significant trends were observed. These include a slight increase in leaf yellowing and some variation in growth across treatments, but these patterns were inconsistent and did not meet thresholds for significance. Increasing sample size and extending experimental duration would improve statistical power and potentially allow for the detection of longer-term effects of magnesium exposure.

Additionally, more specified imaging with larger quantities of available tissue would allow for greater analysis of tissue structures, as well as cross-sectional views of tissue. This would allow for greater understanding of magnesium sequestration, compartmentalization, and localization, as well as determining whether tissues such as the cortex serve as additional sites of magnesium sequestration.

Finally, integrating quantitative measurements of both calcium and magnesium in all experimental systems, not just the greenhouse experiment, would allow for better understanding of how consistent treatments are, as well as how these ions are interacting.

Conclusions

Overall, the studied populations of *M. macrophylla* demonstrate strong tolerance to elevated magnesium, showing minimal signs of the typical phenotypic markers of serpentine syndrome across treatments. This tolerance may be mediated by this species' ability to perform tissue-specific compartmentalization, sequestering excess magnesium to leaf and root tissues. While further studies are needed to see if this sequestration impacts Ca:Mg molar ratios, these findings may suggest the potential of this sequestration as a strategy to maintain suitable calcium-to-magnesium molar ratios in magnesium-toxicity-affected tissues.

Rather than exhibiting classic symptoms of magnesium toxicity, the studied individuals of this species appear capable of managing high-magnesium environments, supporting its classification as a serpentine-tolerant plant. This provides insight into the physiological strategies underlying ultramafic specialization, as well as the species' potential to show ecotypic differentiation between subpopulations.

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