

A Comparison of Theoretical and Actual Coumarin Exudation Under Iron Limitation to Understand Root Exudation Mechanics

By

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B.S. Chemical Engineering  
Massachusetts Institute of Technology, 2025

Submitted to the Department of Civil and Environmental Engineering in partial fulfillment of the requirements for the degree(s) of  
Master of Engineering  
at the

Massachusetts Institute of Technology

May 2025

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May 9, 2025

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Abstract

Nutrient cycling is an important component of plants' immune systems, largely driven by the act of exuding environmentally influential metabolites from roots. Root exudation may be driven by multiple unique mass-transport mechanisms, including active and passive transport types, though the latter is not well-studied despite being labelled a significant driver of low molecular weight metabolite exudation. This research investigates the generally accepted assumption that low molecular weight metabolites, including iron-fixing coumarins (scopoletin, fraxetin, etc.) are primarily exuded passively, and high molecular weight metabolites follow an active exudation approach. Scopoletin and scopolin exudation from *Arabidopsis thaliana* in low-iron and replete conditions is quantified to determine if the hypothesized passive diffusion mechanism is a significant contributor to coumarin exudation. LC-MS analysis suggests that passive diffusion of scopoletin and scopolin from roots plays a significant role in total coumarin exudation values. Further research should include investigating the implications of passive coumarin exudation on long-term iron storage and soil health in addition to the relationship between coumarin production and exudation.

Thesis supervisor: Dave Des Marais

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## **1. Introduction**

### **1.1. Overview**

With agriculture being a critical component to worldwide food production, rising atmospheric temperatures and carbon dioxide levels threaten crop productivity as plants instinctively alter their resource allocation strategies, including the amount of nutrients that plants purge via root exudation (1). Currently, it is known that the rhizosphere, or the nutrient dense region surrounding plants' root systems, plays an important role in plants' immune response (2). Plants are capable of emitting secondary metabolites that control rhizosphere function and diversity primarily through active transport, an exudation mechanism that allows for movement of heavier molecules against concentration gradients via transporters (3). However, non-targeted coumarin exudation's potential as a preventative iron-limitation or infection measure has not been thoroughly investigated. It is critical to understand subsurface nutrient transfer to adequately diagnose potential crop and soil health issues, though poor understanding of root exudation rates, mechanisms, and spatial distributions limit our ability to optimize agricultural yield.

### **1.2. Present knowledge of exudation mechanisms**

The current system for categorizing root exudation mechanisms by exudate species is driven by binary classification. In most cases, exudates are assumed to follow an either active or passive approach based on molecular weight, with low molecular weight (LMW, <1000Da) molecules (amino acids, sugars, phenols, etc) engaging in passive methods and high molecular weight (HMW, >1000Da) molecules (proteins, mucilage, etc.) in active methods (20, 21, 22). However, this weight-based

approach is an oversimplification for ease of classification that potentially ignores the capability of some metabolites to alter their exudation strategy based on environmental conditions. Coumarins, a class of phenolic molecules commonly exuded by a wide variety of plant species, are an example of such metabolites.

### 1.3. Coumarin as an Exudate of Interest

Coumarins, a class of iron-chelating, antimicrobial compounds secreted by *Arabidopsis thaliana* (*A. thaliana*) roots, have been selected as a secondary metabolite of particular interest due to their well-studied ability to mobilize iron in low-availability scenarios (4, 25, 26). At the root-soil interface, coumarins, particularly scopoletin, fraxetin, and sideretin, reduce available Fe(III) through what is known as a strategy-I mechanism (5). Coumarins notably exist in a glycoside form when stored within the plant and undergo deglycosylation, or cleaving of the glucose group, upon extracellular exudation. The exact timing of deglycosylation is not entirely understood, though coumarins generally assume a free-coumarin form once in soil environments (29).

By the current accepted definition, coumarins as LMW molecules should prioritize a passive diffusion-based mechanism. However, studies have identified that in stressed conditions, coumarin exudation assumes an active approach, governed primarily by ATP-binding cassette transporters (19, 27). A key question that arises from coumarin exudation is the difference in the magnitude of coumarin release in non-stressed conditions, potentially following the hypothesized yet poorly studied passive model as a preventative measure against iron loss, and increased exudation as part of

recovery efforts in iron-stressed situations (7). This research hypothesizes that the lack of a stressor signal in the normal iron conditions will result in steady, slower coumarin exudation similar to the expected rate based on passive diffusion. The significance of passive coumarin exudation, particularly scopoletin and scopolin, compared to total coumarin exudation will be investigated. Scopoletin and scopolin were selected as primary coumarin targets due to being notably one of the most abundant coumarin types in *A. thaliana*. Scopoletin and its derivatives play a significant role in *A. thaliana*'s iron reduction strategy (30, 29). Comparison of exudation rates from live plant samples to a theoretical concentration from mathematical modeling would also help determine whether coumarin release occurs at magnitude similar to a diffusion-like scenario.

#### 1.4. Microfluidic approach

“Plant-on-a-chip” polydimethylsiloxane (PDMS) devices, notably the EcoFAB, are becoming increasingly common vessels in which root exudation is studied (8, 28). Such devices are gaining popularity across a variety of fields, particularly the biomedical space for the miniaturization of organ systems (24). Additionally, their low cost and small size is ideal for high throughput experimentation (23). When applied to root exudation analysis, PDMS sampling devices are best suited for mechanistic analyses and limited error from unnecessary variables compared to field or soil setups. The device's small size (50mm x 40mm x 15mm) made sterility maintenance simpler by minimizing the media-air interface and allowing for complete encapsulation from surroundings. Spatial root exudation analyses also benefit from the application of microfluidic techniques because of the ability to perform high resolution root imaging

through the transparent growth chamber walls. Coumarins in particular benefit from this feature as they exhibit a distinct fluorescence upon photo-dimerization from UV light exposure, providing a simple method of confirming the presence of coumarin exudation using a single-chamber device for hydroponic growth (9).

#### 1.5. Manipulation of Iron Concentration

Coumarins' known ability to engage in active exudation in response to iron-depleted environments provides an excellent mechanism for stimulating the active transport of an influential secondary metabolite. Therefore, iron-deficient and replete treatment conditions will be implemented to induce elevated coumarin exudation. Scopoletin and scopolin exudation rates during and after respective iron treatments will be compared to a theoretical passive diffusion-limited exudation model to analyze the efficiency of active coumarin exudation via ABC transporters and determine if coumarin exudation in non-stressed conditions mimics a passive mechanism. Analysis of both free coumarin and coumarin glycoside concentrations in media and roots will provide valuable information regarding coumarin movement in response to low iron availability. Secondary metabolite sampling procedures include the use of a single-chamber microfluidic device coupled with liquid chromatography mass spectrometry (LC-MS) to more precisely identify coumarin concentrations. Overall, quantification of these important parameters will provide insight into the relevance of coumarin diffusion and *A. thaliana*'s iron management strategies, as well as question the validity of a molecular weight-based method of classifying metabolite exudation mechanisms.

## 2. Methodology

### 2.1. Sampling device synthesis and sterilization

The “plant-on-a-chip” device mold for plant growth was designed using Autodesk Fusion360 and synthesized using a FormLabs Form 4BL resin printer. Device features include an approximately 2mL primary growth chamber, a circular port for upwards plant growth, and small inlet channel to optimize the media exchange process (Figure 1). Printed molds were cured under UV light for twelve hours and thoroughly rinsed with 70% ethanol. To create the device, molds were filled with 7.5mL of PDMS and curing agent (10:1) according to manufacturers’ instructions (SYLGARD 184 Silicone Elastomer kit) (Figure 2). After drying for two hours at 80°C, devices were removed from molds and prepared for surface binding. The device and glass base were each treated with an Electro-Technic Products BD-20 high frequency generator for 30s, then bound together for two hours at 90°C for permanent attachment. Prior to the introduction of media, sterilization occurred according to the following protocol: devices were submerged in Milli-Q Type I ultra-pure (Milli-Q) water for four hours to prevent leaching during experimentation, rinsed three times with Milli-Q water, then submerged in 70% ethanol for 30 minutes followed by a second treatment in 100% ethanol for 5 minutes. Residual ethanol was allowed to evaporate off overnight in a sterile space, and devices were further treated with UV light for one hour prior to use.

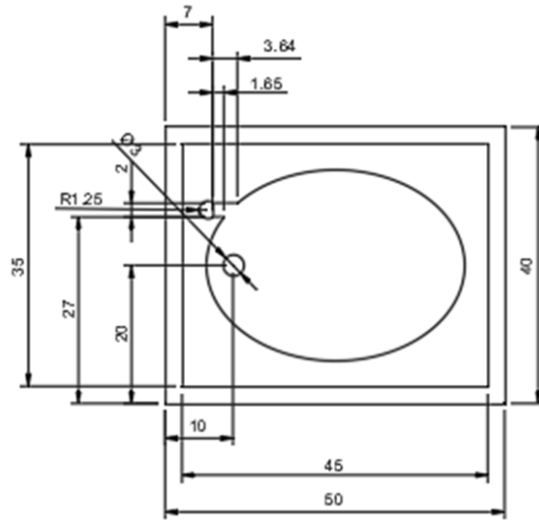


Figure 1: **Sampling device dimensions.** A single-chamber PDMS device was created to maintain sterility and optimize imaging capabilities.

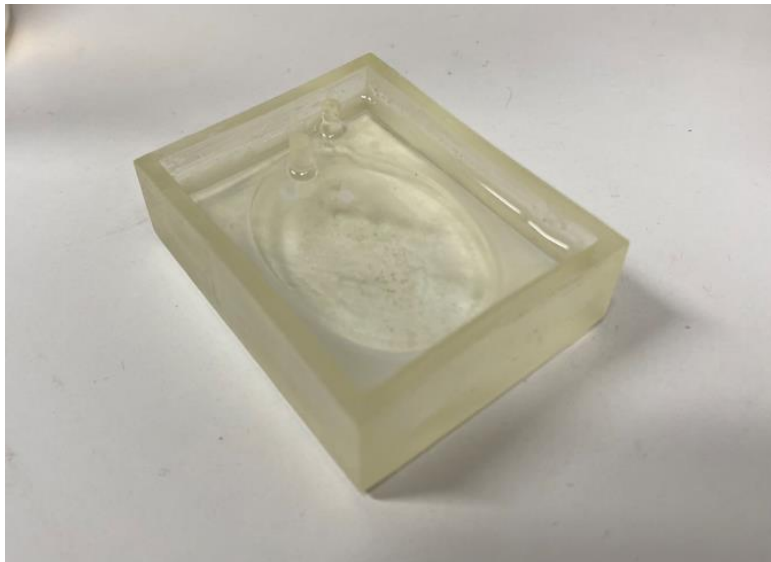


Figure 2: **Filled device mold.** The device manufacturing and sterilization required approximately six hours per device, beginning with the fabrication and setting of PDMS.

## 2.2. Plant growth conditions

*A. thaliana* Col-0 seeds were sterilized using a deionized water solution containing 1% (v/v) Triton X-100 and 10% (v/v) sodium hypochlorite. Seeds were agitated in solution for 15 minutes, then triple-rinsed with sterile deionized water before plating on half-strength Murashige-Skoog (MS) LB+agar plates. Following a 48-hour dark period at 5°C, germinated seeds were grown in standard temperature (~23°C), light (16h:8h, light:dark), and carbon dioxide (400ppm) conditions for 21 days. Preliminary experimentation suggested that 21-day-old *A. thaliana* plants are ideal for implementation in the chosen device due to their medium-length root system (root length = ~25mm), allowing for easier insertion. The selected seedling age's ability to be placed in devices without significant root contact prevents potential stress exudation in response to physical damage. Seedlings with much smaller or larger root lengths led to high rates of plant mortality due to drought conditions from evaporation and excessive handling, respectively. Confirmation of coumarin exudation in the given conditions was confirmed qualitatively during preliminary experimentation using UV imagery. The presence of fluorescence in sample roots after exposure to treatment media, most notably in iron-deficient samples, suggested treatment effectiveness. A shorter exudation period was deemed optimal for coumarin analysis due to images from long-term (>24 hours) exposure showing decreased fluorescence (33).

## 2.3. Treatment media compositions

Iron-deficient and replete half-strength MS media, containing iron concentrations of 0µM and 50µM, respectively, were used as treatment conditions in the hydroponic

setup. All plastic and glassware were first acid-washed in 7% HCl for 30 minutes to remove traces of metals and triple-rinsed in Milli-Q water before use. Iron-deficient and replete medias [0.18g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.22g/L  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ , 100 $\mu\text{L}$  0.5M boric acid stock solution, 15mL nitrogen stock solution (54.0g/L  $\text{NH}_4\text{NO}_3$ , 65.6g/L  $\text{KNO}_3$ ), 2mL phosphate stock solution (100.0g/L  $\text{KH}_2\text{PO}_4$ , 46.2g/L  $\text{K}_2\text{HPO}_4$ ), 10mL 0.5M MES buffer stock solution adjusted to pH 5.5 with 1M KOH] were supplemented with 10mL of the corresponding trace metal stock solution. All the following trace metals were first prepared as individual stock solutions using 0.01M HCl, then combined to form the final trace metal stock solution added to the half-strength MS medias. Concentrations in iron-deficient trace metal stock solution were as follows: 10mM  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 3mM  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5mM KI, 0.1mM  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.01mM  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , and 0.015 mM  $\text{CuCl}_2 \cdot 5\text{H}_2\text{O}$ . Replete trace metal stock solution was created using the same method with the addition of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  at a concentration of 10mM. EDTA was also added to replete media so that the final concentration was 100 $\mu\text{M}$ . All media was adjusted to pH 5.6 using 1M KOH if necessary, sterilized by 0.22 $\mu\text{m}$  vacuum filtration, and stored in dark conditions until use.

#### 2.4. Growth conditions

Coumarin exudates were collected in Milli-Q water after exposure to the two treatment conditions: half-strength MS media with normal iron (50 $\mu\text{M}$ ) and iron-deficient (0 $\mu\text{M}$ ) levels. Milli-Q water was included as a third treatment condition to be used as a baseline since the 0 $\mu\text{M}$  Fe concentration during exudate collection in Milli-Q water was hypothesized to also induce coumarin exudation in all trials. Negative controls, including

sampling devices containing treatment media without plant samples, media samples, and root samples without exposure to any treatment conditions, were also analyzed in triplicate.

Seedlings were placed into sampling devices filled with the respective treatment media (n=2) and sealed inside Magenta™ tissue culture vessels to maintain sterility (Figure 3). Control trials for each treatment type included media without live plant samples (n=3). Device windows were covered by opaque fabric to mimic the natural light conditions of the root environment. Samples were grown in standard temperature (23°C), light (16h:8h, light:dark), and carbon dioxide (400ppm) conditions for two hours. Treatment media was then collected for analysis. Live roots and devices were rinsed and refilled with sterile Milli-Q water. Resulting exudate and fresh root samples were obtained after an additional two hours in collection conditions. Visualization of this process is available in Figure 4. 200uL of Milli-Q water from each sample was plated on agar petri dishes to confirm that sterility was maintained throughout the experimental period. All media and fresh root samples were weighed after collection and immediately flash frozen using liquid nitrogen. Samples were stored in the dark at -80°C prior to freeze-drying.

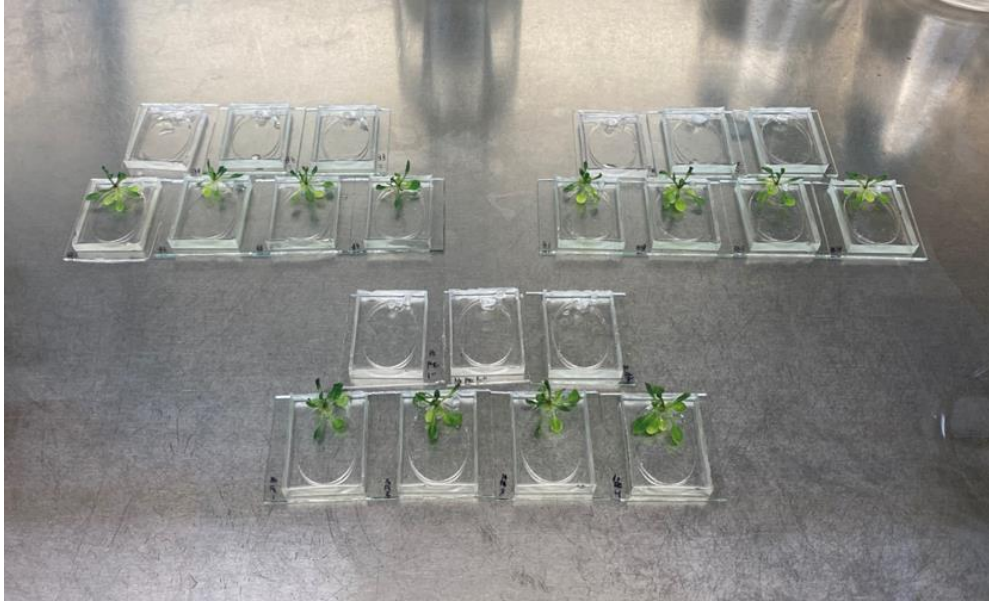


Figure 3: **Experimental and negative control trials.** Preliminary experiments used UV imaging to confirm effectiveness of treatment conditions through coumarin fluorescence.

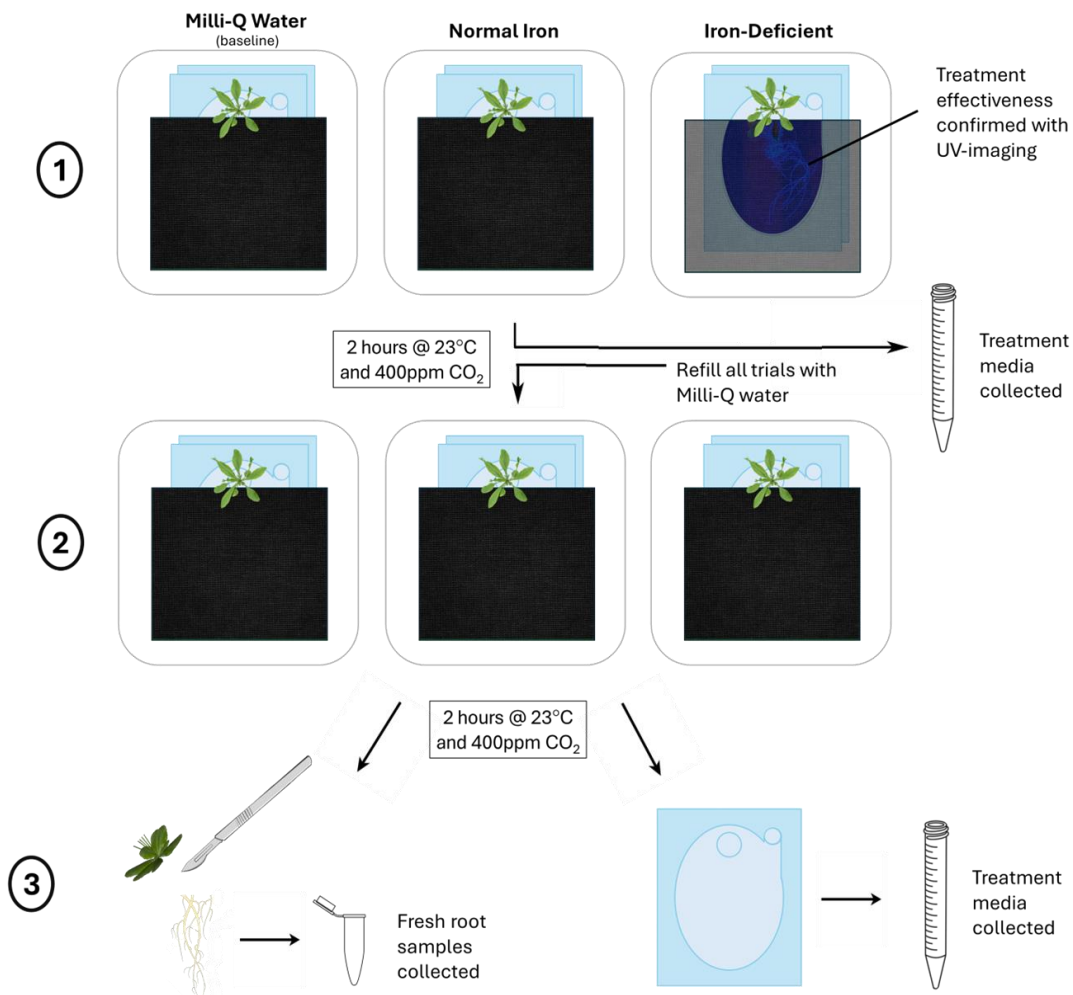


Figure 4: **Overview of experimental design.** 21-day-old *Arabidopsis thaliana* plants were placed in sterile EcoFAB-like devices for two 2-hour collection periods. Devices were covered with opaque fabric to mimic natural root conditions.

## 2.5. Preparation of media and fresh root samples for analysis

To prepare for LC-MS analysis, lyophilized media samples were resuspended in 1mL of 100% LC-MS grade methanol and filtered via 0.22 $\mu$ m syringe filtration. Lyophilized root samples were ground for 20 seconds using a bead mill until a sufficiently fine powder was achieved. To maximize the coumarin yield from root samples, a methanol extraction was performed according to Siso-Terraza et al. with

modification: 0.5mL of 100% LC-MS grade methanol was added to extract all phenolic compounds. Samples were centrifuged at 12,000 x g at 4°C for 5 minutes and the supernatant was collected. Resulting pellet was resuspended in 0.5mL of 100% LC-MS grade methanol and the centrifugation process was repeated. The two recovered supernatants were combined to produce a total sample volume of 1mL, homogenized for 30 seconds, then filtered via 0.22µm syringe filtration. All samples were stored temporarily at -20°C prior to LC-MS analysis.

#### 2.6. Scopolin and scopoletin quantification using LC-MS

Scopolin and scopoletin quantification was performed using an Agilent 1260 Infinity II HPLC system with an Agilent ZORBAX Rapid Resolution SB-C18 threaded column (2.1mm x 50mm x 1.8µm) coupled to an Agilent InfinityLab single quadrupole LC/MSD system. A 5µL injection volume of gradient (0% to 100%) mobile phase acetonitrile containing 0.1% (v/v) formic acid was implemented using an 11-minute method. The mass spectrometer was operated in single ion mode at 40°C with positive polarity and a fragmentor voltage of 135V. Mass-to-charge ratios of 355.1 m/z and 193.2 m/z were targeted in mass spectra for analysis, representing scopolin and scopoletin ([M+H]<sup>+</sup>), respectively. An 8-point standard curve (0.5µM, 1µM, 2.5µM, 10µM, 25µM, 50µM, 75µM, 100µM) was used for scopolin and scopoletin qualification. Standards were prepared following the same methods described earlier.

### 3. Discussion

#### 3.1. Preliminary UV device imaging

Imaging of *A. thaliana* samples within the device chambers after treatment and prior to exudate collection suggested that coumarin exudation was present in all samples in both treatment conditions (Figure 5). This is understandable due to the Milli-Q water collection conditions mimicking a no-iron condition, meaning that a reference coumarin exudation rate of *A. thaliana* in Milli-Q water as a treatment condition was needed as a baseline coumarin value. All samples also displayed blue-green fluorescence in the leaves, though this is more likely a result of sinapoyl malate presence, which acts as protection against UV radiation, rather than coumarin presence based on the time frame (11). The described trials are notably separate from samples collected for LC-MS analysis to prevent coumarin photolysis from affecting results.

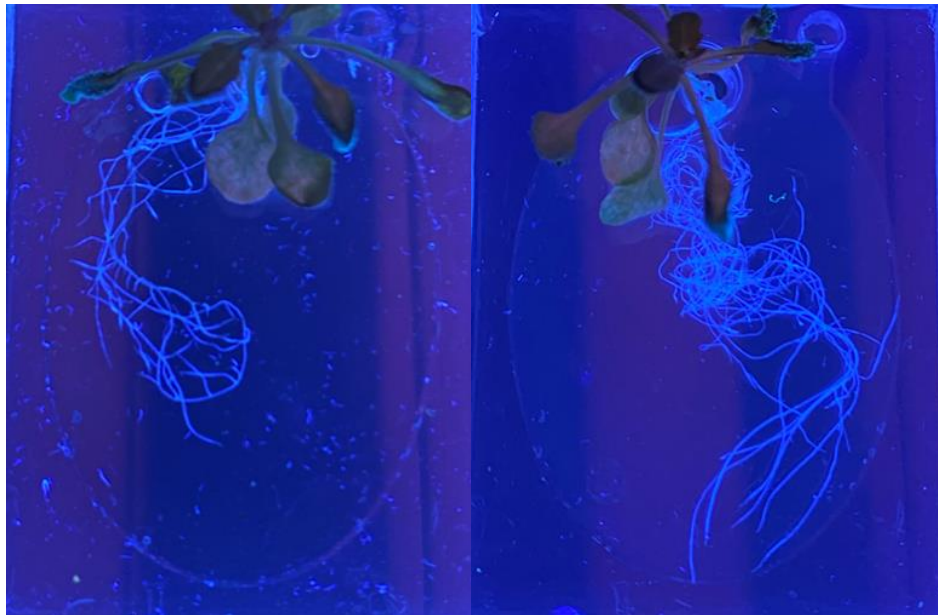


Figure 5: **Roots of iron-deficient samples.** Preliminary experiments used UV imaging to confirm effectiveness of treatment conditions through coumarin fluorescence.

### 3.2. Treatment-induced coumarin exudation effects

Results from LC-MS analysis suggest a variety of coumarin species exist in both root and media samples, with scopoletin and scopolin being the most common. Evidence of a treatment effect from iron manipulation is apparent in both root and treatment media samples (Figure 6), where *A. thaliana* exposed to no-iron conditions have >3.1x more scopolin and >1.3x more scopoletin compared to replete and control conditions.

Analysis of scopoletin and scopolin concentrations in root samples suggests that a non-active mechanism driving coumarin exudation, particularly in normal iron conditions. Control root samples that were not exposed to any treatment had scopolin and scopoletin values that were 2.8-4.2x greater than those of treatment trials. This significant decrease in root coumarin concentration is likely due to the exudation process. If coumarin exudation rate exceeds its production in the epidermal and cortical cells, such a decrease in stockpiled coumarins would occur. Roots from iron-deficient samples contained less total coumarins than samples exposed to replete media. The correlation of less coumarins in root samples with higher exudation rate provides supporting evidence that there is a different mechanism driving scopoletin and scopolin exudation in replete compared to iron-deficient samples. Exudation, despite no known active mechanism trigger being present, suggests that a diffusion-based mechanism may be participating.



**Figure 6: Quantities of scopolin and scopoletin in (a) root and (b) treatment media from LC-MS analysis.** Root samples that were not exposed to any treatment had elevated coumarin concentrations, suggesting that the rate of coumarin exudation exceeds that of production. Greater total coumarin exudation in iron-deficient media suggests that treatment successfully initiated active transport mechanisms.

### 3.3. Implications of scopoletin:scopolin ratios

Analysis of scopoletin and scopolin ratios supports the idea that coumarin glycosides dominate in root environments and free coumarins exist primarily in soil environments (Figure 7). However, a significant number of counts of coumarin glycosides existed within media samples, which contradicts the idea that deglycosylation occurs immediately upon exudation. The short experimental time frame is not conducive for deglycosylation, requiring a timescale of hours to occur, which explains the magnitude difference in free versus glycoside species. Similarly, a significant number of counts of free coumarin existed in root samples. This finding is to be expected as coumarins remain close to the root surface after exudation, as seen in previous fluorescent images, meaning that newly cleaved free coumarins may have been included in collected root samples despite technically being exuded. Further analysis of time dependency on coumarin storage and production to confirm this hypothesis would be useful in understanding *A. thaliana*'s coumarin production strategy, particularly in terms of glycosylation timing.

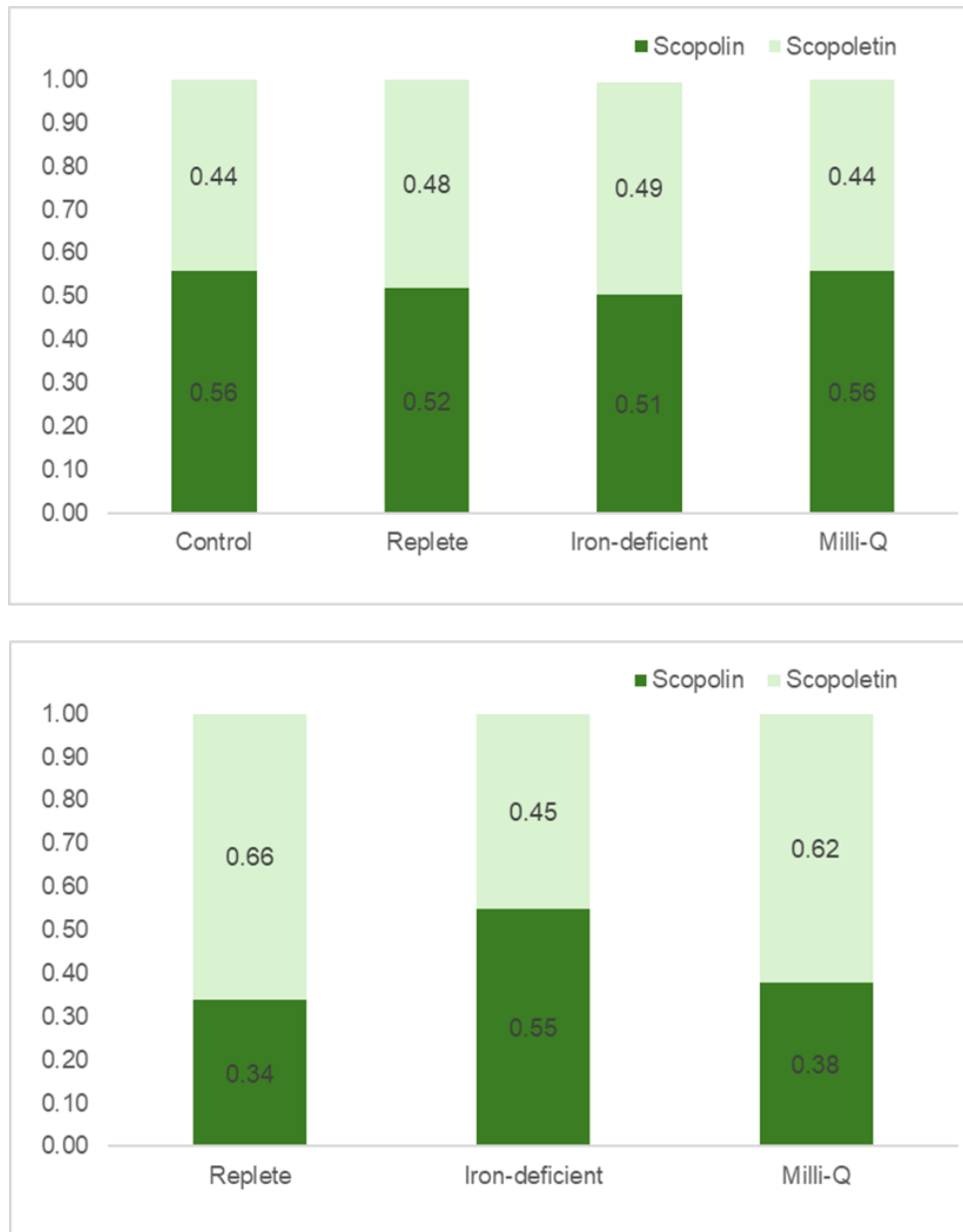


Figure 7: **Ratios of scopolin to scopoletin in (a) root and (b) treatment media samples.** Root and media samples tended to have greater amounts of scopolin and scopoletin, respectively, matching expected molecular behavior.

#### **4. Conclusion**

Analysis of scopoletin and scopolin concentrations in root and liquid exudate samples suggests that *A. thaliana* exudes coumarins scopoletin and scopolin partially through a non-active mechanism. Currently, coumarin diffusion is an often-overlooked root exudation mechanism, though this research suggests that it may play a significant role in *A. thaliana*'s nutrient cycling. Exact timing and conditions for such a passive mechanism to activate and its external implications, including soil pH balancing and microbial diversity manipulation, are unknown. Additional research on coumarin production rates in epidermal and cortical cells and its relationship to a diffusion-like mechanism may provide valuable insight into *A. thaliana*'s iron retention strategy.

## **5. Acknowledgements**

I would like to thank my research advisor, Dave Des Marais, for his mentorship and valuable feedback throughout the project. I am also grateful to my research mentor, Chloe Heitmeier, for her assistance and guidance. Additionally, I would like to acknowledge the McRose group at MIT's Parsons Laboratory for their assistance with sample analysis and supply sourcing.

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