“COMPARATIVE EFFECTS OF NITROGEN FERTILIZATION ON SEEDLING GROWTH IN FOUR BROMELIAD SPECIES”

A Senior Thesis Presented to
The Faculty of the Department of Organismal Biology & Ecology,
Colorado College

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Bachelor of Arts Degree in Organismal Biology & Ecology
25th day of April 2025

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Comparative Effects of Nitrogen Fertilization on Seedling Growth in Four Bromeliad Species

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INTRODUCTION

The Bromeliaceae is a predominantly neotropical monocotyledon family. It includes approximately 80 genera and 3,800 species which differ in morphology, habit, & ecology. Bromeliads can be found across nearly 75 degrees of latitude in the Americas, from the southeastern United States to southern Chile, and occupy a range of ecologies with enormous variations in elevation, climate, and anthropogenic disturbance (Benzing, 2000; Smith and Downs, 1974; Ulloa Ulloa *et al.,* 2017).

The family's broad success in the Americas can largely be attributed to adaptive radiation, when an ancestral lineage quickly diversifies into multiple groups with different forms, ecologies, and functions (Benzing, 2000; Schluter, 2000). The Bromeliaceae are a classic example of this phenomenon (Benzing, 2000). From the ancestral bromeliad – which likely resembled other terrestrial lilioid monocots with an extensive system of absorptive roots and a non-impounding rosulate shoot – emerged three broad groups, each characterized by increasing levels of epiphytism: Pitcairnioideae, Bromelioideae, & Tillandsioideae (Benzing, 2000; Givnish, 2014; Hietz & Zotz, 2001).

To understand the morphological adaptations that bromeliads evolved to enable success as an epiphyte – the habit of a plant that grows on another plant rather than in soil – it is imperative to first consider the unique environmental pressures associated with this growth form. Epiphytism has evolved independently multiple times across angiosperms, including in orchids (Orchidaceae), aroids (Araceae), cacti (Cactaceae), and bromeliads (Bromeliaceae) (Benzing, 2004; Zotz, 2016). Epiphytic plants often experience greater environmental fluctuations than their terrestrial counterparts, including more variable water availability, nutrient supply, and temperature. These fluctuations arise from their reliance on ambient humidity & rainwater, exposure to open air, and limited access to soil nutrients (Benzing, 1990). To cope with these challenges, epiphytic bromeliad groups have developed specialized strategies for water and nutrient acquisition. Unlike terrestrial plants, which primarily absorb nitrogen through their roots, epiphytes often acquire nitrogen from atmospheric sources, rain, or organic debris (Benzing, 2000).

Pitcairnioideae bromeliads most closely resemble the ancestral bromeliad with well-developed root and shoot systems, as most lineages (although not all) are terrestrial. While the group was historically thought to be a single subfamily, more recent molecular technologies have split it into six subfamilies: Pitcairnioideae, Brocchiniodeae, Hechtioideae, Lindmanioideae, Navioideae, and Puyoideae (Givnish *et al.*, 2007; Givnish *et al.*, 2011). The other two bromeliad subfamilies – Bromelioideae & Tillandsioideae – are characterized by high levels of epiphytism, though species in the Bromelioideae tend to be more flexible in habit, often producing functional roots in soil, than those in the Tillandsioideae. Both subfamilies have evolved specialized mechanisms including absorptive trichomes, the phytotelmata of overlapping leaf bases to impound water, and a slow life history strategy to mitigate unique epiphytic pressures (Benzing, 1990; Smith & Downs, 1974; Zotz & Asshoff, 2010). Trichomes, common among plants for UV protection and thermoregulation (MacAdam, 2009), also play key roles in nutrient & water acquisition in epiphytic bromeliads (Benzing, 1976; de Oliveira, 2022). In Bromelioid species, absorptive trichomes are typically located at leaf bases, while in Tillandsioideae, they often cover the entire leaf, reflecting a greater reliance on the shoot system for nutrient uptake. The phytotelmata, a tank-like structure in many epiphytic bromeliads, captures water and organic debris (Benzing, 2000). Additionally, many epiphytic bromeliads form mutualistic relationships within these tanks with bacteria, fungi, and animals that help convert organic matter and atmospheric nitrogen into bioavailable nutrients (Benzing, 2000; Silva et al., 2020; Stryker et al., 2024).

These leaf adaptive traits reflect a shift in nutrient and water absorption from roots to shoots with increasing epiphytism in adult bromeliads. However, there are still many unknowns regarding the role of roots and resource allocation in juvenile bromeliads, particularly before the development of the phytotelmata (Takahashi & Mercier, 2024). This study aims to explore how early nitrogen availability influences growth patterns and root versus shoot investment in four bromeliad species with varying degrees of epiphytism spanning the three broad groups: terrestrial *Puya mirabilis,* flexible *Aechmea recurvata*, flexible *Billbergia brasiliensis*, & epiphytic *Vriesea rafaelii*.

Like many other morphological traits, bromeliad seed and seedling morphology vary significantly across subfamilies and may reflect epiphytism and nitrogen acquisition strategies. Tillandsioideae seeds fall from dry parental capsules and are often characterized by an elaborate flight apparatus called the coma. This coma, composed of coma hairs, serves as the seed testa and is a diagnostic feature for this subfamily (Benzing, 2000). The length, quantity, and mass of the coma hairs vary significantly across species, impacting buoyancy, dispersal ability, and substrate adhesion, all of which contribute to each species'establishment strategy (Benzing, 2000; Chilpa-Galván et al., 2018; Wester & Zotz, 2011). Species often produce fleshy fruits with seeds coated in mucilage, which facilitates zoochorous dispersal (Benzing, 2000; Silva et al., 2019). This dispersal method, typically via animal scat, may indicate nutrient abundance at the seedling's new location. In line with this, Bromelioideae species follow the classic monocot seedling development pattern, with the root radical emerging first, followed by the hypocotyl and cotyledon, and are thought to depend on soil nutrients in the early stages of establishment (Benzing, 2000; Kowalski et al., 2021). Puyoideae seeds, often falling from three-locule capsule fruits, tend to be ovate, winged, and have fairly robust desiccation tolerance (Caladerón-Hernández & Pérez-Martínez, 2018; Varadarajan & Gilmartin, 1988). This may help with dispersal and potential seed banking (Benzing, 2000).

Several seed traits are shared throughout the family. Bromeliad seeds typically have an anatropous morphology, with a two-layered integument that provides a source of starch for developing seedlings. The embryo occupies roughly one-third of the seed, with the remaining space filled by parental endosperm. The outer layer of the endosperm is made of starchless, cubical cells, while the inner layer is rich in starch and contains thin-walled lenticular grains (Benzing, 2000). Most bromeliad species follow early developmental patterns reminiscent of other monocots, which involves initial imbibition, the protrusion of a root radial, followed by the development of the hypocotyl, cotyledon, and finally true leaves (Tomlinson, 1970). However, certain Tillandsioid species deviate from this and first produce a cotyledonary sheath followed by the primary root, which suggests less early reliance on root tissue for growth (Kowalski et al, 2021). The energy to form these initial organs, whether the root radial or the cotyledonary sheath, comes from the metabolism of the parental endosperm, which is triggered by water imbibition in the seed's earliest days (Benzing, 2000; MacAdams, 2009).

In this study, I aim to investigate whether the epiphytic gradient observed across the four bromeliad species as adults influences seedling growth and their response to two different nitrogen treatments. Specifically, I will examine how nitrogen application alters the allocation of resources between root and shoot tissues in seedlings, inferring investment in roots as a proxy for root functionality. I hope to understand whether the degree of epiphytism–ranging from terrestrial to highly epiphytic species–shapes how seedlings allocate resources to root versus shoot tissues when exposed to varying nitrogen levels.

Puya mirabilis is the most terrestrial of the four species, belonging to the solely terrestrial Puyoideae subfamily (formerly Pitcairnioideae, now placed in molecular phylogenies as sister to the Bromelioideae), lacking absorptive trichomes and phytotelmata (Givnish et al., 2014). Native to Bolivia and Argentina, this species forms large grassy clumps and typically grows to about 30 cm tall (GBIF Secretariat, 2023). Its seeds emerge from dry capsules (Smith & Downs, 1974). Due to its reliance on soil for nutrients, *P. mirabilis* is expected to show the highest levels of plasticity in root and shoot growth when exposed to the different nitrogen treatments, with increased root elongation and shoot plasticity as it adjusts its resource allocation in response to the increased availability of nitrogen.

Vriesea rafaelii, the most epiphytic species in this study, belongs to the subfamily Tillandsioideae. Endemic to the Brazilian Atlantic Rainforest, *V. rafaelii* has a distinctive funnel-shaped rosette with wide, overlapping leaves creating a large phytotelmata. This species is characterized by slow growth and minimal root investment, which allows it to acquire nitrogen from atmospheric sources and organic debris rather than soil. Because of this, I expect *V. rafaelii* to exhibit minimal plasticity in root growth when exposed to varying nitrogen levels, as its root system is less involved in nitrogen acquisition, and minimal plasticity in shoot growth due to its slow life history strategy. The seeds emerge from a dry capsule and have plumose-like coma hair appendages (Benzing, 1980).

Billbergia brasiliensis and *Aechmea recurvata* belong to the subfamily Bromelioideae, which includes species with flexible epiphytic strategies. Positioned between *V. rafaelii* and *P. mirabilis* on the epiphytic gradient, these species are capable of growing in both epiphytic and terrestrial conditions. *B. brasiliensis* is native to eastern Bolivia and Brazil, characterized by several banded, succulent, silvery leaves that form a tubular shape and reach about 24 inches in length (GBIF Secretariat, 2023). Its inflorescence has large pink bracts, white ovaries, and purple petals that recoil (Tropiflora). The seeds, around 5 mm in size, emerge from orange to brown trigonous seed pods in orange mucilage. *A. recurvata*, found in southeastern Brazil, Uruguay, &

Paraguay, has a rosette of stiff & succulent lime-green leaves. Its seeds emerge from a shrimp-shaped black berry covered in purple mucilage. These species are expected to demonstrate intermediate responses to nitrogen treatment, reflecting their mixed epiphytic habits.

This study will explore how varying levels of epiphytism impact root functionality and nitrogen sensitivity at the seedling stage by examining the response of these species to nitrogen treatments. Nitrogen is a crucial macronutrient for plants and plays a key role in building biomass through the synthesis of amino acids and proteins, which are essential for growth and development. I expect that the more epiphytic species, such as *V. rafaelii*, will exhibit less sensitivity to nitrogen, relying less on root growth for nitrogen acquisition, and show minimal plasticity in shoot growth due to its slower and more conservative growth strategy. In contrast, the more terrestrial species *P. mirabilis* is expected to show highest sensitivity to nitrogen, with its roots being solely responsible for nitrogen uptake. As a species that relies heavily on soil for nutrients, *P. mirabilis* is expected to exhibit considerable plasticity in its root system, elongating in response to nitrogen deprivation to maximize nutrient absorption. Species with intermediate epiphytic traits, such as *Aechmea recurvata* and *Billbergia brasiliensis*, are expected to demonstrate a mix of these responses. These species are able to grow in both epiphytic and terrestrial conditions, and while they still rely somewhat on their roots for nitrogen uptake, they also employ epiphytic strategies to acquire nutrients. I therefore expect their response to nitrogen to be less pronounced than *P. mirabilis* but greater than *V. rafaelii*, reflecting their flexible nutrient acquisition strategies.

METHODOLOGY

Seed selection: I germinated 2160 bromeliad seeds in total, with 270 seeds per each of the four species, per two trials. Trial 1 began on 07-16-2024, and data collection continued until 07-30-2024, with seedlings transplanted into sterile soil on 08-02-2024. Trial 2 started on 08-27-2024, with data collection running through 09-20-2024, and seedlings transplanted into sterile soil on 10-05-2024.

Before the main trials, I conducted a qualitative "Trial 0" to assess seed viability and determine the ideal seed age for each species. This trial also helped ensure sufficient germination success under my experimental conditions and evaluated whether seeds from fruits of different ages in *Aechmea recruvata* and *Billbergia brasiliensis* had differential viability. *Puya mirabilis* and *Vriesea rafaelii* did not have viable fruits of varying ages at the time. Based on my qualitative observations, I found that *A. recurvata* fruits aged 6-8 months and *B. brasiliensis* fruits around 12 months old produced the highest germination rates, and fruits one year older than those had low rates. Additionally, I observed that seeds from *V. rafaelii* with brittle coma hairs, likely because of previous drying, had no germination success.

Figure 1: Examples of *Aechmea recurvata* individual 095Ba a) intact inflorescence b) fruits and removed inflorescence and c) seeds.

Aechmea recurvata: I selected seeds from plump black fruits (6 to 8 months old) from individual 095Ba for both trials, seen in **Figure 1**. The inflorescence bracts still exhibited green to red coloration, and the inflorescence had not yet senesced. The fruits were easy to remove, and the cone-shaped seeds (approximately 2 mm long and 1 by 1 mm wide at the base) were coated in purple mucilage. This seed and fruit selection was informed by my qualitative Trial 0.

Billbergia brasiliensis: I collected seeds from individuals 119 and 124 in Trial 1, and 016 in Trial 2. The selected fruits were around 12 months old, plump, and ranged in color from light orange to darker brown. **Figure 2** depicts viable *B. brasiliensis* fruits and seeds from individual 112, which were tested in Trial 0. The cone-shaped seeds (approximately 4 mm long and 2 by 2 mm wide at the base) were coated in an orange mucilage.

Figure 2: *B. brasiliensis* with plump, lightly orange colored fruits that are between six and 12 months old. Seeds are covered in an orange mucilage.

Puya mirabilis: Seeds fell from dry, open capsules with three locules, seen in **Figure 3**. Small mesh bags were placed around capsules to catch viable seeds once they fell. I selected seeds from individual 026 for both trials, as this individual had healthy vegetation and an abundance of seeds that successfully germinated in Trial 0. The seeds were flat, winged, and triangular (approximately 4 mm by 2 mm).

Figure 3: *Puya mirabilis* individual 026 reproductive structure

Figure 4: *V. rafaelii* seeds from the capsule and from bagged storage that are ideal to use with 'fluffy' & not brittle coma hairs.

Vriesea rafaelii: Since the capsules on mature plants, as seen in **Figure 4** were not yet open, I used seeds from individuals 020 in Trial 1 and 060 in Trial 2, both of which were collected in July 2023. Germination success seemed to rely more on how "fluffy" the coma hairs were, as noted in my qualitative Trial 0. Seeds had been stored in dark, dry conditions at room temperature in paper bags. The coma hairs measured around 17 mm, with the seed itself measuring around 4 mm.

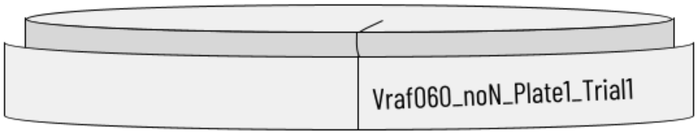

Figure 5: Side view of an example petri dish with label to the right of drawn line.

<u>Seed sterilization and planting</u>: For Trial 1, I prepared 135 *Fisherbrand Petri Dish, Slippable Lid 100mm x 15mm* ™. Each treatment per species had 10 plates, with 15 empty plates (5 for each treatment) used as controls for mass measurements. For Trial 2, I prepared 135 dishes, 105 *Fisherbrand Petri Dish, Slippable Lid 100mm x 15mm* ™ and 30 *Kord-Valmark 100 x 25mm Slippable STERILE Extra Deep Dish Petri Dish with ISO Mark, 340/CS* for *Billbergia brasiliensis*, as seedlings outgrew the smaller dishes by Day 17 in Trial 1.In both trials, I marked the top, side, and bottom of each dish with a permanent marker to help orient the plates and keep track of individual seeds, as seen in **Figure 5**. The plate nomenclature format was:

First-letter-of-genus-first-three-letters-of-specific-epithet-individual-number_Nitrogen-treatment _Plate#_Trial#

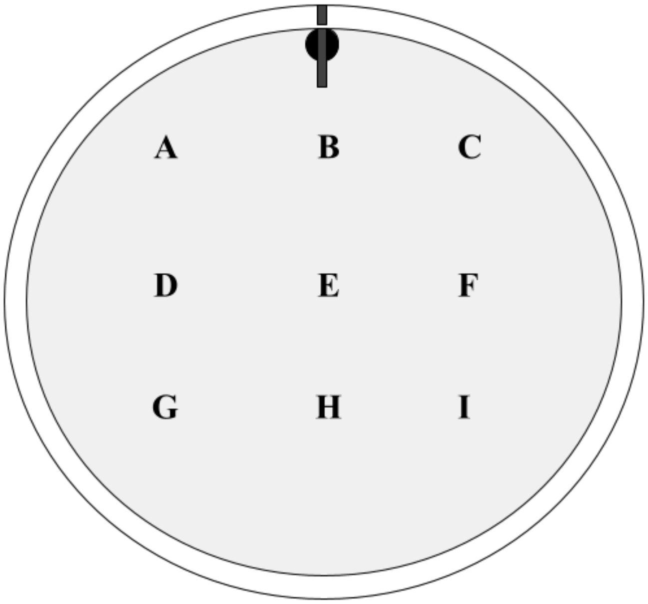

Figure 6: Top view of an example petri dish.

I placed a piece of *Fisherbrand Filter Paper Qualitative P8* ™ into each dish, dotting the edge and aligning it with the pen mark on the dish. Seeds were labeled A through I depending on their placement within the 3x3 grid, as seen in **Figure 6**.

3 mL of appropriate nitrogen treatment (see below) was then pipetted into each dish. This was done before the seeds were placed on them, rather than after, due to issues with static and seeds sticking to the lid when placed on dry filter paper.

To prepare *Aechmea recurvata* and *Billbergia brasiliensis* seeds for sterilization, I cut the fruits open with a razor blade, and placed the plumpest seeds on a *KimTech Science*Brand*™ *Kimwipe*. I used sterile forceps and *Kimwipes*™ to separate individual seeds from any carpel or other fruit tissue. Seeds were patted to remove excess mucilage. Systematic mucilage removal was crucial to reduce the chances of microbial activity and increase the rate of germination, and more was removed during the sterilization process. *P. mirabilis* seeds needed no pre-sterilization preparation. *V. rafaelii* seeds had their coma hairs removed with sterile scissors and forceps, with care taken to not cut any of the seed coat. This was done to reduce microbial activity during germination.

To sterilize the seeds, I synthesized 500 mL of NaOCl by mixing 250 mL DI water with 250 mL ThermoScientific sodium hypochlorite, 5% active chlorine Cat. No: 419550010, Lot: A04478, CAS 7681-52-9 to create a 2.5% NaOCl solution for seed sterilization. I prepared Erlenmeyer flasks with funnels, inside of which I folded *Whatman Filter Papers Qualitative Circles 125mm* into quarters to resemble a coffee filter. The setup can be seen in **Figure 7**. I rinsed seeds with 10 mL 2.5% NaOCl. *B. brasiliensis* and *A. recurvata* seeds were then transferred to a *Whatman Filter Papers Qualitative Circles 125mm* and I removed remaining mucilage, visible with the ‘bleaching’ effect of the NaOCl, with sterile forceps and a *KimTech Science*Brand*™ *Kimwipe*. Once all of the bleach had dripped into the Erlenmeyer flask, I rinsed seeds again with another round of 10 mL 2.5% NaOCl, and removed any remaining mucilage from *B. brasiliensis* and *A. recurvata* seeds in the same manner as before. I then transferred seeds to fresh folded filter papers and rinsed them with two rounds of 10 mL DI water. I placed nine seeds into each plate in a 3 by 3 grid with sterile forceps.

Figure 7: Seed sterilization set up

<u>Nitrogen treatments</u>: Seeds and seedlings were continually fertilized with varying levels of urea as the source of bioavailable nitrogen (no, low, high levels). I used FisherBiotech, NH_2CONH_2, Electrophoresis grade, Lot No. 020975, BP169-500, 500 g, FL-07-09 as my urea source and dissolved 60.062 g Urea in 1L DI water to prepare 1 M Urea stock. To make 5 L of 0 the mM urea treatment (no nitrogen treatment), I prepared 5000 mL DI water. To make 5 L of the 0.01 mM urea treatment (low nitrogen treatment), I mixed 0.05 mL of the 1 M Urea stock with 499.95 mL DI water. To make 5 L of the 0.1 mM urea treatment (high nitrogen treatment), I mixed 0.5 mL of the 1 M Urea stock with 4995 mL DI water.

<u>Seed germination:</u> I set the germination chamber to 12-hour photo periods and temperatures that were 30 °C during the ‘day’ and 25 °C during the ‘night.’ A tray of DI water was placed at the bottom of the chamber for humidity.While there are no germination studies for the four species used in this study, several studies on closely related genera informed my protocols. Rios et al. (2016) successfully germinated *Aechmea constantinii* at temperatures between 25°C and 35°C with air relative humidity around 60%. They also used paper towels to remove mucilage, preventing microbial activity, and employed autoclaved filter paper moistened with DI water as a substrate. Duarte et al. (2018) germinated *Vriesea frigurgensis*, *V. bituminosa*, *V. pardalina*, *Tillandsia gardneri*, and *Racinaea aerisincola* at temperatures between 25°C and 30°C. Maiky & Cláudio (2018) achieved 96% germination success with *Aechmea bambusoides* seeds on Germitest paper using an 8-hour photoperiod and temperature regimes of 20°C and 30°C. Carvalho et al. (2022) washed *Quesnelia quesneliana* seeds with 10% NaOCl and used similar mucilage removal techniques to those I employed. Duarte et al. (2024) germinated *Aechmea nudicaulis*, *Dyckia sacatilis*, *Tacinaea aerisincola*, *Tillandsia gardneri*, *Vriesea bituminosa*, *V. friburgensis*, and *V. pardalina* using 12-hour photoperiods and a constant 25°C temperature. Estevan et al. (2010) successfully germinated *Dyckia pectinata* and *Billbergia zebrina* seeds using a 25°C day/21°C night temperature regime in substrates such as sphagnum, coconut fiber, washed river sand, carbonized rice husks, and sawdust. Gaismov (2024) found optimal germination of *P. mirabilis* at 18-20 °C and 75% to 80% humidity. The sterilization techniques, photoperiods, and temperature regimes used in these studies directly informed my sterilization protocol and the chosen germination chamber settings.

Each species was assigned a separate shelf in the germination chamber, with each treatment (no N, low N, high N) occupying one-third of the shelf. Plates 1-10 within each treatment were randomly arranged in a 2 x 5 grid. Every time I collected data (approximately every 3.5 days), I rotated species up one shelf, and shifted the treatments to the right. This rotation ensured that all species and treatments were exposed to varying positions in the chamber, minimizing any positional growth bias.

I applied the appropriate nitrogen treatment to the dishes every other day. Instead of lifting the petri dish lids, I used a micropipette to dispense treatment into the ‘lip’ of the plate to reduce any microbial exposure. I pipetted 3 mL of treatment on Day 0 and the days on/after imaging & plate mass data collection. This was because the data collection necessitated the plates to be outside of the germination chamber and in the dry lab for several hours. On all other days, I pipetted 2 mL of treatment into each dish.

Data collection: I measured dry plate mass on Day 0, and wet mass on Days 7, 14, and 21 with an analytical balance. The plate served as the unit of replication. I recorded Day 0 dry mass with the sterilized seeds on the dish, but before any treatment was applied. Plates were removed from the germination chamber for wet mass measurements. Due to the dry lab environment, moisture evaporation from the plates was so rapid that there was a noticeable decrease in mass as each plate was weighed. As a result, I suspect that the order in which plates were weighted–which was not deliberately randomized and often ordered by species and treatment–had a greater impact on mass measurements than the actual seed and seedling mass did. These data were not included in this project’s analysis.

I collected organ score data on days 3, 7, 10, 14, 17, and 21, with the seedling serving as the unit of replication. On each specified day, I assigned each seedling an ‘organ score’ based on their progress in organ development. This discrete scoring system tracked seedling progression through various stages of organ development. These data were not included in this project’s analysis. Scoring system: 0: no sign of any germination; 1: seed coat split; 2: presence of a root radical; 3: presence of non-true leaf tissue (i.e. cotyledon and/or hypocotyl); 4: presence of one true leaf; 5: presence of two true leaves; 6+: presence of $\#\, true\ leaves + 3$

I calculated germination percentage on days 3, 7, 10, and 14, with the plate as the unit of replication. It was calculated by dividing the number of seedlings in a plate that had progressed past an organ score of 0 by 9, the total number of seedlings in each plate. Summary statistics of data were included in this project's analysis.

I took plate imaging data on days 7, 14, and 21, with the seedling as the unit of replication. ImageJ 1.54 was used to quantify root and shoot length each week (Schindelin et al., 2012). Images were taken on an iPhone with a measuring tape in the background and then uploaded to ImageJ where a pixel:mm ratio was calculated. Roots and shoots were then traced with the 'segment' feature in ImageJ. Length was then calculated with the 'measure' tool and inputted into Excel. Only these imaging data were used in the project's analysis.

Data analysis: To assess how nitrogen treatment impacted seedling growth (root length, shoot length, and the root-to-shoot ratio) over time for each species, I used a Linear Mixed-Effects Model (LMM). The model accounted for repeated measurements over time and the nested structure of the data, with treatment nested within trial, plate ID nested within treatment, and seed ID nested within plate ID. I ran a separate LMM for each species. My response variables included root length, shoot length, and the root-to-shoot ratio (root length ÷ shoot length) measured at Days 7, 14, and 21. The fixed effects in the model were nitrogen treatment (no N, low N, high N), time (Days 7, 14, 21), and the interaction between the two (treatment:day). Trial number and Plate ID were included as random effects. Seedling ID (i.e., A-I position in each plate) was not included as a random effect, as it added complexity and redundancy without contributing substantially to the observed variation in growth. I determined this through a visual inspection of the model residuals and by examining the variance components. In future analyses, I plan to conduct more quantitative tests, such as residual analysis or Levene's test, to assess the homogeneity of variance across groups.

Outliers were removed using the Interquartile Range (IQR) method. I did this due to the skewed nature of my data and the resilience of the IQR method to extreme values, particularly since I suspected that some of these outliers were a result of data entry errors rather than true biological variation. The formula for my model was:

$$Value \sim Nitrogen * Day + (1|Trial) + (1|PlateID)$$

I tested the normality of my data distribution with the Shapiro-Wilk test, as a LMM assumes a normal distribution of data. Although my data were non-normal, time constraints made the LMM the most appropriate analysis tool and the alignment of results with my hypothesis supported its use. I used the *lme4* and *lmrTest* packages in R for model fitting. I used pairwise comparisons with a Holm-Bonferroni adjustment applied for my post-hoc testing to explore significant differences between nitrogen treatments at each time point. I used the *emmeans* package in R for this analysis.

RESULTS

I had several questions about how nitrogen impacts early bromeliad seedling growth and development. The first focuses on treatment-based developmental differences within a particular species, but not across species. Growth is continuous, and therefore we must not only look at what is happening **at** time points, but the growth that happens **between** them as well. I did this with a linear mixed effect model, and used the following question and subquestions as a framework.

How does nitrogen treatment impact the growth of root length, shoot length, and root-to-shoot ratio between time points within each species?

a) How do low and high nitrogen treatments impact the growth of root length and shoot length between time points (days 7, 14, 21) compared to the no N treatment? Does the magnitude and/or significance of these change over time?
b) How do shifts in organ investment between time points change based on nitrogen treatment?

Germination Results

On Day 7, the mean percentage of *Aechmea recurvata* germination was 98.1% in both Trials 1 and 2. *Billbergia brasiliensis* had 73.8% seeds germinate by this time in Trial 1, and 98.1% germinate by this time in Trial 2. *Puya mirabilis* saw 98.9% germination by Day 7 in both Trials 1 and 2. *Vriesea rafaelii* had 72.2% germination by Day 7 in Trial 1, but only 33.3% germination by this time in Trial 2.

***Aechmea recurvata* LMM**

Root Length

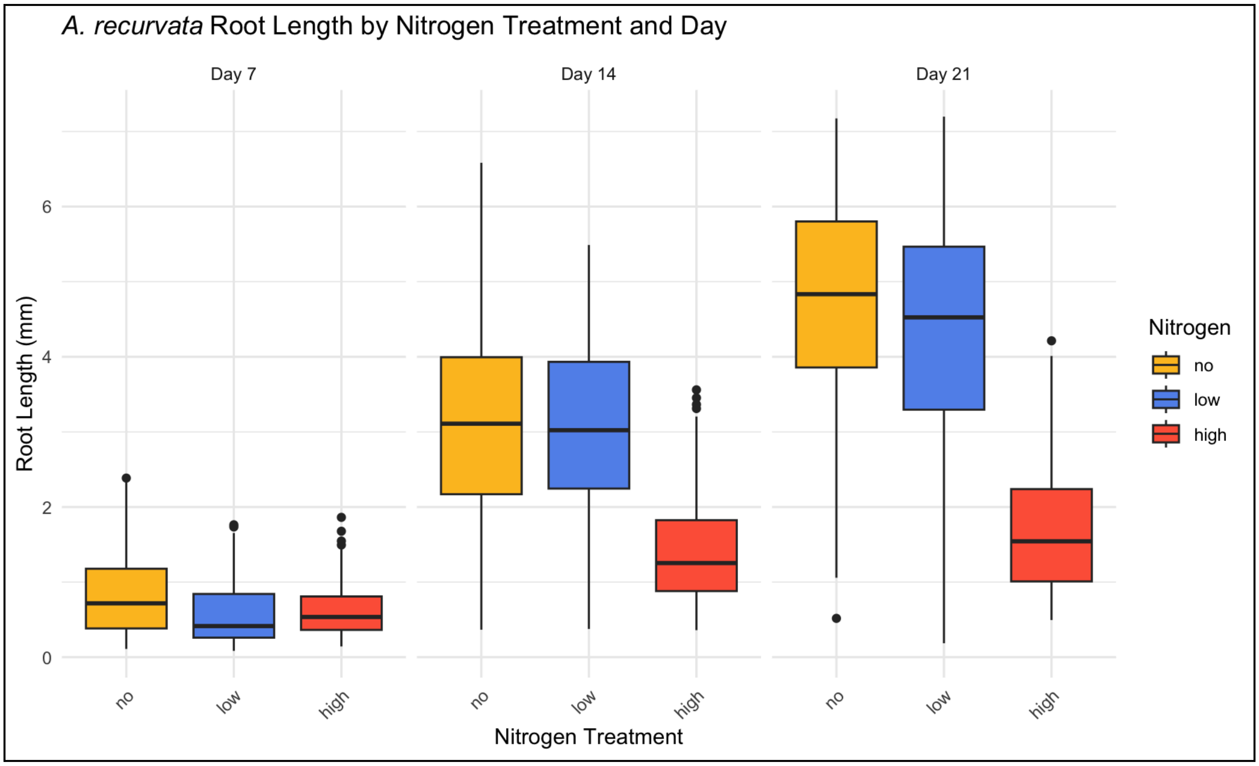

Figure 8: *A. recurvata* root lengths under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

Figure 8 shows how high nitrogen significantly reduces root growth at later time points. On Day 14, seedlings under high nitrogen show a significant reduction in root growth compared to those receiving no nitrogen, with an estimate of **-1.4168** (p-value = **3.18e-16**). This negative effect is even stronger on Day 21 with an estimate of **-2.7141** (p-value < **2e-16**), confirming that high nitrogen suppresses root growth over time. The estimates and p-values indicate that the negative impact of high nitrogen on root growth becomes more pronounced and significant as time progresses. In contrast, the low nitrogen treatment did not significantly alter root growth for *A. recurvata* compared to the no nitrogen treatment.

The day effects show that seedlings under all treatments experience significant root growth over time. Between Day 7 and Day 14, root growth increases significantly, with an estimate of **1.2028** (p-value < **2e-16**), and by Day 21, growth remains significantly greater than Day 7 with an estimate of **3.9818** (p-value < **2e-16**). This shows a steady increase in root growth across all treatment groups as seedlings develop.

Shoot Length

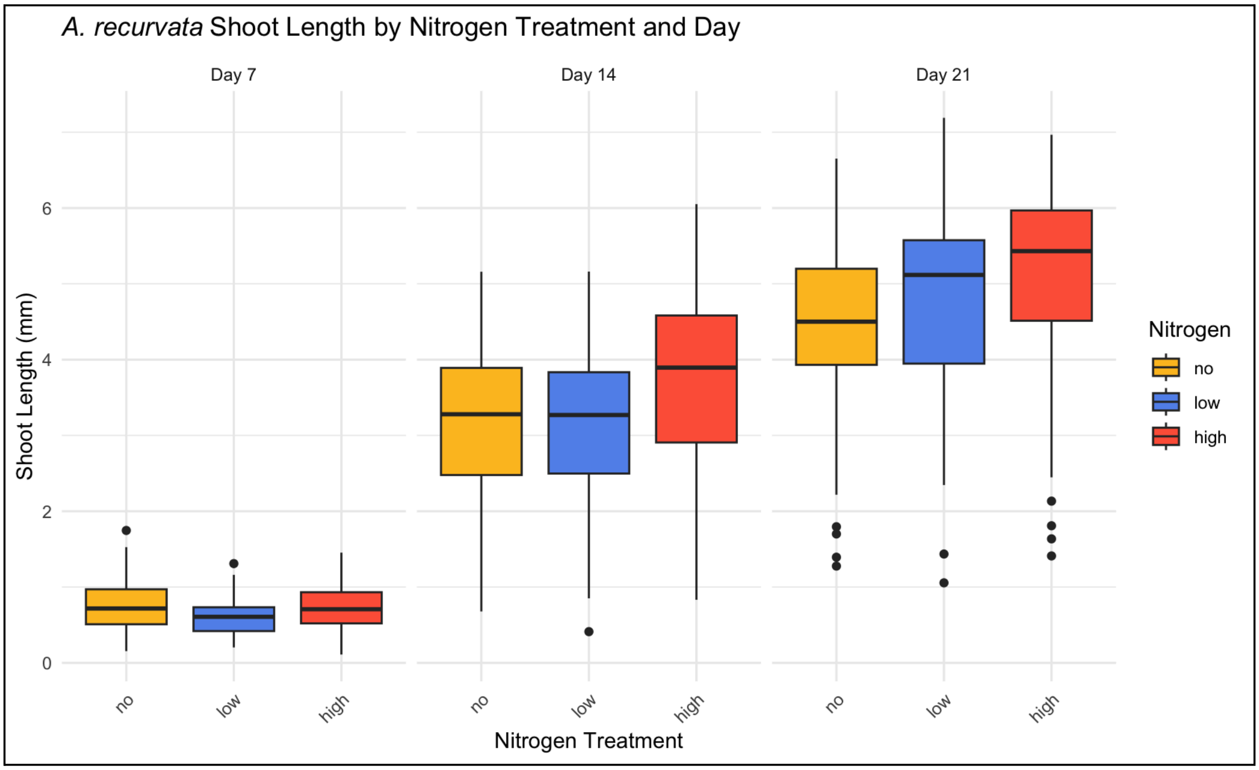

Figure 9: *A. recurvata* shoot lengths under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

Figure 9 illustrates that high nitrogen significantly promotes shoot growth at later time points. On Day 14, seedlings under high nitrogen have a significantly increased shoot length, with an estimate of **0.61375** (p-value = **0.000341**), and this effect continues on Day 21 with an estimate of **0.69448** (p-value = **0.000499**). The increasing magnitude of the estimates indicates that high nitrogen's positive impact on shoot growth strengthens over time, and consistently significant p-values across time points suggest that this promotion remains robust. Low nitrogen had no significant impact on shoot length.

The day effects show that seedlings, regardless of nitrogen treatment, experience significant shoot growth over time. Between Day 7 and Day 14, shoot length increases significantly with an estimate of **2.2806** (p-value < **2e-16**), and by Day 21, the growth continues with an estimate of **3.8011** (p-value < **2e-16**). These effects demonstrate that shoot growth is generally progressing throughout the early stages of development.

Root to Shoot Ratio

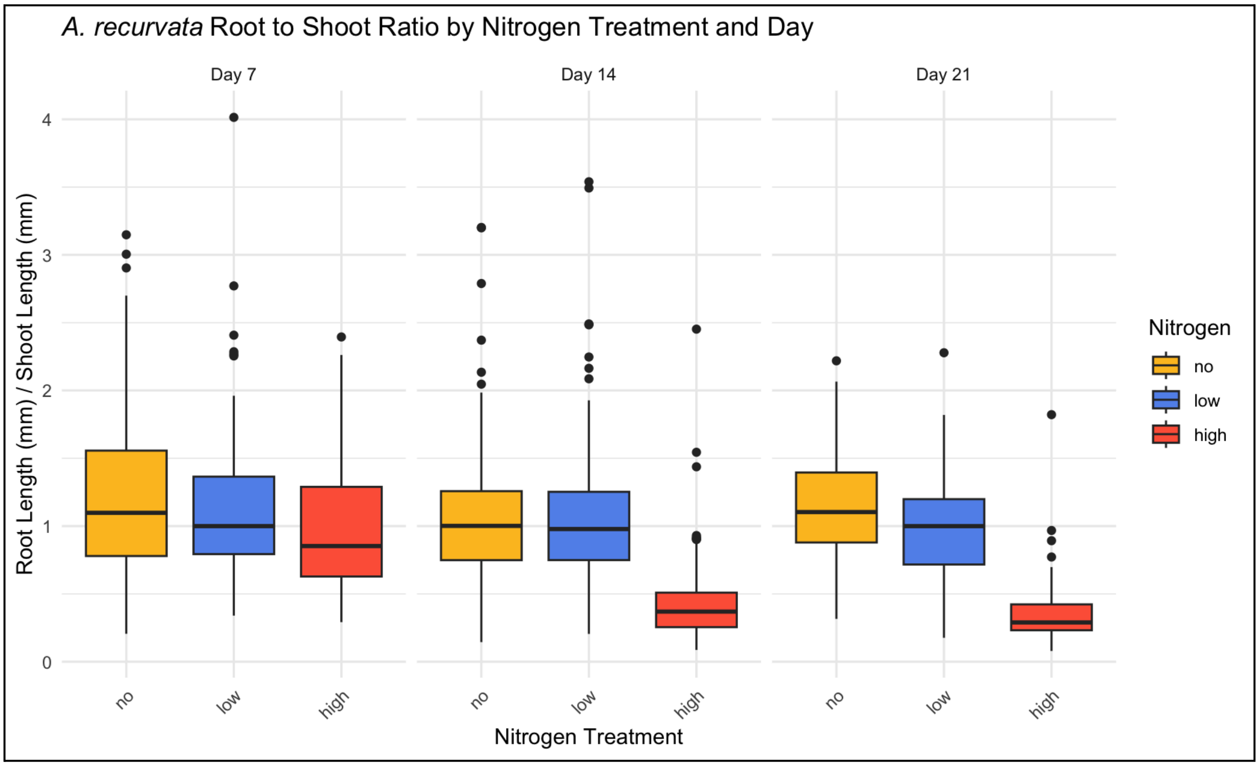

Figure 10: *A. recurvata* root-shoot-ratios under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

High nitrogen significantly reduces the root-to-shoot ratio independent of time, with a fixed effect estimate of **-0.27164** (p-value = **3.09e-05**). However, the effects of high nitrogen become more pronounced with time. On Day 14, high nitrogen has an estimate **-0.34056** (p-value = **2.49e-05**), and on Day 21, the estimate is **-0.50452** (p-value = **7.78e-08**). The estimates and p-values suggest that the reduction in root-to-shoot ratio strengthens over time, indicating a shift of biomass allocation towards shoot tissue under high nitrogen treatment. These trends can be seen in **Figure 10**.

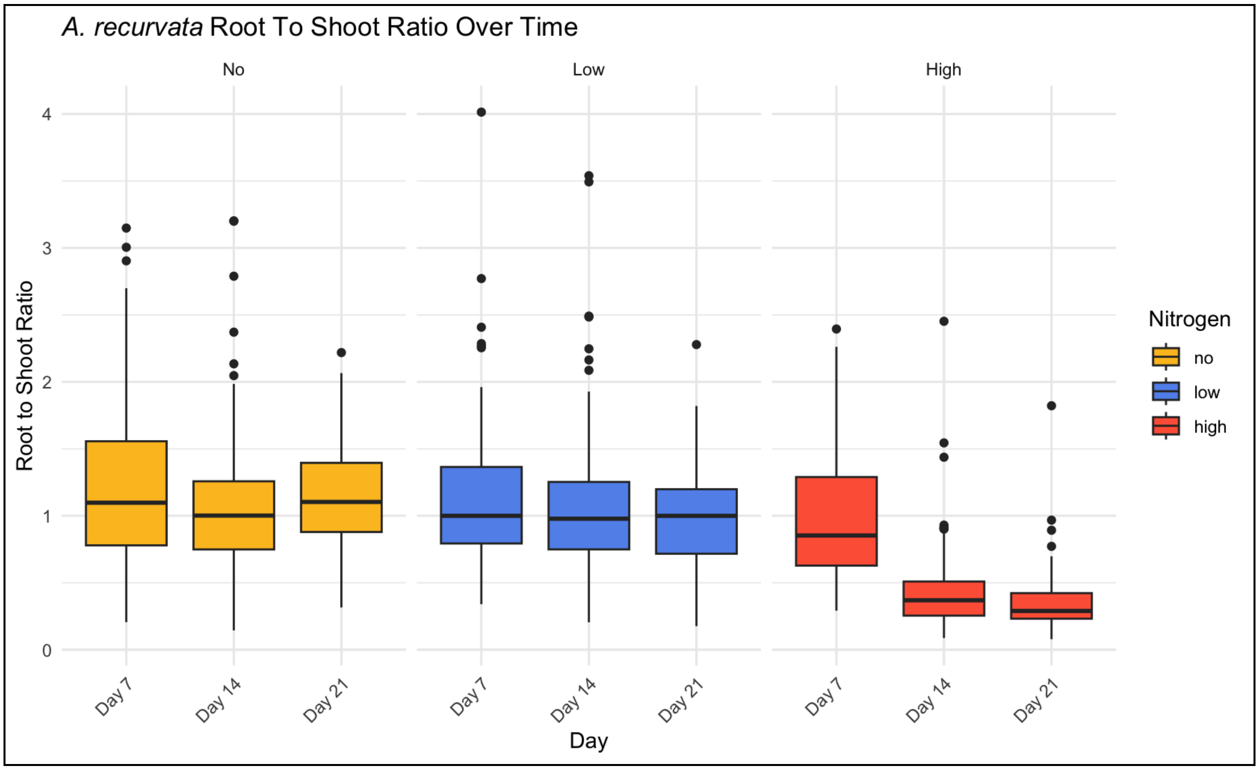

Figure 11: *A. recurvata* root-shoot-ratios under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

The day effects in the LMM, depicted in **Figure 11**, show how *Aechmea recurvata* allocates biomass over time, independent of nitrogen treatment. On average, the root-to-shoot ratio significantly decreases between Day 7 and Day 14, with an estimate of **-0.22654** (p-value = **0.000107**), but remains relatively stable between Days 14 and 21. This suggests that in the second week of growth, *A. recurvata* seedlings invest more in their root systems relative to their shoots compared to the first week of growth, and this pattern stabilizes by Day 21 and is no longer significant. However, seedlings under high nitrogen deviate from this trend, showing a more pronounced reduction in the root-to-shoot ratio over time.

***Billbergia brasiliensis* LMM**

Root Length

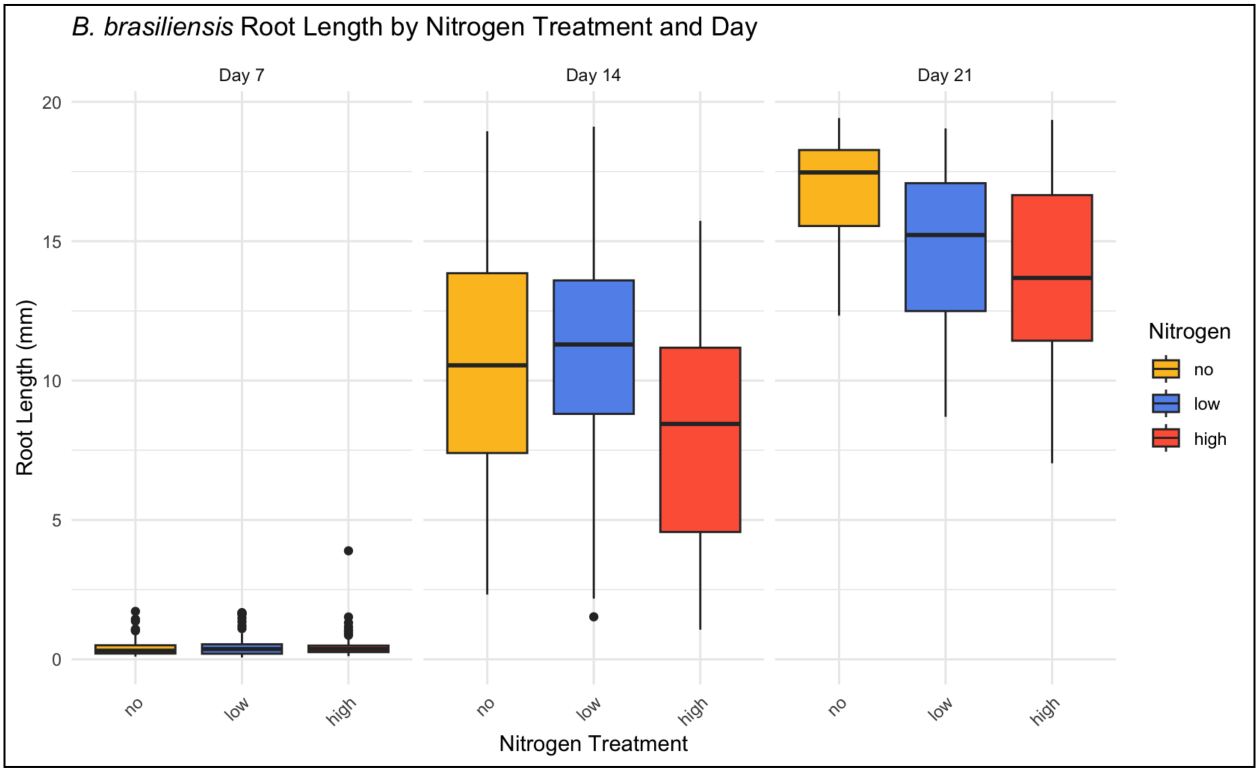

Figure 12: *B. brasiliensis* root lengths under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

High nitrogen significantly stunts root growth in *B. brasiliensis* seedlings, with the effect becoming more pronounced and significant over time, as seen in **Figure 12**. On Day 14, the estimate is **-2.5104** (p-value = **2.48e-05**), and on Day 21, the estimate is **-3.8487** (p-value = **1.84e-06**). This shows that root growth between both Days 7 & 14 and Days 14 & 21 is significantly reduced under high treatment compared to seedlings receiving no nitrogen. In contrast, there is no difference in root growth between seedlings receiving low nitrogen and those reveiving no nitrogen.

The model's day effects indicate that seedlings experience significant root growth over time, regardless of nitrogen treatment. Between Day 7 and Day 14, root growth increases significantly, with an estimate of **9.8613** (p-value **< 2e-16**), and between Day 14 and Day 21, growth continues with an estimate of **18.4094** (p-value **< 2e-16**). This shows a steady increase in root growth across all treatment groups as seedlings develop.

Shoot Length

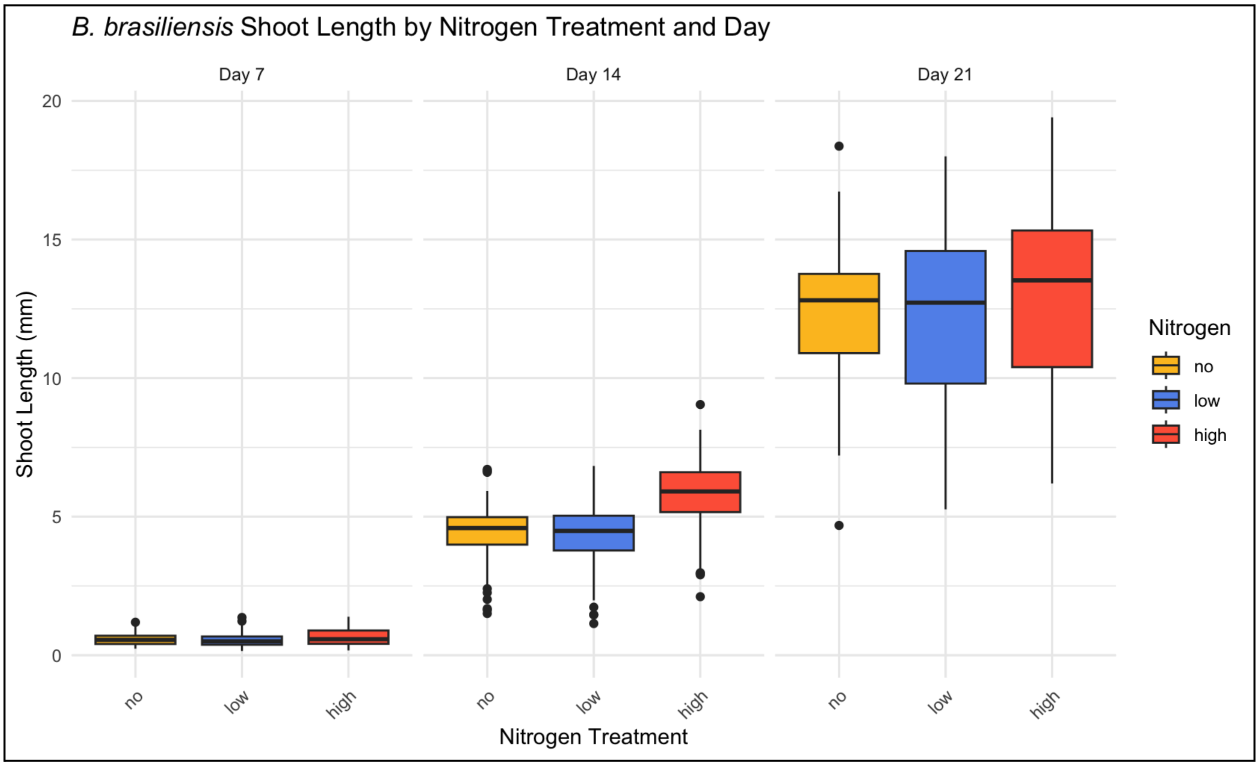

Figure 13: *B. brasiliensis* shoot lengths under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

High nitrogen similarly promotes shoot growth in *Billbergia brasiliensis* seedlings, with the effect becoming more pronounced over time, as illustrated in **Figure 13**. On Day 14, seedlings under high nitrogen show a substantial increase in shoot length, with an estimate of **1.3612** (p-value = **0.0002**). The positive impact continues between Days 14 & 21, with an estimate of **1.1806** (p-value = **0.0045**), indicating that while the positive impact of high nitrogen remains strong, it becomes slightly less pronounced by Day 21. The p-values indicate that this promotion remains robust across both time points, with a consistent significant effect on shoot growth.

The day effects indicate that seedlings under all treatments experience significant shoot growth over time. Between Day 7 & Day 14, shoot length increases substantially, with an estimate of **3.8647** (p-value < **2e-16**), and growth continues through Day 21 with an estimate of **11.8492** (p-value < **2e-16**). This shows a steady, significant increase in shoot length across all treatments, with shoot growth accelerating as seedlings develop.

Root to Shoot Ratio

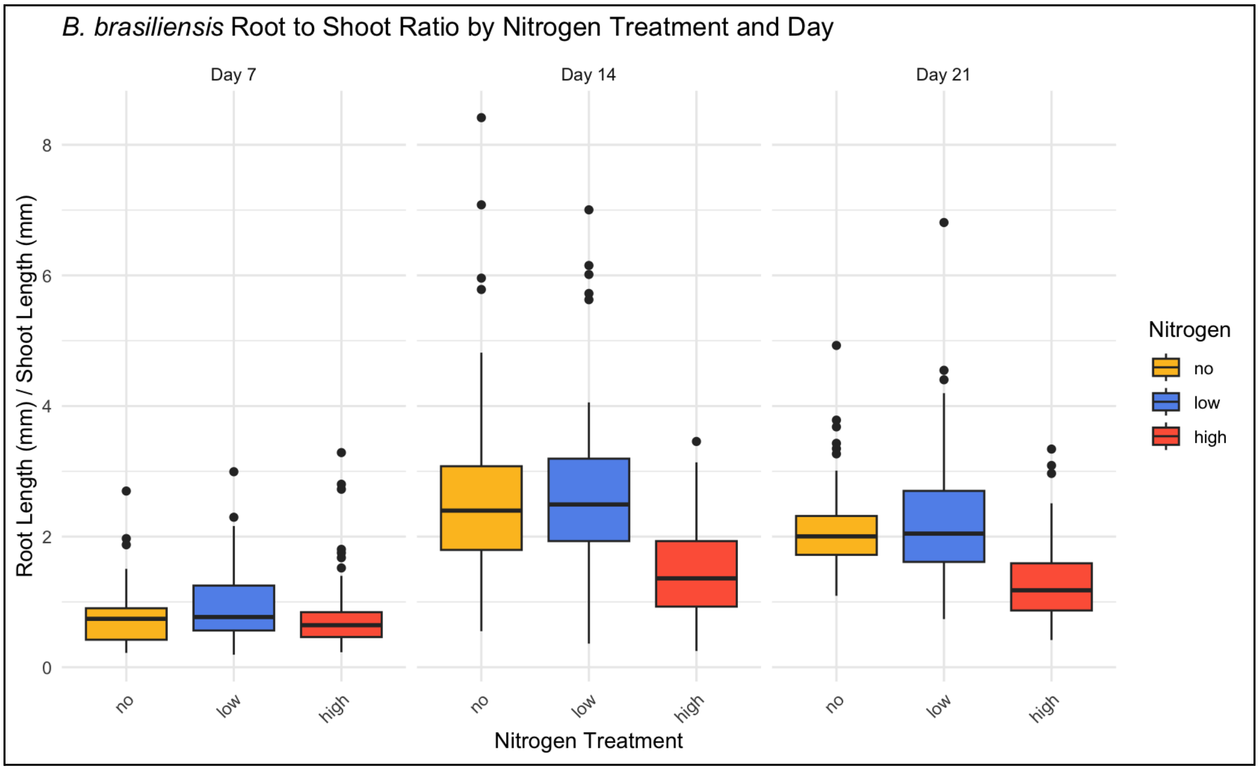

Figure 14: *B. brasiliensis* root-to-shoot ratios under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

High nitrogen significantly reduces the root-to-shoot ratio over time, with an effect that becomes more pronounced as seedlings develop. These trends can be seen in **Figure 14**. On Day 14, high nitrogen has an estimate of **-1.08830** (p-value = **1.87e-10**), indicating a strong reduction in the root-to-shoot ratio compared to the ratio of seedlings receiving no nitrogen. By Day 21, the estimate is **-0.81300** (p-value = **1.49e-05**), suggesting that while the effect of high nitrogen remains significant, the magnitude of reduction is slightly less pronounced than on Day 14. These results indicate that high nitrogen promotes a shift in biomass allocation toward shoot growth, particularly early in development. In contrast, low nitrogen does not significantly impact the root-to-shoot ratio at either time point, with estimates of **-0.02511** (p-value = **0.886**) on Day 14 and **0.02639** (p-value = **0.891**) on Day 21.

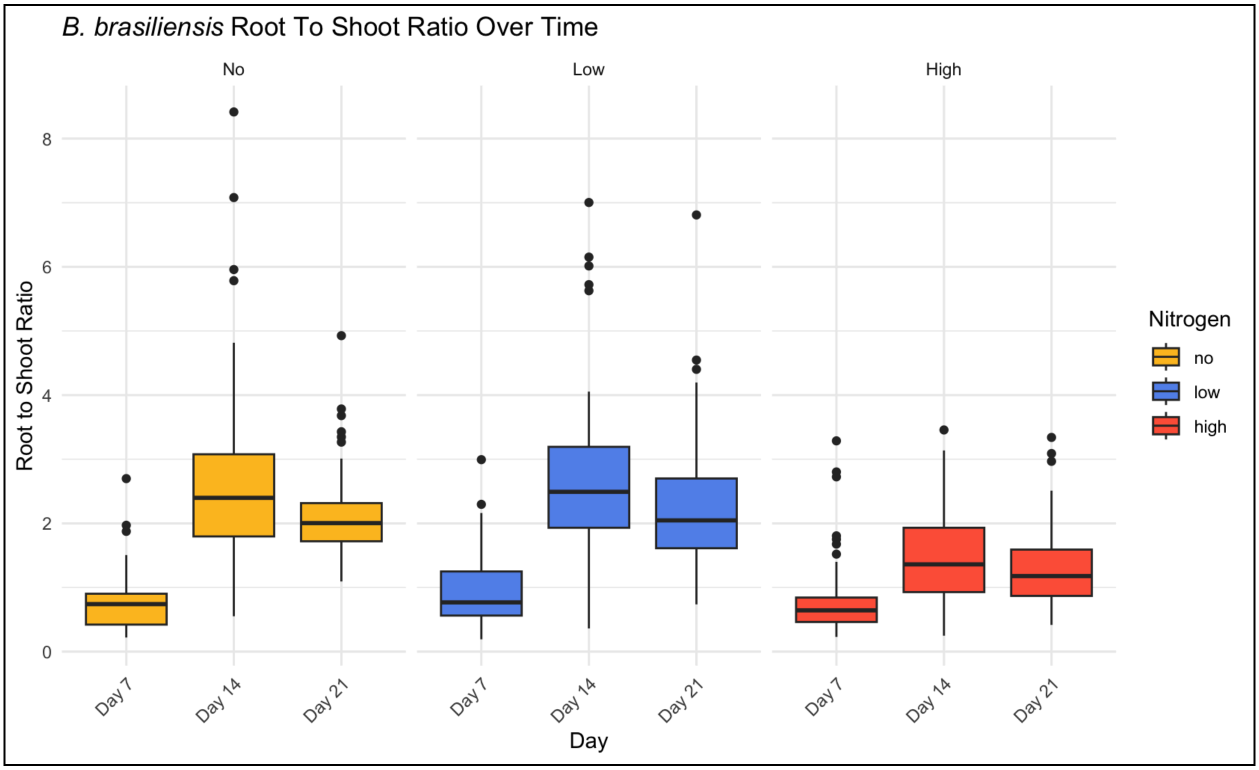

Figure 15: *B. brasiliensis* root-to-shoot ratios under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

The day effects in the LMM show how *Billbergia brasiliensis* allocates biomass over time, independent of nitrogen treatment and are illustrated in **Figure 15**. The root-to-shoot ratio significantly increases from Day 7 to Day 14 (estimate = **1.72956**, p-value < **2e-16**), and remains high on Day 21, although slightly lower than on Day 14 with an estimate of **1.31653**. (p-value < **2e-16**). This suggests a strong developmental shift toward shoot investment. However, seedlings under high nitrogen deviate from this pattern, showing a substantial reduction in root-to-shoot ratio, particularly on Day 14. This could mean that while *B. brasiliensis* seedlings naturally invest more in shoot growth as they develop, high nitrogen accelerates this shift, with the strongest effect observed during early seedling growth.

Puya mirabilis **LMM**

Root Length

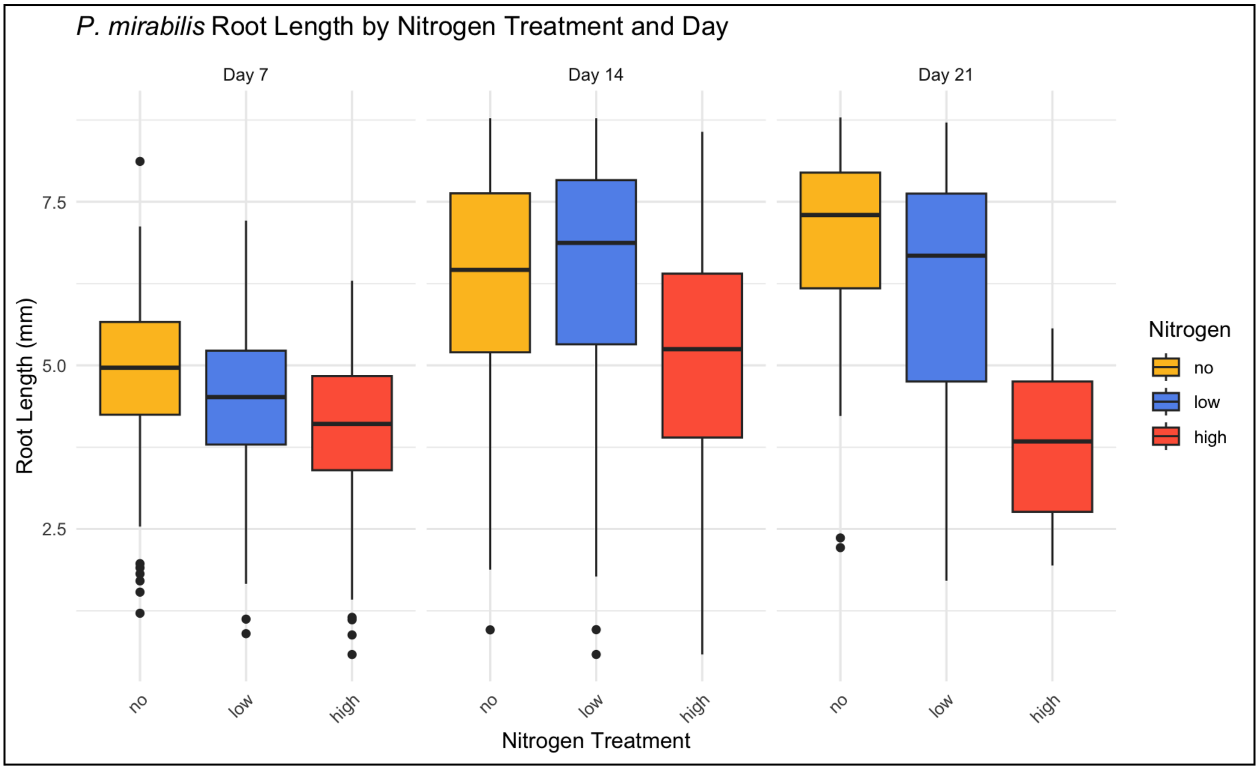

Figure 16: *P. mirabilis* root lengths under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

Independent of time points, both low and high nitrogen treatments suppress *Puya mirabilis* seedling root growth. The estimate for low nitrogen is **-0.45245** (p-value = **0.0027**), and the estimate for high nitrogen is **-0.83517** (p-value = **4.12e-08**). High nitrogen therefore has a greater magnitude and significance of impact. Roots also grow significantly over time, with a Day 14 estimate of **1.56428** (p-value < **2e-16**) and a Day 21 estimate of **2.00831** (p-value = **1.82e-11**).

Under low nitrogen, root length increases at Day 14 (estimate = **0.54604**; p-value = **0.02068**), which is a pattern inconsistent with the overall pattern of root length suppression with nitrogen treatment. However, this effect disappears by Day 21, as there is no significant difference in root growth compared to seedlings receiving no nitrogen.

Under high nitrogen, root length is suppressed both at Day 14 (estimate = **-0.48084**; p-value = **0.03265**) and Day 21 (estimate = **-1.68585**; p-value = **0.00372**). The suppression effect of high nitrogen is seen to increase in significance and magnitude as time progresses. These trends can be seen in **Figure 16**.

Shoot Length

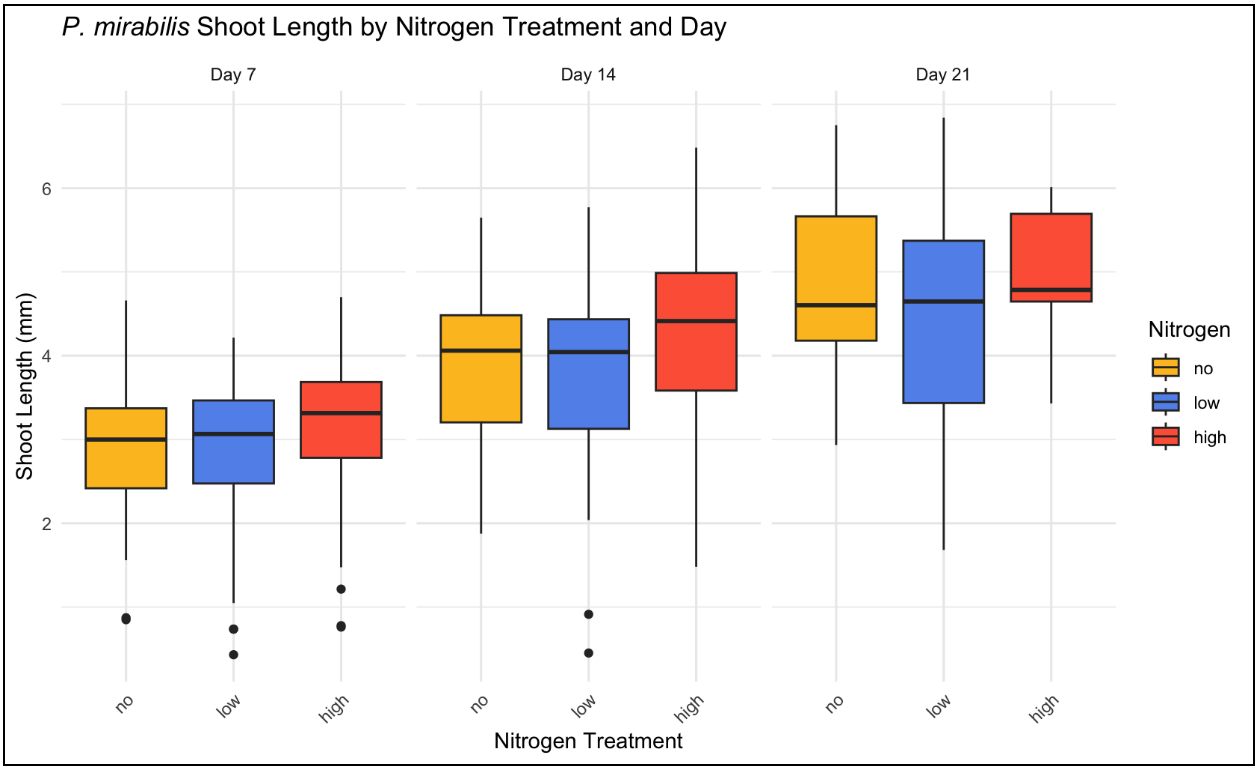

Figure 17: *P. mirabilis* shoot lengths under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

Independent of time point, high nitrogen increases *P. mirabilis* shoot length (estimate = **0.30647**; p-value = **0.000376**) while low nitrogen has no significant effect, visually depicted in **Figure 17**. Shoot length also significantly increases by Days 14 (estimate = **0.97724**; p-value **< 2e-16**) and 21 (estimate = **2.18351**; p-value **< 2e-16**).

While high nitrogen does not significantly affect shoot length at either of the later time points, low nitrogen reduces shoot length by Day 21 (estimate = **-0.41195**; p-value = **0.026574**). This indicates that high nitrogen promotes shoot growth early on, while low nitrogen induces shoot suppression by Day 21.

Root to Shoot Ratio

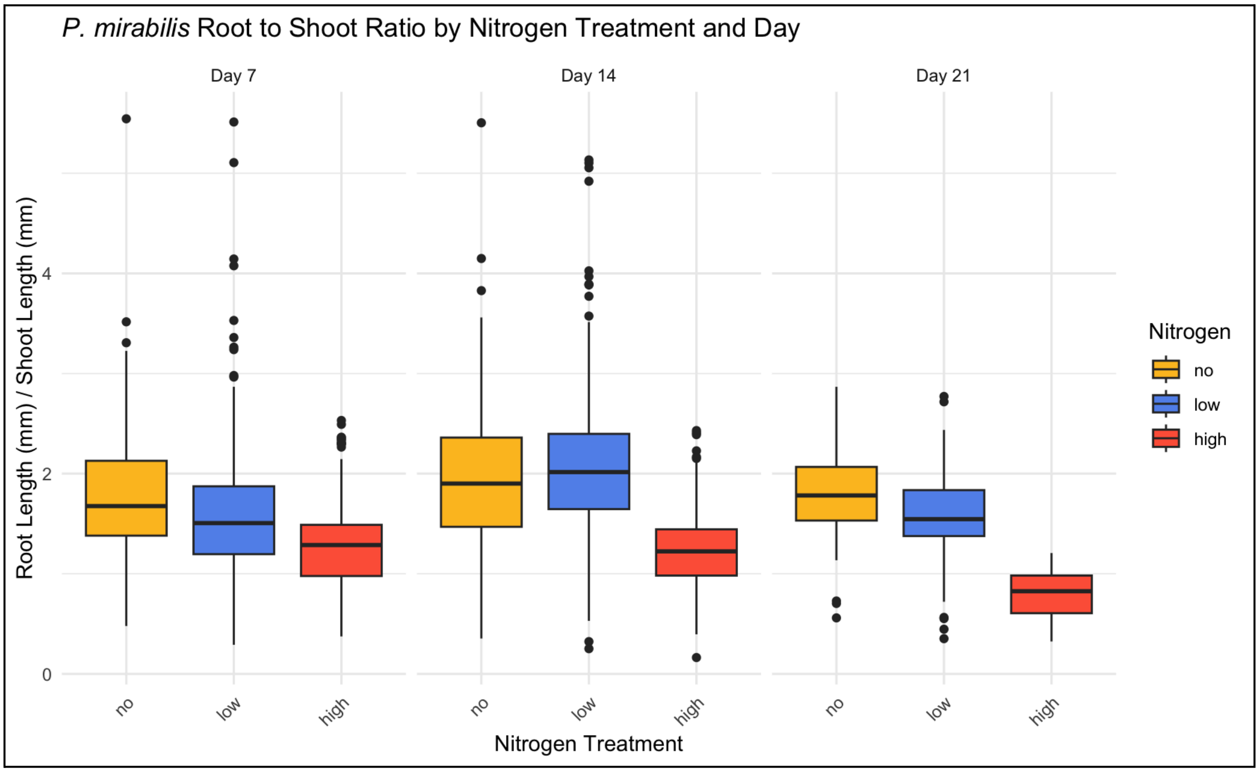

Figure 18: *P. mirabilis* root-to-shoot ratios under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

Under low nitrogen, the root-to-shoot ratio increases at Day 14 (estimate = **2.535e-01**; p-value = **0.00694**), indicating a shift towards root investment. Under high nitrogen, the root-to-shoot ratio decreases at Day 14 (estimate = **-0.2772**; p-value = **0.00330**) and Day 21 (estimate = **0.461**; p-value = **0.04972**). Independent of nitrogen treatment, high nitrogen strongly decreases the root-to-shoot ratio with an estimate of **-0.4526** (p-value = **1.58e-11**), indicating a stronger shift towards shoot tissue in seedlings receiving high nitrogen treatment. These trends can be seen in **Figure 18**.

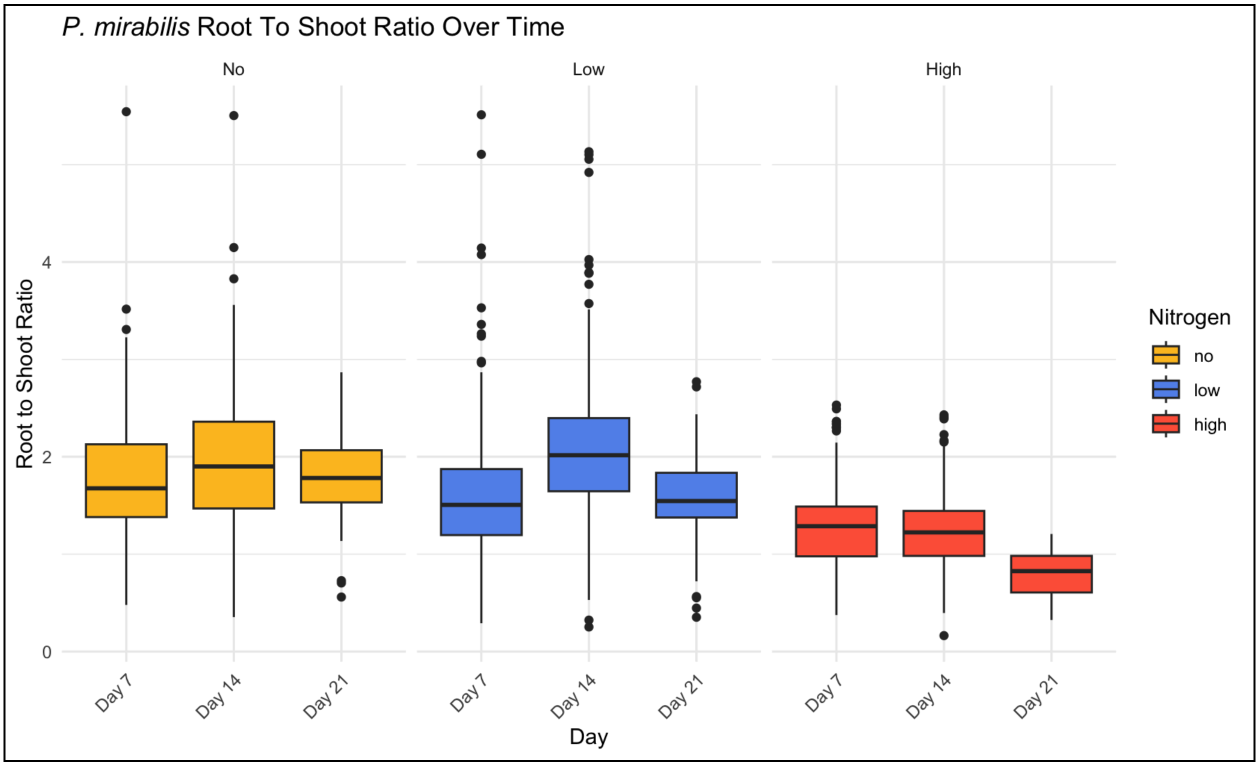

Figure 19: *P. mirabilis* root-to-shoot ratios under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

The root-to-shoot ratio increases slightly at Day 14 (estimate = **0.2083**; p-value = **0.00172**), but there is no significant difference between the root-to-shoot ratio between Days 7 & 21, as depicted in **Figure 19**. This shows a greater overall relative investment in root tissue at Day 14 followed by a return to the Day 7 root-to-shoot ratio.

***Vriesea rafaelii* LMM**

Root Length

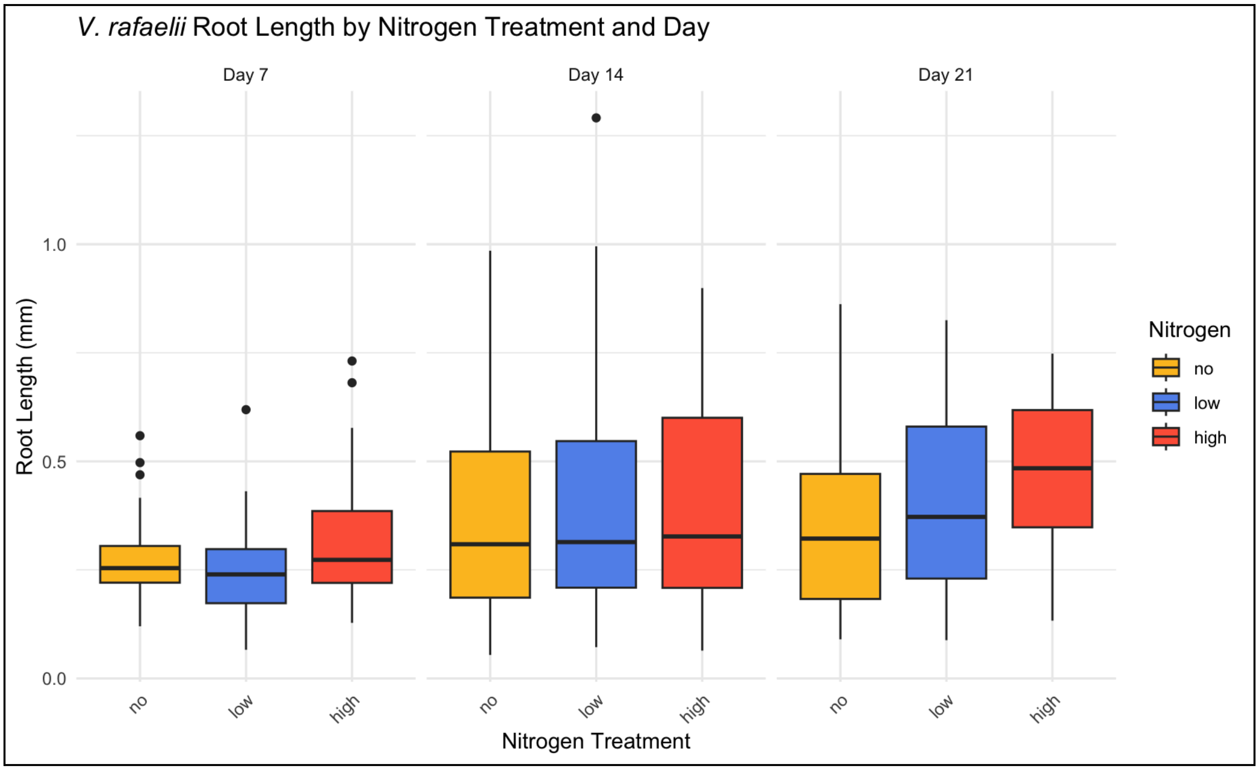

Figure 20: *V. rafaelii* root lengths under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

The day effects for *Vriesea rafaelii* suggest that root length significantly increases over time. Day 14 has an estimate of **0.0982** (p-value = **0.0035**), and Day 21 has an estimate of **0.0789** (p-value = **0.0426**). Nitrogen has no significant effect on root growth by itself or interacting with time. These trends are seen in **Figure 20**.

Shoot Length

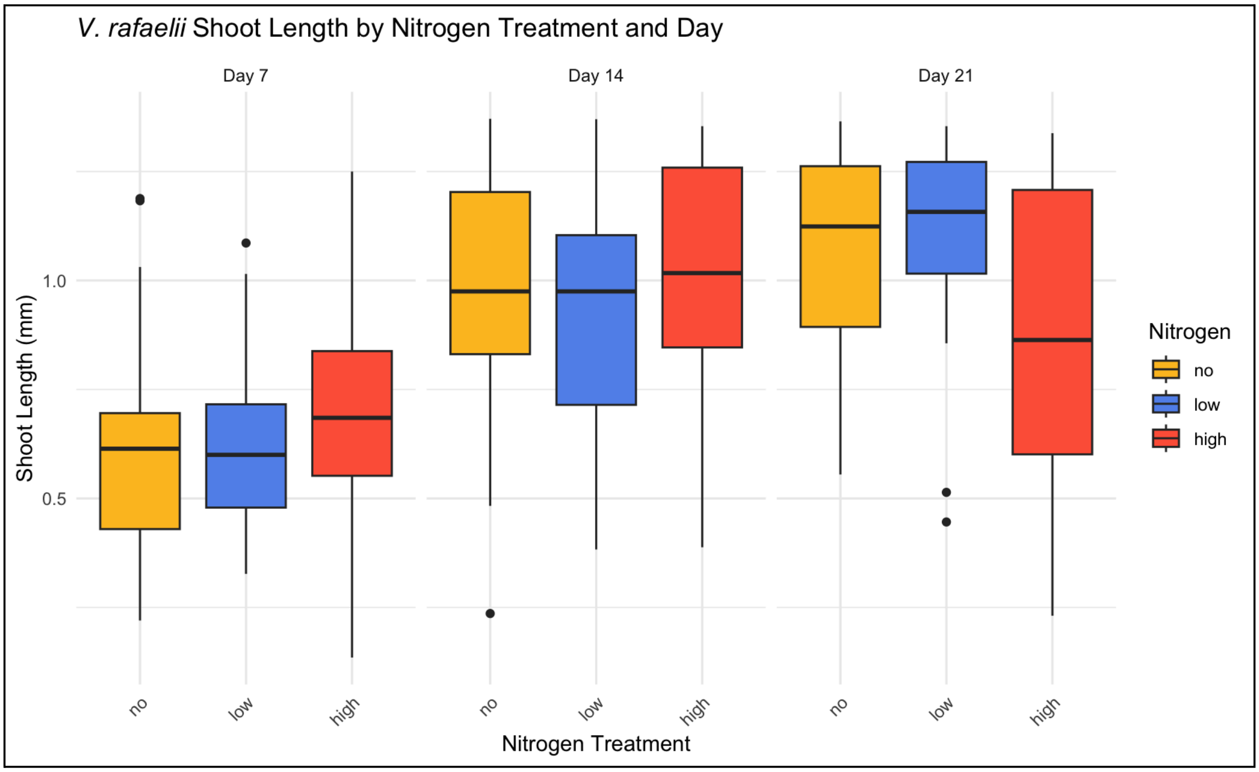

Figure 21: *V. rafaelii* shoot lengths under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

Similarly to root length growth in *Vriesea rafaelii*, the only significant effect on shoot length growth is time by itself. At Day 14, the estimate is **0.6771** (p-value < **2e-16**) and at Day 21 is **0.8773** (p-value < **2e-16**). There is no significant effect of nitrogen, both independent of and interacting with time. These trends are shown in **Figure 21**.

Root to Shoot Ratio

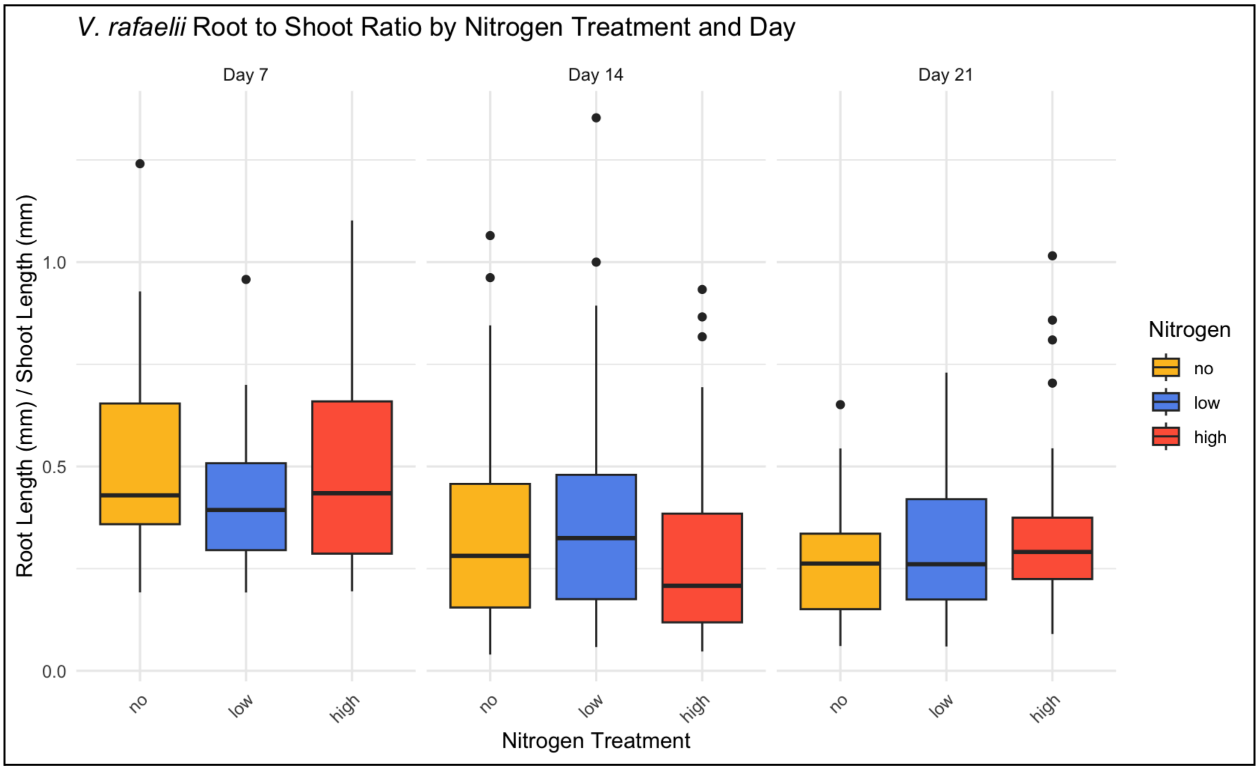

Figure 22: *V. rafaelii* root-to-shoot ratios under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

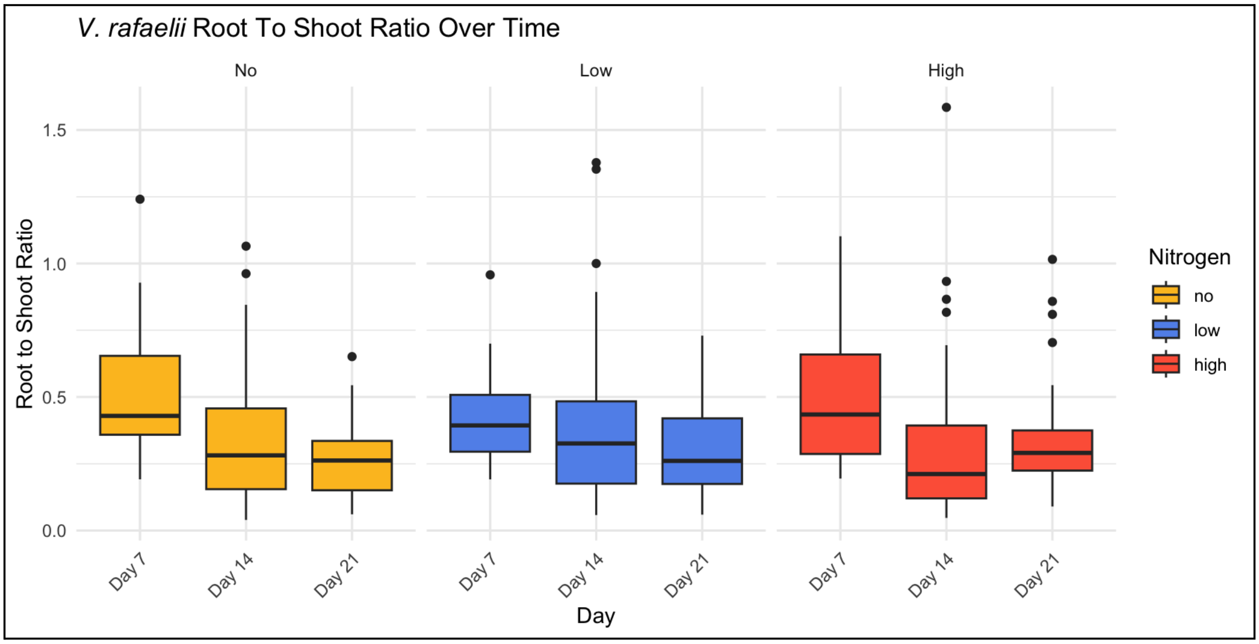

Figure 23: *V. rafaelii* root-to-shoot ratios under no nitrogen, low nitrogen, and high nitrogen at Days 7, 14, and 21.

Day is the only factor that has an impact on the root to shoot ratio of *Vriesea rafaelii*. At Day 14, the estimate is -0.2050 (p-value < 2e-16) and at Day 21 the estimate is -0.2204 (p-value < 2e-16), indicating a general trend towards shoot investment over time for the species. Nitrogen has no effect on the root to shoot ratio independent of time and at each time point. These trends are seen in **Figure 22** and **Figure 23**.

DISCUSSION

These experiments lead to three major conclusions. First, the literature-informed germination protocol led to robust germination (> 50%, often closer to 100%) in all species by Day 14. It is clear, however, that *Vriesea rafaelii* germinated both less reliably and later than the other three species. The lower rates of germination may be due to the seeds' storage for several years while the other species had viable seeds from greenhouse specimens. The slow growth may also reflect *V. rafaelii*'s slow growth as the most epiphytic species.

Second, all nitrogen sensitivity appears to decrease as species become more epiphytic. All species, except the highly epiphytic *Vriesea rafaelii*, responded to high nitrogen by shortening their roots and lengthening their shoots. However, only the fully terrestrial *Puya mirabilis* exhibited differential growth under the low nitrogen treatment, with a response similar to that under high nitrogen, albeit smaller in magnitude. Further examination of *P. mirabilis*' growth at individual time points under low nitrogen reveals an unexpected reversal in this overall trend, resembling a nitrogen scavenging response.

The third major conclusion is that shifts in root-to-shoot investment over time appear to be species-specific, with each species following a distinct pattern in how resources are allocated between root and shoot tissues during early development. While high nitrogen treatment generally shifts investment towards shoot tissue, the root-to-shoot ratio remains consistent with each species' baseline allocation pattern.

I propose that the relationship between decreasing nitrogen sensitivity and increasing epiphytism arises from the highly variable epiphytic environment and resulting conservative growth strategies. While epiphytic pressures are not uniform, it is generally accepted that vascular epiphytes face greater drought and nitrogen stress compared to soil-based terrestrials (Benzing, 1990; Zotz & Hietz, 2001; Zotz, 2016). Due to their intermittent and often limited nitrogen & water supplies, many vascular epiphytes adopt a slower, more conservative growth strategy (Laube & Zotz, 2003). In fact, epiphytes have been seen to have as much as 45% less leaf nitrogen content than their terrestrial counterparts (Hietz et al., 2022). Liu et al. (2022) further found these lower nutrient concentrations to actually enhance fitness & drought resilience.

This literature contextualizes the responses of *Aechmea recurvata*, *Billbergia brasiliensis*, and to some extent, *V. rafaelii*, to the three nitrogen treatments in this study. It seems risky for these epiphytes, particularly as seedlings, to drastically alter their biomass allocation in response to the smaller nutrient fluctuations typical of epiphytic habitats. Seedlings in general are much less resilient than adult plants, and recent research suggests that even highly epiphytic bromeliads rely on their roots for nutrient and water acquisition before tank formation (Carvalho et al., 2017; Mooney & Hobbs, 1986; Rankenberg et al., 2022; Takahashi et al., 2022; Takahashi & Mercier, 2024; Vanhouette et al., 2017). Were these seedlings to engage in root-shortening in response to lower levels of nitrogen, it could compromise their ability to acquire water and nitrogen given the unpredictability of epiphytic environments. However, I suspect that the 0.1 mM urea applied surpasses a threshold of nitrogen abundance, signaling to *A. recurvata* and *B.*

brasiliensis that the environment is sufficiently nutrient-rich to warrant a shift in biomass allocation towards shoot growth. *Vriesea rafaelii* showed no differential growth under any of the nitrogen treatments. This lack of response may be due to its slower growth rate – *V. rafaelii* produced its first true leaf around Day 21, while other species did so around Day 10 – a trait characteristic of many other epiphytic Tillandsioideae. Alternatively, it could indicate that *V. rafaelii* requires a higher nitrogen threshold to alter its root vs. shoot investment.

Terrestrial *Puya mirabilis* was the only species for which low nitrogen significantly impacted growth. Although the overall response was consistent – albeit smaller in magnitude – with the high nitrogen root-stunting & shoot-promoting, a more nuanced response emerges in the nitrogen:day interactions. I observed an unexpected root *promotion* at Day 14 and shoot *stunting* at Day 21. I hypothesize that the level of nitrogen applied in the low treatment (0.01 mM urea) creates a "Goldilocks" nitrogen stress, where plants are sufficiently stressed to trigger nitrogen scavenging (e.g., root elongation), but still have enough nitrogen available to support biomass production. Other monocots have been seen to engage in the same behavior under low nitrogen stress (Gao et al., 2015; Gaudin et al., 2011; Liu et al., 2008; Tian et al., 2008). I also suspect that this low grade nitrogen stress this Day 14 root growth is indicative of is also responsible for the significantly shorter shoot growth seen by Day 21.

Each species exhibits a unique pattern in how it invests between root and shoot tissues over time, reflecting distinct strategies even in the earliest growth. In particular, *Aechmea recurvata* and *Billbergia brasiliensis*, two closely related Bromelioid species, show different baseline patterns in biomass allocation. This divergence may be linked to their distinct vegetative growth strategies. Jabaily et al. (2021) classifies *A. recurvata* as a leaf adder and *B. brasiliensis* as a leaf lengthener. The pixel analysis in this study primarily captures leaf length but does not account for the quantity of leaves, which could explain the relatively stable observed investment in *A. revcurvata* and the fluctuations in *B. brasiliensis*. The integration of organ score data with this pixel analysis is a crucial next step to explore how early tissue investment aligns with adult patterns and whether this early investment comes at the expense of other developmental processes.

Early bromeliad nitrogen sensitivity remains poorly understood. While these four species exhibit a clear gradient of responsiveness to three urea concentrations, a more comprehensive study is needed to assess whether these patterns persist throughout the phylogeny, especially before, during, and after key developmental milestones such as tank formation. Additionally, the influence of organic versus inorganic nitrogen sources on growth remains unclear. The timing of nitrogen exposure also warrants further inquiry, specifically whether post-endosperm nitrogen exposure has a more significant impact than exposure after later developmental stages. A more thorough understanding of early nitrogen-induced developmental responses across the family will provide critical insight into the plasticity of bromeliad nutrient acquisition strategies throughout development.

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ACKNOWLEDGEMENTS

I want to thank the many people who have made this project possible. Thank you to Dr. Jabaily for your unwavering support, and the numerous hours you put into helping me think through experimental design, methodology, and analysis as well as edit this body of writing. Thank you to Ms. Constance Filling and Mr. Robert Hevey, Jr. who funded this project during the summer of 2024. Thank you to Dr. Shane Heschel for reviewing my methodology, being a second reader on this thesis project, and caring for the bromeliads. Thank you to Ali Keller for caring for the bromeliads as well. Thank you to Dr. Carrie Moon for helping with soil sterilization, and to Donna Sison for institutional and project organization support. Thank you to Dr. Kate McGinn and Dr. Erin Bodine for helping me analyze my data, and to Dr. Brad Oberle & Dr. Karolina Heyduk for helping me think through nitrogen related phenomena. Thank you to Magdalyn Rowley-Lange for helping with seedling care and methodology review.
A final thanks to the Colorado College Organismal Biology & Ecology department.