Function, phylogeny, and the fabulous Fabaceae:

Evolutionary insights from a greenhouse experiment

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Abstract

How do plants thrive (ultimately acting as carbon dioxide sinks) in the face of nutrient limitation? While plants can adopt a wide variety of traits to acquire different nutrients, they are often lumped into functional categories based on a single trait, such as the ability to fix nitrogen, which neglects the potential effect of evolutionary history on trait expression. This is of special interest in tropical Fabaceae, which include nitrogen fixers. These functional groupings (whether a species fixes nitrogen) are assumed to influence nutrient trait expression and are represented as such in earth system models used to predict global change. Recent studies of tropical nitrogen fixing Fabaceae challenge the use of oversimplified functional groupings, but none include enough species to properly assess the influence of evolutionary history on nutrient trait expression in this family. Here, we test the effect of evolutionary history on nutrient traits across 12 diverse tree genera within Fabaceae. We also test a debated hypothesis about the relationship between fixation and other plant nutrient acquisition strategies, specifically that nitrogen fixing species have more nitrogen to spend on root phosphatase enzymes. We grew 22 tropical Fabaceae tree species in a greenhouse for six months and measured key nutrient acquisition traits including nitrogen fixation rate, root phosphatase activity, root morphology and chemistry, and carbon metabolism. Overall, we found strong evidence that evolutionary history shapes nutrient traits in Fabaceae. We also describe possible evidence for systemic differences associated with the ability to fix nitrogen. Finally, we observed that root phosphatase activity increased over 4-fold with every unit increase in nitrogen fixation rate, providing strong evidence for a mechanistic relationship between these two traits. This relationship supports the assumption that species with more fixed nitrogen can ‘trade’ it for root phosphatases to enhance phosphorus acquisition. We stress the importance of considering the effect of evolutionary history in nutrient

trait analyses in Fabaceae –especially when making assumptions about trait relationships that are represented in earth system models.

Résumé

Comment les plantes grandissent-elles (agissant en tant que puits de carbone) face à la pauvreté des sols en éléments nutritifs? Alors que les plantes peuvent démontrer une grande variété de caractéristiques pour acquérir différents nutriments, elles sont souvent regroupées en catégories fonctionnelles basées sur une seule caractéristique, telle que la capacité à fixer l'azote. Ces catégories négligent l'effet potentiel de l'ascendance commune sur l'expression des caractéristiques nutritives. Ceci est d'un intérêt particulier pour les légumineuses tropicales (Fabaceae), une famille qui comprend des fixateurs d'azote. Ces groupements fonctionnels (soit composés d'espèces fixatrices ou non) sont supposés influencer l'expression des caractéristiques nutritives et sont représentés comme tels dans les modèles du système terrestre qui prédisent le changement global. Des études récentes sur les légumineuses fixatrices d'azote tropicales remettent en question l'utilisation de groupements fonctionnels trop simplifiés, mais aucun n'inclut suffisamment d'espèces pour évaluer l'influence de l'ascendance commune sur l'expression des caractéristiques nutritives dans cette famille. Nous évaluons l'effet de l'ascendance commune sur les caractéristiques nutritives à travers douze divers genres au sein des légumineuses. De plus, nous évaluons l'hypothèse controversée selon laquelle les espèces fixatrices d'azote ont plus d'azote à dépenser en enzymes phosphatases racinaires. Nous avons cultivé 22 espèces de légumineuses tropicales dans une serre pendant six mois et mesuré une variété de caractéristiques nutritives clés, notamment le taux de fixation de l'azote, l'activité de phosphatases racinaires, la morphologie et la chimie des racines ainsi que le métabolisme du carbone. En somme, nous avons constaté que l'ascendance commune prédit l'expression des caractéristiques nutritives des légumineuses. Nous décrivons également de possibles différences systémiques associées à la capacité de fixer l'azote. Enfin, l'activité de la phosphatase racinaire a

augmenté de plus de 4 fois avec chaque augmentation unitaire du taux de fixation de l’azote, indiquant une relation mécaniste entre ces deux caractéristiques. Cette relation soutient l'hypothèse selon laquelle les espèces qui fixent plus d’azote "l'échange" pour des phosphatases racinaires afin d’améliorer l'acquisition du phosphore. Nous soulignons l'importance de considérer l'effet de l'ascendance commune dans les analyses des caractéristiques nutritives chez les légumineuses, particulièrement lors de la formulation d'hypothèses sur les relations entre les caractéristiques qui sont représentées dans les modèles du système terrestre.

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Contribution of Authors

Mia Marcellus wrote the present document and manuscript (Chapter 1), ran the greenhouse experiment, and performed all laboratory and statistical analyses.

Dr. Ellie Goud created the phylogenetic tree used in the analyses in Chapter 1, advised on statistical analyses, and provided code and tutorials to execute evolutionary analyses.

Natalie Swartz (undergraduate Research Assistant) and Emily Brown (NSERC USRA) assisted with greenhouse and laboratory methods, and helped write the Methods section of Chapter 1.

Dr. Fiona Soper advised, helped, edited, and provided suggestions throughout the entire process (from study conception and lab work to manuscript writing).

Thesis Format

This single-chapter thesis is written in a manuscript-based format. Citations are provided in the style consistent with the scientific journal *Ecology*. This manuscript is being prepared for submission to the journal *New Phytologist.*

List of Abbreviations

A: Photosynthetic assimilation rate

AMF: Arbuscular Mycorrhizal Fungi

ANOVA: Analysis of Variance

ARA: Acetylene Reduction Assay

ARACAS: Acetylene Reduction Assay by Cavity ring-down laser Absorption Spectroscopy

BM: Brownian Motion

C_2H_4: Ethelyne

CaC_2: Calcium carbide

DW: Dry Weight

FUN: Fixation and Uptake of Nitrogen

K_m: the substrate concentration at which half of the enzyme active sites are saturated

MAG: Modified Arabinose Gluconate

MUB: 4-Methylumbelliferone

N: Nitrogen

OLS: Ordinary Least Squares

P: Phosphorus

PC: Principal Component

PCA: Principal Component Analysis

PIC: Phylogenetic Independent Contrast

PGLS: Phylogenetic Generalized Least Squares

RPA: Root Phosphatase Activity

RT: Room Temperature

SE: Standard Error

SLA: Specific Leaf Area

SRL: Specific Root Length

USDA: United States Department of Agriculture

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General Introduction

Traits are heritable characteristics exhibited by living organisms which underlie functions that form the basis of ecological interactions and ecosystem functioning (Bolnick et al. 2011, Freschet et al. 2021, Laughlin et al. 2021). In plants, both above and belowground traits are important in ecosystem functioning and dynamics, but the latter has traditionally received significantly less study due to technical challenges, and the current literature is biased toward studies of aboveground plant traits (Messier et al. 2010, Laliberté 2017).

Plants can invest in diverse traits to acquire different nutrients in order to grow, the expression of which ultimately influences plant fitness, community dynamics, and ecosystem functioning (Warren et al. 2015). Often referred to as the "black box" of ecology, these nutrient acquisition traits are expressed underground and hidden from the naked eye. They include a diversity of fine-root morphological features, physiological processes, phenological patterns, and belowground symbioses with soil microbiota (Willis et al. 2013, Fernandez and Kennedy 2015, Mishra et al. 2022). To access chemically diverse and often limiting soil nutrients (in particular nitrogen; N and phosphorus; P), plants may exhibit a spectrum of acquisitive fine root architecture, change their degree of exchange with symbionts, and/or engage in exudation of a variety of compounds including enzymes (for example, root phosphatases; RPA). Phosphomonoesterases, specifically, are enzymes exuded by plant roots that mineralize phosphomonoesters which constitute the vast majority of soil organic P available for plant uptake (Cabugao et al. 2021). Plants may also outsource nutrient uptake functions by forming belowground symbioses between their roots and mycorrhizal fungi (including arbuscular mycorrhizal fungi; AMF) and N fixing bacteria which can be extremely important for

overcoming nutrient limitation (Wieder et al. 2015, Laliberté 2017). Specifically, AMF associate with over 80% of land plants and significantly increase the soil volume effectively exploited by roots and increase acquisition of nutrients, namely inorganic P, while also providing protection against pathogens and drought stress (Bonfante and Genre 2010). N fixing symbioses, representing the largest N input in many ecosystems, convert gaseous N to a bioavailable form via nitrogenase enzymes. The majority of N fixing species worldwide are found within one plant family, the Fabaceae (legumes). Together, N fixing bacteria (e.g., rhizobia) and AMF activate joint signaling pathways required to mediate infection by the symbiont and build internal structures within the host plant to serve as sites of nutrient exchange, ultimately allowing plants to reduce the severity of N and P co-limitation (Wang et al. 2022).

Trait-based ecologists have sought to consolidate vast amounts of data across diverse plant lineages and species to characterize broad patterns in trait coordination (Valverde-Barrantes et al. 2020). These patterns in turn may be used as "golden rules" to inform modeling and global change predictions (Anderegg et al. 2021). For instance, Wright et al. (2004) identified six key leaf traits (leaf mass per area, photosynthetic capacity, leaf N, leaf P, dark respiration rate, and leaf lifespan) that broke out into a worldwide spectrum of strategies: plants with quick returns on their investment of photosynthate and mineral nutrients into leaves were characterized by high leaf nutrient concentrations, high photosynthetic/respiration rates, lower dry-mass investment, and lower leaf lifespan (short-lived and acquisitive leaves). The opposite was observed for leaf types at the slow end of the trait continuum. This leaf economics spectrum has since served as a classic null hypothesis upon which to base plant studies (e.g., Donovan et al. 2011, Osnas et al. 2013, Onoda et al. 2017). More recently, attention has focused on identifying whether root traits can also be understood in a similar framework. Absorptive root

traits were hypothesized to fall along an economic spectrum parallel to leaf traits. Thin fast-growing short-lived roots were classified as ‘acquisitive’ whereas thicker longer-lived roots were ‘conservative’ (Reich 2014). Under the supposed unidimensional root economic spectrum, we expect a positive relationship between root tissue density and root diameter and for the latter to negatively correlate with nutrient acquisition rates (Kong et al. 2019). However, support for such a spectrum is lacking. As it turns out, there is some evidence root trait coordination is dependent on whether microbial symbionts (e.g., mycorrhizal associations) are considered along with the root functional traits in question (Weemstra et al. 2016, Kong et al. 2019). For instance, McCormack and Iversen (2019) found that mycorrhizal colonization rate increased with increasing root diameter, contrary to what was expected under the acquisitive-conservative root spectrum. They also found a negative relationship between root tissue density and root diameter. Kong et al. (2019) showed evidence for allometric nonlinear relationships and phylogenetic conservatism shaping root trait syndromes, contrary to the linear nature of the leaf economic spectrum. Literature reviews have tried to consolidate such data into global patterns of nutrient strategy (e.g., conservative vs. acquisitive), including the influence of mycorrhizal colonization strategy and found conflicting results. For example, Averill et al. (2019) found that mycorrhizal type was a significant predictor of traits within a phylogenetic framework whereas another review found the opposite (Valverde-Barrantes et al. 2017). Empirical studies simultaneously and directly testing the assumptions of both the leaf and root economic spectrum are rare, and evidence for leaf and root trait coordination is lacking (e.g., Laughlin et al. 2017). Since absorptive roots and leaves are essential to plant nutrient uptake and ultimately ecosystem functioning, understanding how these are coordinated and shedding light on the black box of

root-trait space is important for our knowledge of nutrient cycling, especially under global change.

In the tropics, P is often highly limiting to plant growth and heterogeneously distributed in soils (Townsend et al. 2008). Consequently, tropical plants use a variety of symbiotic, morphological, and physiological strategies to access P. Widely distributed in the tropics, the Fabaceae family is of special interest as some (not all) plants in this family can fix N and thus already possess one potential mechanism to respond to nutrient limitation. One highly debated hypothesis stipulates that N fixing species are abundant in P-poor/N-rich tropical soils (Hedin et al. (2009) because N fixers can invest more N in P-acquisition mechanisms such as phosphatase enzyme production allowing them to overcome P limitation (Houlton et al. 2008, Nasto et al. 2014, but see Batterman et al. 2018, Soper et al. 2019). Despite the controversy, N fixing and non-fixing Fabaceae species have historically been treated as two functional groups (e.g. in earth system models; Allen et al. 2020). For instance, an N cost for N-rich phosphatase enzymes is incorporated into some commonly used earth system model components, such as the FUN model, with fixers assumed to have more N to 'spend' (Allen et al. 2020). Yet investment in nutrient acquisition traits such as RPA may be phylogenetically conserved in related species and occur regardless of N fixation ability (Zalamea et al. 2016, Png et al. 2017, Sun et al. 2021). Indeed, there is evidence that even within Fabaceae, a variety of strategies for balancing investment in different nutrient acquisition traits may occur unrelated to fixation ability (Soper et al. 2019), but these experiments are based on comparisons of only a small number of species. It remains to be properly tested whether species vary in nutrient acquisition traits and strategy regardless of functional group in Fabaceae.

So what ultimately underlies the observed variation in plant functional traits? It seems that the key to consolidating and understanding plant trait coordination lies in the answer to this question. Leaf traits are often predictable across clades and environments, and largely follow one pattern (Wright et al. 2004, but see Heberling and Fridley 2012), while root traits seem to be expressed according to a different set of rules (Weemstra et al. 2016, Kong et al. 2019, Dallstream et al. 2022). Interestingly, there is growing evidence that root traits may be better predicted by evolutionary history and thus subject to finer levels of variation compared to the coarse patterns observed in leaf functional traits (Valverde-Barrantes et al. 2017, 2020, Hoeksema et al. 2018). However, this has mostly been investigated across diverse species assemblages spanning multiple plant groups and clades, has rarely (if ever) included more than a handful of functional traits (particularly root traits) like AMF and N fixing bacteria, and, importantly, has rarely been tested at the species level, and never in Fabaceae (Valverde-Barrantes et al. 2017, 2020, Hoeksema et al. 2018). Nutrient limitation is anticipated to limit Fabaceae's capacity to act as carbon sinks (Wieder et al. 2015). Thus, testing whether evolutionary history predicts a representative variety of above- and belowground (growth and resource supply) traits in leaves and acquisitive traits in roots in Fabaceae should be prioritized.

"Nothing in biology makes sense except in the light of evolution" (Dobzhansky 1973). While we agree that understanding the underlying functions of traits, the variation in expression therein, and how these scale to shape biological communities and ecosystems is central to understanding life on Earth (Garnier et al. 2015), why has trait-based ecology traditionally operated in an isolated silo from the study of evolution (Johnson and Stinchcombe 2007)? This problem was identified just over a decade ago (Johnson and Stinchcombe 2007), and although evolutionary history is starting to be incorporated in studies of variation in root functional traits

in plants at large scales (e.g., Hoeksema et al. 2018, Valverde-Barrantes et al. 2020, Sun et al. 2021), the synthesis between the two fields has only begun to permeate finer scales of study (i.e., using a phylogenetic backdrop to understand species-level variation in a large number of plant traits; Goud et al. 2019, 2021). Phylogenetic data is now much more readily available to incorporate into analyses and help us avoid the statistical biases inherent to studying life (the non-independence of related organisms). Merging the fields of ecology and evolution and accounting for evolutionary history only risks unmasking valuable patterns and relationships in traits (e.g., Valverde-Barrantes et al. 2017) and allowing us to better predict the outcomes of ecosystem processes and global change (Cavender-Bares et al. 2009, Anderegg et al. 2021).

Evolutionary history predicts nutrient strategy in tropical legume trees

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Abstract

Plants can invest in a variety of traits to acquire nutrients, but it is unclear how trait combinations at the species level are determined by either the legacy of evolutionary history (phylogeny) or trade-offs in resource investment. One trait in particular– N fixation ability– is often assumed to correlate with expression of multiple other traits and used to define plant functional groups, without considering the potential confounding effect of evolutionary history. Here, we test the effect of phylogeny, function (N fixation ability) and trait co-ordination on nutrient trait expression across 12 diverse genera (22 species) of tropical Fabaceae (legume) trees, including both N fixers and non-fixers. We measured above- and below-ground morphological and physiological traits including N fixation rate, root phosphatase activity, root morphology and chemistry, and carbon metabolism in greenhouse-grown seedlings. We found a phylogenetic signal in most traits, suggesting that evolutionary history shapes nutrient trait expression at the species level. We also found some evidence for systemic differences in root trait expression between N fixing and non-fixing species, though for other traits apparent differences in group means were not significant when analyses accounted for phylogenetic non-independence. We identified wide variation in trait expression even in closely related species, with evidence for trait co-ordination including positive correlations between root respiration, phosphatase activity, specific root length, and root N content. Finally, we tested a debated hypothesis of trait co-ordination; that N fixing species are able to 'trade' N for root phosphatases to enhance P acquisition. We found evidence for a mechanistic relationship between these two traits; across 15 species root phosphatase activity increased over 4-fold with every unit increase in N fixation rate, and this relationship held true regardless of phylogeny. We conclude that evolutionary history, trait co-ordination, and function all play a role in determining the nutrient

trait expression of Fabaceae, and stress the importance of explicitly considering phylogeny in future analyses.

Introduction

Plant nutrient acquisition strategies underlie important ecological processes such as community niche differentiation and influence ecosystem functions such as carbon sequestration (De Deyn et al. 2008, Freschet et al. 2021). Because plant growth is frequently constrained by limited nutrient availability, particularly of nitrogen (N) and phosphorus (P; Norby et al. 2010, Wieder et al. 2015), plants invest in a variety of traits related to acquiring these nutrients. Such traits can include the relative surface area and architecture of roots, their rate of physiological activity, the exudation of specialized enzymes, and symbioses with mycorrhizal fungi and N fixing bacteria (van der Heijden et al. 2016, Laliberté 2017). While plants can express a wide range of trait values across clades and environments (Valverde-Barrantes et al. 2020), we do not understand what the major drivers of nutrient trait expression are at the species level.

The specific combination of trait values expressed by a given individual or species (hereafter, nutrient strategy) may be shaped by several processes, namely trade-offs in investment between traits, co-ordination of complementary functions, and evolutionary legacies. As plants have a limited amount of energy to invest in root structures and functions, not all traits may be optimized at once, resulting in trade-offs defined by negative correlations between traits. For example, increased resource capture surface area could be achieved either by allocating carbon directly to root construction, or indirectly to mycorrhizal fungi (McCormack and Iversen 2019). It is also possible that specific aspects of function play a mechanistic role in determining other aspects of nutrient strategy, resulting in observed coordination (positive relationships). For example, whether a plant is capable of fixing N is thought to determine investment in other acquisitive traits (Houlton et al. 2008). Overall nutrient trait expression may be conserved (i.e., constrained by evolutionary history), or plastic (i.e., traits expressed differently depending on the

environment; Comas et al. 2012, Ma et al. 2018). These possible determinants of are not necessarily mutually exclusive and may not operate in isolation. For instance, evolutionary history is often closely linked to certain functions (e.g. the capacity for N fixation is highly evolutionarily conserved within leguminous N fixers, Oldroyd et al. 2011, Martin et al. 2017) and may also be related to the degree of plasticity expressed in certain traits (e.g. Gifford et al. 2013, Melino et al. 2015). So what ultimately underlies the observed variation in plant functional traits? To address this question, some studies have individually addressed the ability of certain functional traits (e.g. N fixation ability or mycorrhizal type) to explain overall nutrient strategy in small groups of species (e.g. Nasto et al. 2014, Zalamea et al. 2016), or looked for broad patterns in trait correlation across divergent clades (Hoeksema et al. 2018, Valverde-Barrantes et al. 2020). However, there remains a need to combine evolutionary and functional considerations to explain nutrient trait expression at the species level.

The large body of work that has sought to identify co-ordination of strategies for above-ground traits does not neatly transfer to the belowground realm (Carmona et al. 2021). Leaf traits tend to largely obey a uni-dimensional acquisitive-conservative spectrum: ranging from thin and fast-growing (quick returns on their investment of photosynthate and mineral nutrients) to thick and slow-growing (lower nutrient concentrations and photosynthetic rates; Wright et al. 2004). It is now widely accepted that belowground root traits do not follow this acquisitive-conservative spectrum (Weemstra et al. 2016, McCormack and Iversen 2019). Rather it has been proposed that roots have at least two axes of dimensionality that capture trade-offs in morphology (strong, thick roots versus investment in acquisitive area) and symbiotic associations (‘do-it-yourself’ versus ‘outsource to mycorrhizae’) and possibly more (Weemstra et al. 2016, McCormack and Iversen 2019). Finally, patterns in above- and belowground economic spectra can be masked by

ignoring phylogenetic conservatism– relationships between traits have been shown to significantly strengthen after accounting for phylogenetic noise (Valverde-Barrantes et al. 2017).

Defining root trait space– and identifying coordination between above- and belowground strategies– has been hampered by the fact that the majority of available root trait data focuses only on a small subset of easily-measured, predominantly morphological traits that do not adequately capture the full spectrum of root function. While these morphological traits are assumed to correlate well with many aspects of physiological functioning, these assumptions are not broadly and rigorously tested (Dallstream et al. 2022). Measuring a suite of above- and belowground traits (especially those that span morphology, symbiotic associations, and physiological functioning) simultaneously under the same conditions offers the best opportunity to investigate trait coordination (Freschet et al. 2017, Soper et al. 2021).

Due to their rich biological and functional diversity, the widely distributed Fabaceae plant family offers an ideal system to test the effect of evolutionary history on nutrient acquisition traits. Common throughout the tropics, it is predicted that nutrient limitation in the this region may limit woody Fabaceae's ability to act as carbon sinks (Wurzburger and Hedin 2016), highlighting the urgency of understanding what determines nutrient strategy in this family. The Fabaceae include many plants capable of fixing N – a distinctive mechanism to respond to nutrient limitation (Afkhami et al. 2017). This specific functional trait (whether or not a species fixes N) is generally assumed to strongly influence overall nutrient strategy, and fixers are represented as a distinct functional group in many commonly used earth system models without considering the potential conflated effect of evolutionary history (Allen et al. 2020, Anderegg et al. 2021). For example, a common hypothesis represented in some models posits that N fixing species can 'trade' fixed N to invest more in root phosphatase enzymes that

enhance P acquisition (Houlton et al. 2008), although it remains to be tested whether this relationship is in fact mechanistic, or simply that higher phosphatase production is instead just an evolutionarily conserved trait in this group and occurs regardless of the degree of N fixation (Condit et al. 2013, Zalamea et al. 2016, Png et al. 2017, Sun et al. 2021). High foliar N, for example, seems common across all Fabaceae, regardless of whether or not they fix N (Martinelli et al. 2021).

Recent studies hint that evolutionary history may be an important predictor of nutrient strategy in Fabaceae (and potentially within and across other plant families, e.g. Hoeksema et al. 2018, Averill et al. 2019), but none include a large enough species sample size for a comprehensive phylogenetic analysis of trait structure (Zalamea et al. 2016, Png et al. 2017, Batterman et al. 2018, Soper et al. 2019, Sun et al. 2021). For instance, Soper et al. 2019 distinguished two nutrient strategies across tropical Fabaceae species characterized by a trade-off between RPA and AMF investment. While one N fixing and non-fixing species both favoured investment in RPA over AMF, the other N fixing species showed the opposite. In addition, Batterman et al. 2018 found that four N fixing tropical Fabaceae species did not have higher RPA than three non-fixers. This finding contradicts the hypothesis that N fixers have more N to spend on root phosphatase enzymes (Houlton et al. 2008). Both studies highlighted an important issue: species may vary in nutrient trait expression regardless of functional group. If this is the case, what does determine the level of trait expression within this family?

Here, we build on previous research by explicitly testing the effect of evolutionary history on nutrient strategies in the Fabaceae family by contrasting a diversity of woody species grown under common conditions. We characterize strategies based on a variety of physiological, morphological and symbiotic traits that better capture the full spectrum of root function, in

addition to aboveground traits that capture growth and resource supply (e.g., photosynthetic rate). To do this, we grew 22 species from across the tropics and subtropics, representing diverse clades (American, Australian, and African lineages), genera (12), growth forms (phyllodes, simple, or compound leaves), and both N fixing and non-fixing functional groups. This marks a departure from studies which examined trait differences among Fabaceae using a small species sample size or compared species mostly within the same genus (Batterman et al. 2018, Soper et al. 2019, Taylor and Ostrowsky 2019, Dovrat et al. 2020, Jaquetti et al. 2021). While it is challenging to clearly tease apart the effects of function and phylogeny on trait expression (owing to the fact that N fixers tend to be more closely related than non-fixers, LPWG 2017), this does not preclude testing evolutionary history as a predictor of nutrient strategies in Fabaceae. This data set enabled us to 1) test for phylogenetic conservatism of individual nutrient acquisition traits, 2) establish differences in trait expression in N fixing and non-fixing Fabaceae, and 3) look for evidence of trade-offs and co-ordination between key nutrient acquisition traits indicative of diverging nutrient strategies.

Methods

Study system and plant growth conditions

We grew 22 species (7-10 individuals each) of tropical legume trees/shrubs in the Fabaceae family (*Caesalpinioideae* subfamily as this group consist of tropical species), 15 of which are documented to fix N (Table S1), under common greenhouse conditions in a pot experiment for a period of 4-7 months (n = 215 plants total). The species varied in their degree of relatedness (i.e., some species within the same genus, some from closely related genera, some outgroups; Figure 1). These species generally occur in dry forests or savannas within the tropics and subtropics and are native to Australia, Africa, and the Americas (Table S1). Seeds came from Sheffield's Seed Company (NY, USA).

For analyses, we used a maximum likelihood multi-gene phylogenetic tree constructed for the 22 species using three gene regions (Figure 1). Specifically, this tree was constructed of 22 sequences each of *trnL* gene and *trnL-trnF* intergenic spacer; *psbA-trnH* intergenic spacer; and *matK*. Sequences were aligned using MUSCLE v3.8 multiple sequence alignment default parameters (Edgar 2004).

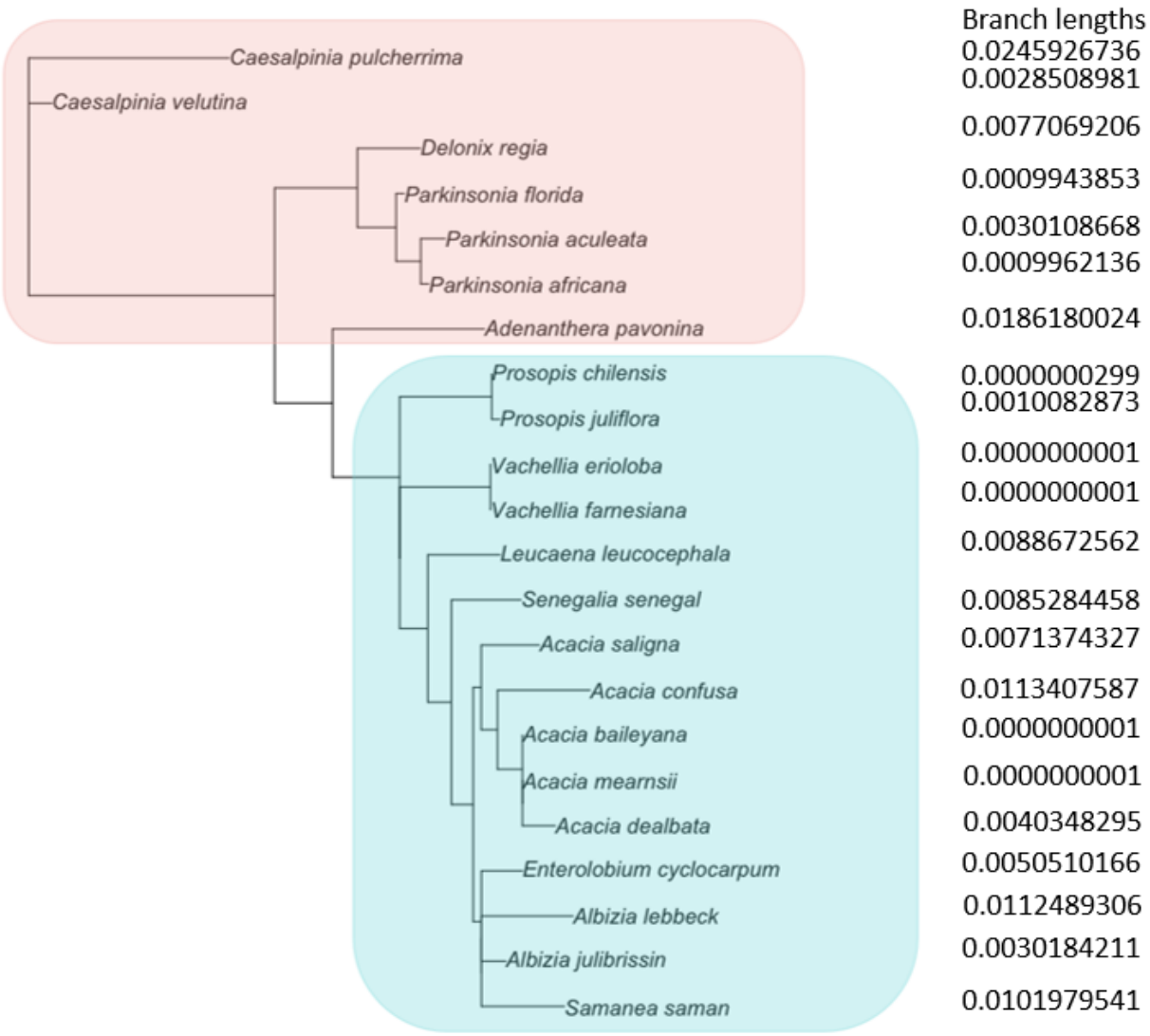

Figure 1. Phylogeny of the 22 studied tropical Fabaceae tree/shrub species, with N fixing species shaded in light blue and non-fixing in light red. Maximum likelihood tree of 22 sequences each of *trnL* gene and *trnL-trnF* intergenic spacer; *psbA-trnH* intergenic spacer; and *matK*. Sequences were aligned using MUSCLE multiple sequence alignment. Terminal branch lengths shown on the right.

We treated seeds by sanding or submerging in boiling water and germinated them in randomly assigned trays filled with 2.5 cm of 1:1 mixed sand:black earth (soil pH_{Ca} 6.7). Once germinated, we transferred 10 plants per species into 2.6 L tall tree pots (Stuewe and Sons) filled with the same soil mixture. Two species, *Parkinsonia africana*, and *Caesalpinia pulcherrima* had 7 and 8 replicates only, respectively, due to high mortality. Pots were arranged randomly in the greenhouse and rotated monthly to minimize any environmental heterogeneity. Each individual was assigned a unique barcode label using the baRcodeR package in R (Wu et al. 2020). Plants grew under Fluence LED lights plus ambient sunlight, with 14 h day length of at least 600 µmols $m^{-2}s^{-1}$ light intensity, 28 ºC day/24 ºC night temperature, and ~30% relative humidity. Plants that didn't survive transplanting within the first month were replaced with healthy same-aged plants from the trays. We fertilized the plants once per week (increased to twice per week beginning week 8) with 50 mL of a modified Hoagland's nutrient solution (Hoagland 1920) with 30% total N and P supplied as $Ca(NO_3)_2$ and KH_2PO_4.

We inoculated all plants with a commercial multi-species (endo- and ecto-) mycorrhizal inoculum (Root Rescue Transplanter, OMRI Canada, Table S2) by mixing 2 g of inoculant throughout each pot prior to transplanting and adding 1 g to each transplantation hole at the time of transfer. We also inoculated seedlings twice at 28 and 35 days after transplanting with 5 mL of a mixture of 25 strains of *Rhizobia* provided by the USDA National Rhizobium Germplasm Collection (Table S3) along with crushed nodules from a subset of previously-inoculated plants. The majority of these strains had been isolated from the same species or genera used in this experiment. Freeze-dried rhizobial strains were grown in modified arabinose gluconate (MAG) broth. Non-fixers were inoculated with 5 mL of MAG-only control.

Because growth rates differed markedly between species, plants were harvested in four stages, when each species reached a minimum size required for analysis but before plants became pot bound (4-7 months). After analyses described below, plants were separated into coarse root (>2 mm diameter), fine root (<2 mm diameter), stem and leaf tissue, dried at 60℃ for 3 days, weighed, and ground (foliar and fine root tissue only).

Belowground traits

Root physiology, chemistry, and morphology

We measured root respiration immediately after harvesting (<5 mins), using a LI-6800 with an Insect Respiration Chamber (LI-COR, Lincoln, NE, USA). Approximately 1 g of fine root tissue was placed in the chamber and allowed to stabilize for 5 mins. Chamber temperature was controlled between 26-28 ℃ (except for *P. juliflora*, where high ambient temperatures precluded accurate temperature control and measurement temperatures ranged from 27-30 ℃). Relative humidity was controlled at 75%, and CO_2 at 400 μmol mol^{-1}. We measured root phosphomonoesterase enzyme activity (RPA) using the 4-methylumbelliferone (MUB) method as per Soper et al. 2019. Ground fine roots were analyzed for C and N content using a Carlo Erba NC2500 elemental analyzer at the Cornell University Stable Isotope Laboratory. We scanned whole root systems using an Epson v800 flatbed scanner and used Rhizovision Explorer (version 2.0.3) to calculate total and fine root length, total root surface area and average diameter (Seethepalli et al. 2021). We calculated specific root length (SRL) of fine roots by scanning, drying, and weighing one subset of fine roots per plant.

Nitrogen fixation

We measured N fixation in whole, intact root systems within 2 h after removal from soil. We used the Acetylene Reduction Assay by Cavity ring-down laser Absorption Spectroscopy

(ARACAS) method (Cassar et al. 2012) with a custom 750 ml transparent flow-through chamber (Figure S6). In measuring the roots intact and with a short incubation time (<12 mins), this approach minimizes known artefacts of the ARA method such as disruption of nodule oxygen permeability and reduction in nitrogenase activity (Soper et al. 2021). Ethylene (C_2H_4) production was measured with a Picarro G-2106 ethylene analyzer (Picarro, Santa Clara, CA, detection limit of 2 ppb per 5 sec) interfaced to a recirculating pump with stainless steel tubing and fittings to minimize leakage and an LI-850 CO_2/H_2O analyzer (Li-Cor Biosciences, Lincoln, NE). Following gentle washing to remove soil, we stored roots at RT in tap water, severing the shoot immediately prior to measurement. The root system and any nodules detached during washing were placed in a recirculating closed loop of volume 850 ml with a flow rate of 0.4 L min^{-1} at ambient temperature (24-26 ℃), and any endogenous ethylene production recorded. Acetylene (derived from CaC_2) was injected into the loop maintaining ambient pressure (Bytnerowicz et al. 2019). N fixation was measured under a headspace of either 10% (saturating, 9 sp.) or 2.5% acetylene (6 sp.). For the latter, values were corrected to be equivalent using species-specific K_m values generated using plants grown under the same conditions (Bytnerowicz et al. 2019). After ethylene production had stabilized (within ~7 mins), the change in ethylene was recorded over a 2 min interval (ethylene production was highly linear; r^2 =0.99). Repeated tests indicated a maximum leak rate of 0.25% of headspace ethylene per hour, extremely low relative to measured production rates. Rates of N fixation (μmol C_2H_4 g^{-1} h^{-1}) were calculated as per Bytnerowicz et al. (2019) accounting for endogenous ethylene production and system leaks. Nodules were detached, counted, and weighed after drying.

Aboveground traits

We measured photosynthetic assimilation rates (A) the week before harvest using a LI-6800 Portable Photosynthesis System (LI-COR, Lincoln, NE, USA), with reference 424 µmol mol^{-1} CO_2, 600 µmol m^{-2} s^{-1} light, 33 °C temperature and 60% relative humidity. We took three measurements per individual, using the three youngest fully expanded sun leaves. Since the compound leaves of most species did not fill the 6 cm^2 chamber aperture, we photographed leaf area to correct values. We analyzed foliar P concentrations in ground leaf tissue using the malachite green spectrophotometric method (Robertson et al. 1999). We calculated specific leaf area (SLA) by weighing a representative subset of leaf tissue with petioles removed, photographing it, and analyzing the images in ImageJ. For species that produced both true leaves and phyllodes (some *Acacia*), only values for phyllodes are presented as they made up the majority of leaf tissue.

Phylogenetic and statistical analyses

All statistical analyses were performed in R version 4.1.2.

1) The effect of evolutionary history on nutrient acquisition traits

To test for a phylogenetic signal in the continuous traits, we determined both Blomberg's K (Picante R package, Kembel et al. 2010) and Pagel's Lambda (λ) (Geiger R package, Harmon et al. 2008) in all traits because they test for signals in different ways (Münkemüller et al. 2012). Briefly, Pagel's λ and Blomberg's K both compare trait evolution relative to a Brownian motion (BM) model of evolution (i.e., where traits vary randomly over evolutionary time, with no directional trends). K compares the variance of phylogenetic independent contrasts (PICs) to what we would expect under BM, where K = 1 means that trait variation among relatives is as

similar as we would expect under BM, and $K < 1$ means that there is less similarity among species than expected under BM. In contrast, λ is not calculated - it is estimated from a maximum likelihood distribution. Values of λ between 0 and 1 represent a scale between a model where all traits are equally distributed (star phylogeny) and a BM model. Significance for a phylogenetic signal in Blomberg's K and Pagel's λ was assessed using chi-square tests (alpha = 0.05), corrected Akaike information criterion (AICc), and log-likelihood values.

2) Differences in trait expression in fixers and non-fixers

To observe differences between N fixers and non-fixers in multi-dimensional trait space, we performed a PCA (described below) using nine traits (fine root fraction, root respiration, root N, RPA, SRL, SLA, assimilation rate, foliar P, absolute growth rate) and included 95% confidence ellipses to distinguish N fixers and non-fixers. To compliment the confidence intervals calculated for the PCA, we performed a linear discriminant analysis (LDA) to classify observations of the 22 Fabaceae species as non-fixers (No) or N fixers (Yes) based on the same nine traits. We then calculated the proportion of correct classifications that were predicted using LDA which were used to determine model accuracy.

Next, we computed trait means and standard deviations to compare differences between N fixers and non-fixers in eight traits (RPA, root respiration, SRL, root N, photosynthetic assimilation rate, SLA, foliar P, and absolute growth rate). To control for phylogenetic non-independence of the data, we tested whether functional group significantly influenced trait values in these traits by performing a phylogenetic ANOVA for each trait using phylANOVA (package phytools), a simulation-based phylogenetic ANOVA method (Garland et al. 1993). We compared these results to regular ANOVAs. We used Holm-Bonferroni corrected p-values for ANOVA analyses.

3) Nutrient acquisition trade-offs and strategies

To observe which traits co-varied, we created a correlation matrix and assessed coefficients. Next, we performed a principal components analysis (PCA) to compute the proportion of variance explained by each component for eight measured traits. These traits were foliar P concentration, photosynthetic assimilation rate, SLA, SRL, root respiration, RPA, fine root fraction, and absolute growth rate. We chose these traits based on their ability to represent key aspects of strategy (root physiology, morphology, growth) while avoiding the use of highly correlated redundant measures (e.g., we did not include both fine root fraction and root to shoot ratio in the PCA).

Finally, we tested whether N fixation rate was correlated with RPA in the 15 N fixing species using a PGLS model which accounts for phylogenetic structure in the residuals of the regression. Lambda was estimated from a maximum likelihood distribution (package caper in R, Orme et al. 2012). We compared this PGLS model to an ordinary least squares (OLS) model that does not take phylogeny into account. We also tested this relationship using all 22 species and included these results in the supplement (Table S5).

Results

Several root traits showed a high degree of interspecifc variation between related Fabaceae species. This was the case for root physiological traits, where mean RPA varied over 4-fold across species (between 5.1 and 21.9 μmol g DW^{-1} h^{-1}), mean respiration varied 4-fold (between 33.8 and 135.4 μg CO_2 g^{-1} min^{-1}), and N fixation rate varied more than two orders of magnitude (from 0.013 to 1.725 μmol C_2H_4 g^{-1} h^{-1}), as well as for some structural traits: e.g., mean SRL varied 8-fold (between 9.8 and 80.3 mm mg^{-1}; Figure 2, S2, and S3).

1) The effect of evolutionary history on nutrient acquisition traits

Evolutionary history significantly influenced most nutrient acquisition traits. We observed a phylogenetic signal in 10 of 13 traits measured (Table 1). All root physiological traits (SRL, root respiration, RPA) and N fixation showed evidence for phylogenetic conservatism (Table 1, Figure 2). Both Pagel's λ and Blomberg's K agreed in nine of 13 traits. However, as Pagel's λ and Blomberg's K did not agree in total root respiration, absolute growth rate, and root C and N %, the signals in these traits should be interpreted with caution. Root fraction, SLA, and foliar P showed no evidence for phylogenetic conservatism.

Table 1. Phylogenetic signal in traits among 22 Fabaceae species. Values in bold are significant ($\alpha = 0.05$). Phylogenetic signal is significant at Pagel's $0<\lambda<1$ and Blomberg's $K>0$, which indicates traits that tend to be more similar among closely related species than expected by chance. Corrected AIC for Lambda and log-likelihood values for each model shown.

Trait	Pagel's λ	Blomberg's K	AICc (λ)	Log-lik (λ)	Log-lik (K)
Absolute growth rate (g/day)	0.98	**0.96**	0.7113	3.311	52.93
Assimilation rate ($\mu mol/m^2/s$)	**0.75**	**0.40**	133.3	-62.99	-78.16
Foliar P (mg/g)	<0.01	0.44	88.012	-40.34	-40.67
N fixation rate (as ARA; µmol C_2H_4/g total biomass/h)	**0.99**	**0.65**	128.9	-60.78	-7.457
Root C (%)	0.94	**0.71**	82.26	-37.47	-40.30
Root mass fraction	<0.01	0.09	59.61	-26.14	15.20
Root N (%)	0.98	**0.59**	24.34	-8.504	-8.881
Root phosphatase activity (µmol /g DW/h)	**0.80**	**0.67**	124.5	-58.57	-61.88
Root respiration (µg CO_2/g/min)	**0.49**	0.20	209.0	-100.9	-109.0
Root tissue density (g/cm^3)	**0.83**	**0.69**	-7.592	7.463	6.344
Specific leaf area (mm/mg^2)	0.82	0.13	143.2	-67.91	-76.54
Specific root length (mm/mg)	**0.71**	**0.35**	191.6	-92.12	-97.02
Standardized nodule biomass (nodule g/ total plant g)	**0.99**	**1.14**	-2172	1090	63.34

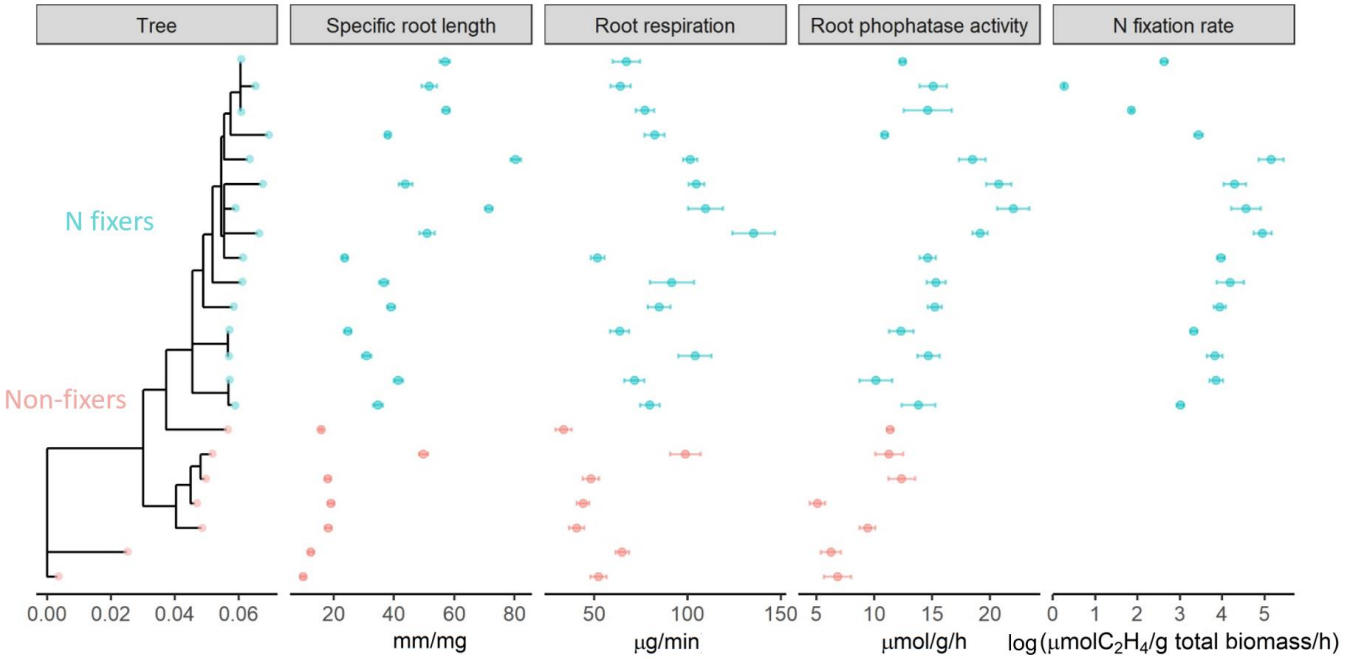

Figure 2. Specific root length (mm mg^{-1}), root respiration (µg CO2 g^{-1} min^{-1}), root phosphatase activity (µmol g DW^{-1} h^{-1}) and N fixation rate (as acetylene reduction, ln µmol C_2H_4 g total plant $biomass^{-1}$ h^{-1}) (mean ± 1 S.E.) for 22 Fabaceae species as related via the maximum likelihood phylogenetic tree (detail in Figure 1). Values for N fixers in light blue and non-fixers in light red.

2) Differences in trait expression in fixers and non-fixers

Trait means appeared to differ more substantially between non-fixers and N fixers for root than for leaf traits (Figure 3). We found significant differences ($p<0.05$) between functional groups for SRL and root N (which were higher in fixers) when analysed with phylogenetic ANOVAs that account for the non-independence of related species. For other root traits (RPA and root respiration), differences were significant when analysed with normal ANOVA, but not phylogenetic ANOVA (Table S4). Many N fixing species had RPA values magnitudes higher than many non-fixers (non-fixer mean = 8.9 ± 3.7 sd, fixers = 15.3 ± 4.7 sd; Figure 2) yet there was still overlap between values regardless of functional group (phylogenetic ANOVA; $p=0.06$).

The PCA (described below) revealed a cluster of non-fixers and N fixers based on 95% confidence ellipses (Figure 4A and S4). Despite variation and overlap in traits among species observed in the PCA, there was evidence that some of this trait variation may be based on functional group, given the observed separation between confidence ellipses. This observation (of the ability to separate functional groups based on measured root traits) was further bolstered when comparing groups through LDA classification results (Figure 4B). The proportion of correct classifications that were predicted using LDA as non-fixer and N fixer were 87.9% and 95.6%, respectively, compared to initial classification in the dataset. Model accuracy was 93.3%.

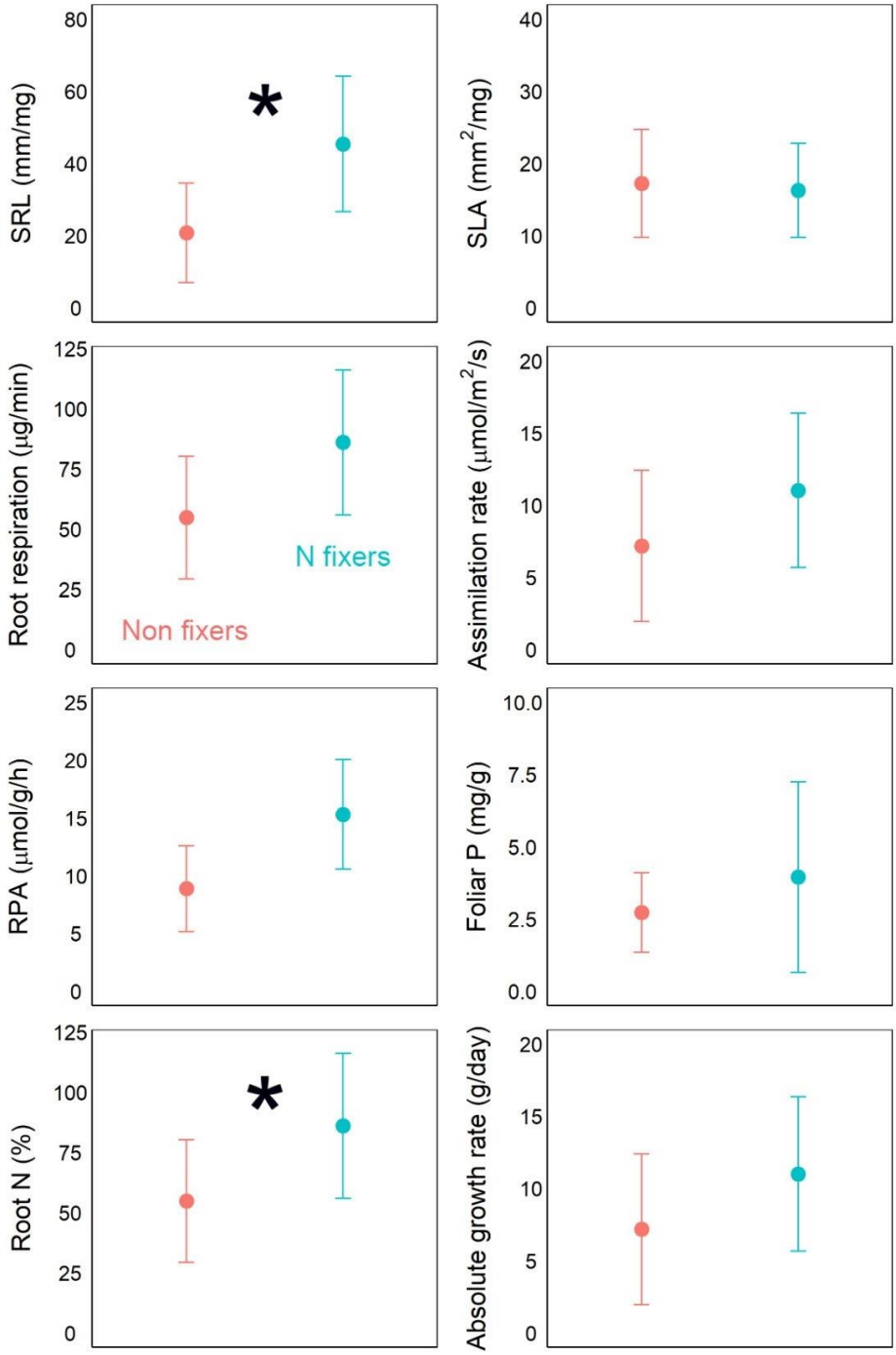

Figure 3. Comparison of eight trait means for non-fixers (light red) and N fixers (light blue), 1 ± S.D. Left column shows root physiological traits (SRL, root respiration, RPA, root N) and the right column shows leaf and growth traits (SLA, assimilation rate, foliar P, whole plant absolute growth rate). Phylogenetic ANOVA performed for each trait (* indicates $p<0.05$).

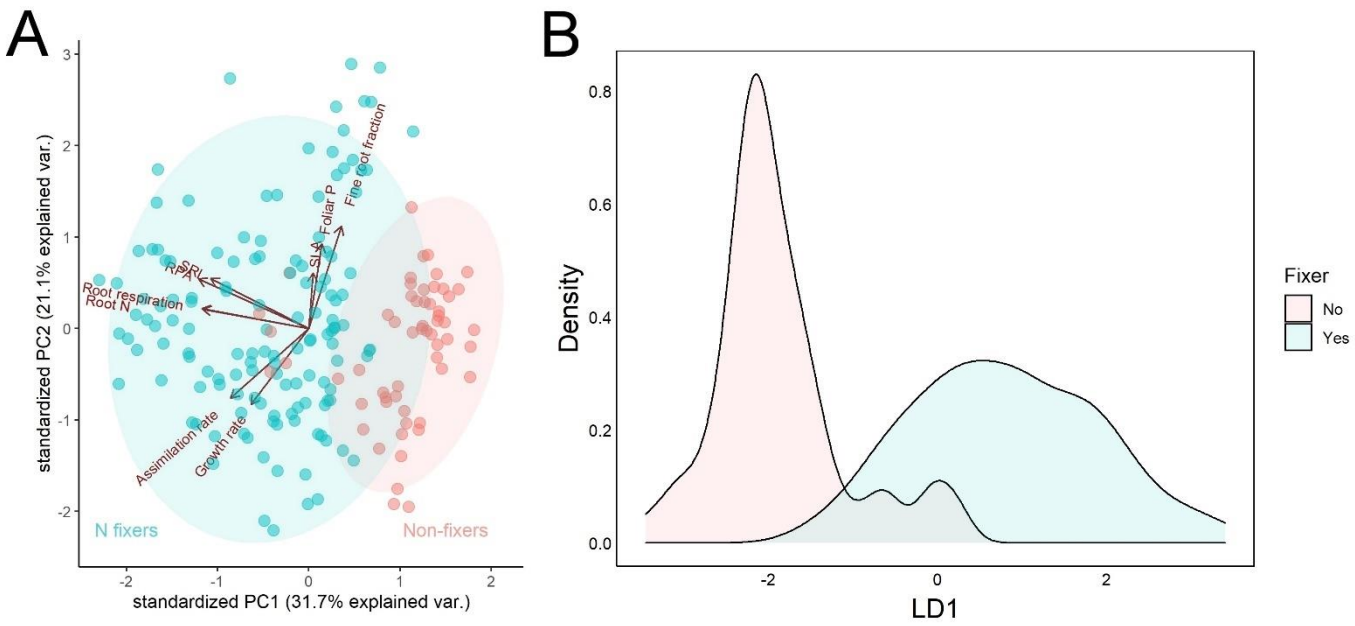

Figure 4. A) Principal Components Analysis of nine traits related to growth and nutrient acquisition for 22 Fabaceae species, including both N fixers (light blue) and non-fixers (light red). 95% confidence ellipses shown in light red and blue. PC1 and PC2 explain over 50% of the variation in the data. Abbreviations: specific leaf area (SLA); specific root length (SRL); root phosphatase activity (RPA). **B)** Linear discriminant analysis classifying observations of 22 Fabaceae species as non-fixers (No) or N fixers (Yes) based on the same nine traits used in A (root fraction, root respiration, RPA, SRL, assimilation rate, root N, foliar P, absolute growth rate). The proportion of correct classifications that were predicted using LDA as non-fixer and N fixer were 87.9% and 95.6%, respectively, compared to initial classification in the dataset. Model accuracy was 93.3%.

3) Nutrient acquisition trade-offs and strategies

Overall, we found evidence that several nutrient acquisition traits were correlated with each other across the 22 study species (Figure S1). Three root physiological traits were positively correlated: RPA, root respiration, SRL, along with root N (which is often considered a proxy for physiological capacity), and two traits related to growth were positively correlated: photosynthetic assimilation rate and growth rate. There was also a significant positive correlation between foliar P, fine root fraction, and SLA.

Multiple traits broke out into main axes as revealed by the PCA (Figure 4A). Among PC 1 and 2, which explained over 50% of the variation in the data, we observed two main trait axes: One axis defined as coordination between RPA, SRL, root respiration, and root N; the second, orthogonal axis defined as assimilation rate and growth rate acting in opposition to foliar P, fine root fraction, and SLA (Figure 4A).

We identified a positive linear relationship between N fixation rate and RPA across the 15 N fixing species which held true regardless of whether phylogeny was accounted for via PGLS ($p<0.05$ for both OLS and PGLS, $\lambda = 0$; Figure 5 and Table S5). In both the OLS and PGLS model, RPA increased over 4-fold with every unit increase in N fixation rate. Thus, as Pagel's Lambda approached zero, indicating no significant effect of phylogeny on model residuals, we report the OLS results only in Figure 5. Interestingly, the effect of phylogeny on the relationship between RPA and N fixation rate was significant when tested in all 22 species (non-fixers assigned N fixation rates of 0; Figure S5). As the OLS definition of R^2 does not carry over easily into GLS, it is to be interpreted with caution (i.e., higher R^2 in OLS than PGLS does not necessarily mean OLS explains variation in the regression better; Symonds and Blomberg 2014).

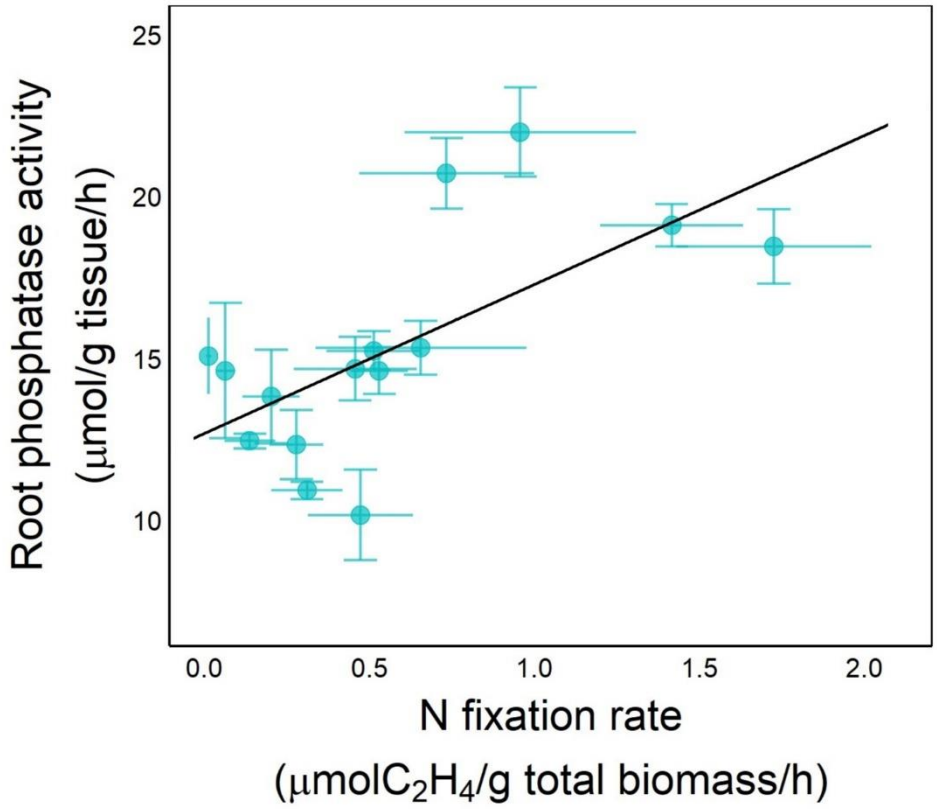

Figure 5. The relationship between mean root phosphatase activity (µmol g DW^{-1} h^{-1}) and mean N fixation rate (µmol C_2H_4 g total plant $biomass^{-1}$ h^{-1}) for 15 Fabaceae species. Ordinary least squares (OLS) regression line shown: slope = 4.6, intercept = 12.7 adjusted R^2= 0.38, $p<0.001$. Statistics for both OLS and PGLS shown in Table S5.

Discussion

Across a morphologically and geographically diverse selection of 22 tropical Fabaceae tree species, we found evidence that nutrient acquisition trait expression is shaped by evolutionary history (phylogenetic conservatism) and co-ordination between traits, including functional relationships between nitrogen fixation and phosphatase enzyme activity. We also found evidence for a general trend towards more physiologically active roots in N fixers, though some apparent differences between functional groups were non-significant after accounting for shared ancestry.

1) Evidence for the effect of evolutionary history on nutrient acquisition traits

Overall, our results show that evolutionary history shapes many functionally significant nutrient acquisition traits in Fabaceae, as evidenced by a phylogenetic signal in most traits tested. This finding supports the idea that phylogenetic structure is important at small scales (within plant families) even though it is predominantly tested at larger scales (across clades and families). Hence, there is functional insight to be gained by explicitly considering evolutionary history within groups of species (such as Fabaceae) rather than only limiting analyses to plants across coarse scales of relatedness (e.g. Hoeksema et al. 2018, Valverde-Barrantes et al. 2020). We found evidence for a phylogenetic signal using two different estimation methods (λ and K) for nitrogen fixation rate, RPA, SRL, and RTD, while root %C, %N and respiration rate were significant for one of the two metrics (Table 1). Photosynthetic assimilation rate (which reflects carbon supply for investment in nutrient acquisition) was also phylogenetically conserved. Previous studies have explored a subset of these traits in a phylogenetic context within mixed-family assemblages in the subtropics in China (Kong et al. 2014, Sun et al. 2021). At that

taxonomic scale, authors variously identified phylogenetic signals for some of the same traits identified here: root respiration, N concentration, root diameter, and SRL. This highlights that some similar patterns of phylogenetic trait conservatism can be expressed at both the between and within-family scale in tropical woody plants.

2) Differences in trait expression in fixers and non-fixers

Fixers had significantly higher root N and SRL (reflecting thinner roots) than non-fixers. This was consistent with an overall suggestion of more physiologically active roots in N fixing species studied, and an overall offset in the multi-trait space. For example, a linear discriminant analysis based on nine traits has an above 90% accuracy in distinguishing the two groups regardless of specific phylogeny. However, we note that some specific trait differences between functional groups that would have been considered significant if analysed without explicitly considering relatedness (e.g., RPA) were no longer statistically significant once phylogenetic non-independence was accounted for. Despite root physiological differences, both groups presented similarly in terms of traits related to growth and carbon metabolism (SLA, photosynthetic assimilation rate, foliar P). Differences between groups may be associated directly with function (e.g., fixers may have more N to allocate to build roots with higher %N and physiological capacity), or it may be that more closely related N fixers tend to behave more similarly and are more physiologically active due to phylogenetic conservatism, although the two scenarios are not mutually exclusive. This may also be explained by a feedback loop; the possible costs associated with increased root physiological activity in N fixers to maintain a more 'active' strategy may be able to be maintained if these species forage and acquire nutrients more efficiently than their less active counterparts (Chen et al. 2016, Dallstream et al. 2022).

Though we acknowledge that there are limitations to interpreting differences using an unbalanced design (e.g., we had twice as many N fixers as non-fixers in the study, and fixation status and phylogenetic structure are not independent, LPWG 2017), we also emphasize that many traits can only be compared effectively in a common garden study, inherently limiting the number of species that can be considered simultaneously. This is because certain traits (especially physiological traits such as RPA) are known to be plastic in response to soil nutrient availability (Nasto et al. 2019), limiting the ability to draw inferences by combining data from multiple sources. Overall, we conclude that even within the Fabaceae, there is evidence that species with the ability fix N share other functional differences that may support the use of fixation to define functional groups (for example, for modeling applications) regardless of the ultimate drivers.

3) Nutrient acquisition trade-offs and strategies

We identified two main trait coordination axes and suggest that these are indicative of diverging nutrient strategies (Figure 4A). The first axis showed strong coordination between three root physiological traits (RPA, SRL, and root respiration), which supports mounting evidence of a highly active and acquisitive root foraging strategy (summarized in Dallstream et al. 2022). These also correlated well with root %N, supporting the common assumption that this trait can be a useful proxy for physiological capacity (Laliberté 2017). However, the second axis where growth and carbon metabolism traits acted in opposition to each other (photosynthetic assimilation rate and growth rate versus foliar P, fine root fraction, and SLA) did not fully correspond to patterns observed in global trait analyses across diverse clades (Díaz et al. 2016). Across a large assembly of vascular plants, plant-size (growth rate and root fraction) were found to act opposingly to leaf metabolic traits (photosynthetic assimilation, foliar P) (Díaz et al.

2016). This difference in findings could be due to the scale of analysis, in which case it highlights the importance of identifying trait coordination at the species level– especially as ignoring species differences can mask patterns in plant economic spectra (Valverde-Barrantes et al. 2017).

4) Positive relationship between N fixation rate and RPA across species

Among the 15 N fixing species, we found a strong positive relationship between N fixation rate (which varied almost two orders of magnitude across species expressed per unit plant biomass) and root phosphatase activity (Figure 5). This provides the strongest evidence to date in support of the debated hypothesis that N fixing plants have more N to invest in the production of N-rich phosphatase enzymes (Houlton et al. 2008), a mechanism to alleviate the P limitation that might predominate especially when N is non-limiting. While some previous studies have noted that RPA can be higher in N fixing than in non-fixing plants (typically comparing N fixing Fabaceae with non-fixers from other groups, e.g., Nasto et al. 2019), the continuous relationship we observed provides evidence for a mechanistic link in which supply of fixed N correlates with ability to invest in greater P acquisition across N fixing species. The spectrum of activity within N fixers may also help explain previous studies that have *not* identified differences based on functional group; these have typically used a small number of species (which may vary in fixation capacity) and concluded that the traits may be species-specific (Guilbalt-Meyers et al, Soper et al. 2019). Both N fixation and RPA also displayed a phylogenetic signal (Table 1) and are therefore influenced by evolutionary history; such conservation of traits makes sense given expected co-ordination between N and P demand. Overall, our findings provide empirical support for plant nutrient uptake models (such as FUN

3.0, Allen et al. 2020) that have begun including a N cost for synthesizing phosphatase enzymes and are increasingly incorporated into larger earth system models (e.g., Braghiere et al. 2022).

Broad implications

In conclusion, we observed differences between N fixers and non-fixers which support the idea that these classic functional groups may differ in more than just N supply, at least for leguminous N fixers. Furthermore, we provide evidence for nutrient trading, as found through the positive influence of N fixation rate on RPA. These results support the general current representation of plant nutrient uptake in earth system models, although we could not completely tease apart the effect of function and phylogeny in trait expression and note that evolutionary history did significantly influence individual trait values. We demonstrate important species differences across Fabaceae, nonetheless. It would be interesting to test whether these nutrient relationships and effects of evolutionary history are upheld across other groups of non-related N fixers (e.g., actinorhizal fixers) outside Fabaceae.

Our analyses demonstrate that failing to explicitly consider evolutionary history when comparing related species can change research conclusions. For example, the phylogenetic vs. regular ANOVAs comparing traits among functional groups yielded different outcomes. We thus highlight the overall importance of accounting for evolutionary history in nutrient trait analyses – especially when making assumptions about plant trait relationships that are implicated in global change predictions (Anderegg et al. 2021). Phylogenetically conserved traits may be less plastic and therefore constrained in their potential responses to changing environments (Liu et al. 2022).

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General Conclusion

Our study addressed a fundamental question in trait-based ecology: what underlies the observed variation in functional traits? We specifically sought to answer this question as it pertains to the often-overlooked 'black box' of ecology – root functional traits.

We considered the effect of evolutionary history on plant nutrient acquisition traits within a broad and diverse sampling of Fabaceae species. We demonstrated that evolutionary history indeed shaped individual nutrient traits. There were also some differences attributable to function (the ability to fix N) in this group. Although we could not completely tease apart the effect of function and phylogeny in trait expression, we nonetheless demonstrated important species differences across Fabaceae. Furthermore, we showed that species with higher N fixation rates also had higher RPA which supports the hypothesis that RPA increases mechanistically with increasing N fixation rate (as hypothesized by Houlton et al. 2008 but contested in Batterman et al. 2018, Guilbeault-Mayers et al. 2020).

Evolutionary history, function, and trait coordination underlie a representative variety of above- and belowground acquisitive traits in Fabaceae. As nutrient limitation is anticipated to limit Fabaceae's capacity to act as carbon sinks (Wieder et al. 2015), understanding the controls and drivers of nutrient acquisition traits in this group is of great importance. In light of our results, we highlight the value of accounting for evolutionary history in nutrient trait analyses – particularly when representing trait relationships to model global change (Anderegg et al. 2021).

We suggest using a phylogenetic backdrop to understand species-level variation in plant functional traits –especially in root acquisitive traits. Future studies may indeed benefit from an evolutionary approach to represent plant, and particularly root, function which can help us better understand and predict processes governed by plant traits.

Appendix

Table S1. Greenhouse study species grouped by subfamily with their native geographic origin, seed source location, and whether the species is reported to fix N (functional group). Functional group classifications were confirmed in this experiment.

Subfamily	Species	Geographic origin	Seed origin	Functional group
CAESALPINIOIDEAE (mimosid)	*Acacia baileyana*	Australia	California, USA	N-fixer
	Acacia confusa	Southeast Asia	China	N-fixer
	Acacia dealbata	Australia	India	N-fixer
	Acacia mearnsii	Australia	India	N-fixer
	Acacia saligna	Australia	Italy	N-fixer
	Adenanthera pavonina	India and Malaysia	India	Non-fixer
	Albizia julibrissin	Korea, Africa, Asia	Louisiana, USA	N-fixer
	Albizia lebbeck	Southern Asia	India	N-fixer
	Enterolobium cyclocarpum	Neotropical	Guatemala	N-fixer
	Leucaena leucocephala	Mexico and Central America	India	N-fixer
	Prosopis chilensis	South America	India	N-fixer
	Prosopis juliflora	Mexico, South America, Carribean	Arizona, USA	N-fixer
	Samanea saman	Neotropical	India	N-fixer
	Senegalia senegal	Africa	India	N-fixer
	Vachellia erioloba	Africa	South Africa	N-fixer
	Vachellia farnesiana	Central America, pan tropical	USA	N-fixer
	Caesalpinia pulcherrima	American tropics	India	Non-fixer
CAESALPINIOIDEAE (non-mimosid)	*Coulteria velutina*	Central America	Guatemala	Non-fixer
	Delonix regia	American tropics, Madagascar	India	Non-fixer
	Parkinsonia aculeata	Neotropical, USA	India	Non-fixer
	Parkinsonia africana	Africa	South Africa	Non-fixer
	Parkinsonia florida	USA and Mexico	California, USA	Non-fixer

Table S2. Mycorrhizal species and propagule density in Root Rescue Transplanter (Root Rescue, Waterdown, ON) used to inoculate plants.

Mycorrhizal species	Content in inoculum (propagules/g)
Glomus intraradices	18
Glomus mosseae	18
Glomus aggregatum	18
Glomus etunicatum	18
Glomus clarum	14
Glomus deserticola	14
Gigaspora margarita	14
Paraglomus brasilianum	14
Glomus monosporum	14
Rhizopogon villosullus	104,375
Rhizopogon luteolus	104,375
Rhizopogon amylopogon	104,375
Rhizopogon fulvigleba	104,375
Pisolithus tinctorius	626,000
Laccaria bicolor	41,750
Laccaria laccata	41,750
Suillus granulatas	130,465
Suillus punctatapies	130,465

Table S3. Rhizobia strains and respective source host species used to inoculate plants.

USDA code	Host plant
3001	*Acacia decurrens*
3003	*Acacia linifolia*
3004	*Albizia julibrissin*
3328	*Acacia pennatula*
3352	
3404	*Leucaena leucocephala*
3406	*Leucaena leucocephala*
3427	*Prosopis juliflora var. juliflora*
3475	*Acacia melanoxylon*
3489	*Gliricidia sepium*
3490	
3499	*Prosopis chilensis*
3517	*Acacia albida*
3667	*Enterolobium ellipticum*
3841	*Acacia constricta*
4361	*Acacia angustissima*
4400	*Senegalia senegal*
4440	
4838	*Leucaena leucocephala*
4869	*Senegalia senegal*
HM1-HM8	*Prosopis glandulosa var glandulosa*

Table S4. Results of phylogenetic and regular ANOVAs for 8 traits in 22 Fabaceae species according to fixation status, 'fixer'. P-values are Holm-Bonferroni corrected for both types of ANOVA.

Traits	**Fixer (regular)**	**Fixer (phylogenetic)**
	(F, p)	(p)
SRL*	21.9, **p = 0.002**	**p = 0.037**
Root respiration	9.7, **p = 0.006**	p = 0.166
RPA	17.9, **p<0.001**	p = 0.065
Root N	18.8, **p<0.001**	**p = 0.047**
SLA*	0.063, p = 0.804	p = 0.917
Assimilation rate	3.7, p = 0.068	p = 0.392
Foliar P*	1.4, p = 0.255	p = 0.598
Absolute growth rate*	8.8, **p<0.01**	p = 0.200

Bold indicates $p < 0.05$
***** Analyzed on a log scale

Table S5. PGLS and OLS results to assess the effect of N fixation rate on RPA. Results shown for analyses using only the 15 N fixing species and analyses using all 22 species with non-fixers given N fixation rates of 0.

Statistic	**15 N fixers**		**All 22 Species**	
	OLS	PGLS	OLS	PGLS
Pagel's Lambda		0		0.42
P	**p<0.01**	**p<0.01**	**p<0.001**	**p<0.002**
R^2	0.38	0.38	0.51	0.36

Bold indicates p<0.05

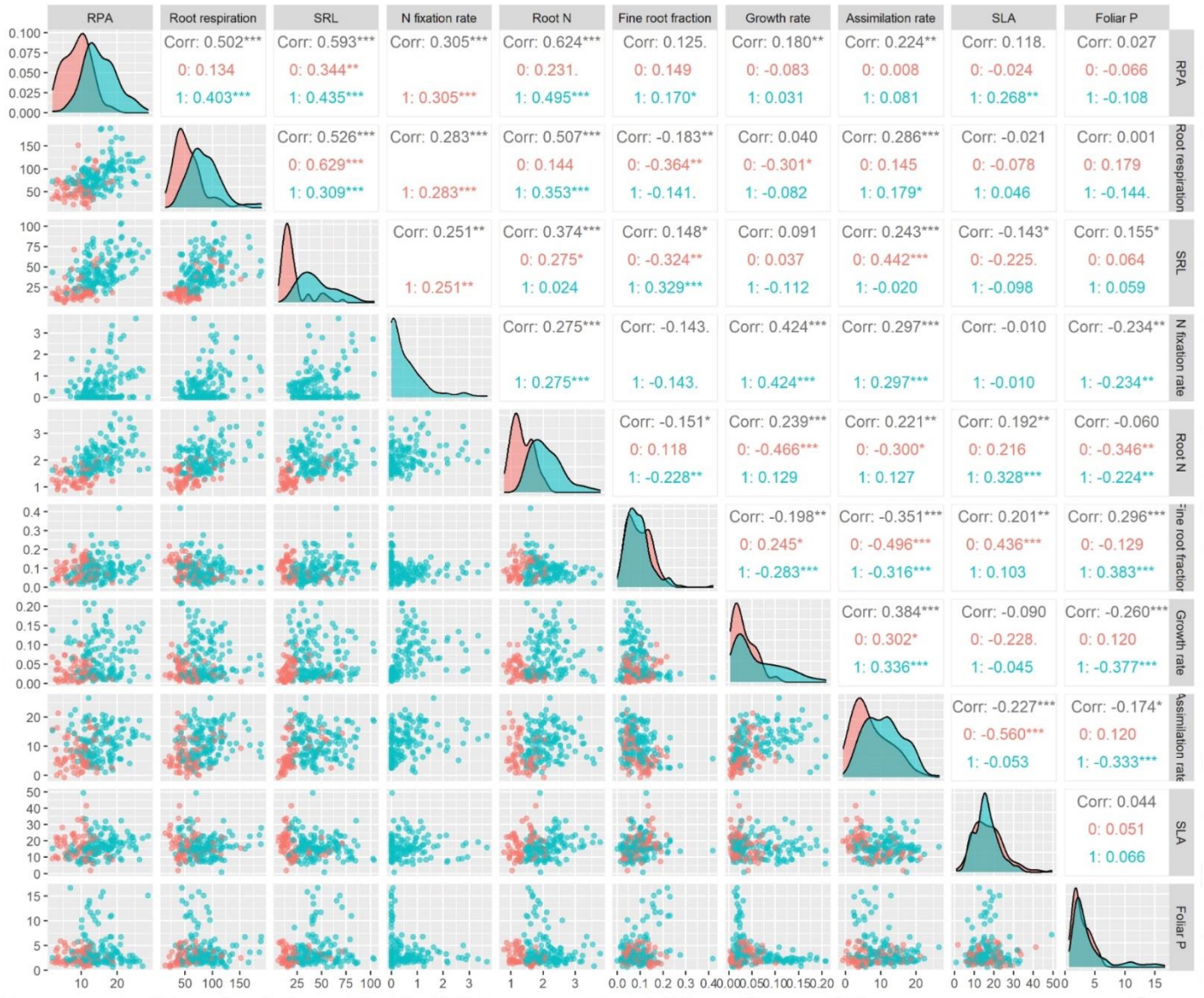

Figure S1. Pearson correlation matrix of 10 nutrient acquisition/growth traits across 22 Fabaceae species. Data points, distributions, and values for N fixing species shown in light blue (1); shown in light red for non-fixers (0). Asterisks next to correlation coefficients denote significance: ***p<0.001; **p<0.01; *p<0.05. Overall correlation coefficients shown in black. Units: root phosphatase activity (RPA, μmol g DW^{-1} h^{-1}), root respiration (μg g^{-1} min^{-1}), specific root length (SRL, mm mg^{-1}); N fixation rate (μmol C_2H_4 g^{-1} h^{-1}); root N (%); fine root fraction (g

fine root/ g total plant); growth rate (g day^{-1}); photosynthetic assimilation rate (μmol m^{-2} s^{-1}); specific leaf area (SLA, mm^2 mg^{-1}); foliar P (mg/g).

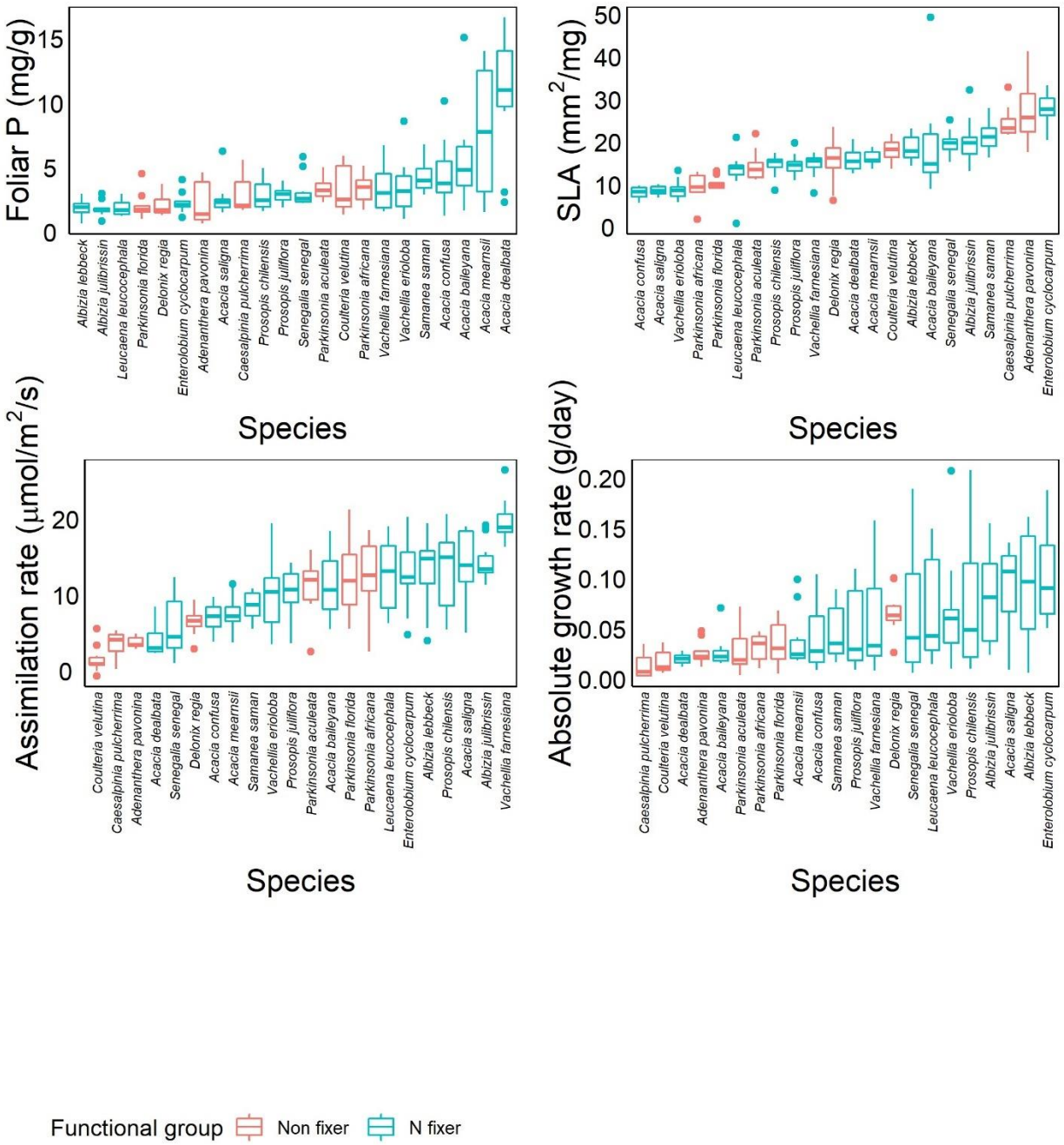

Figure S2. Aboveground traits across 22 Fabaceae species. Non fixers shown in light red, N fixers in light blue.

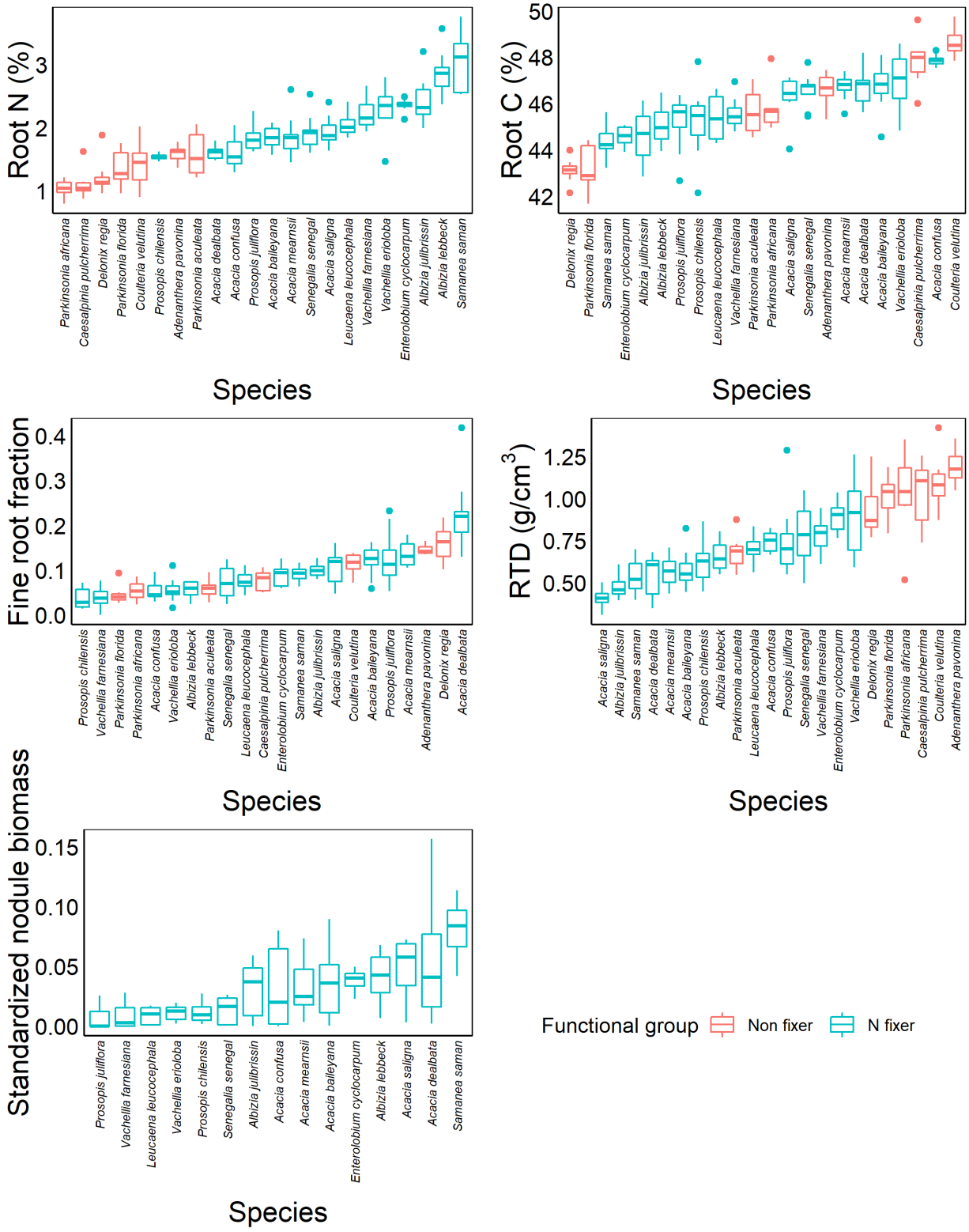

Figure S3. Belowground traits across 22 Fabaceae species. Non fixers shown in light red, N fixers in light blue.

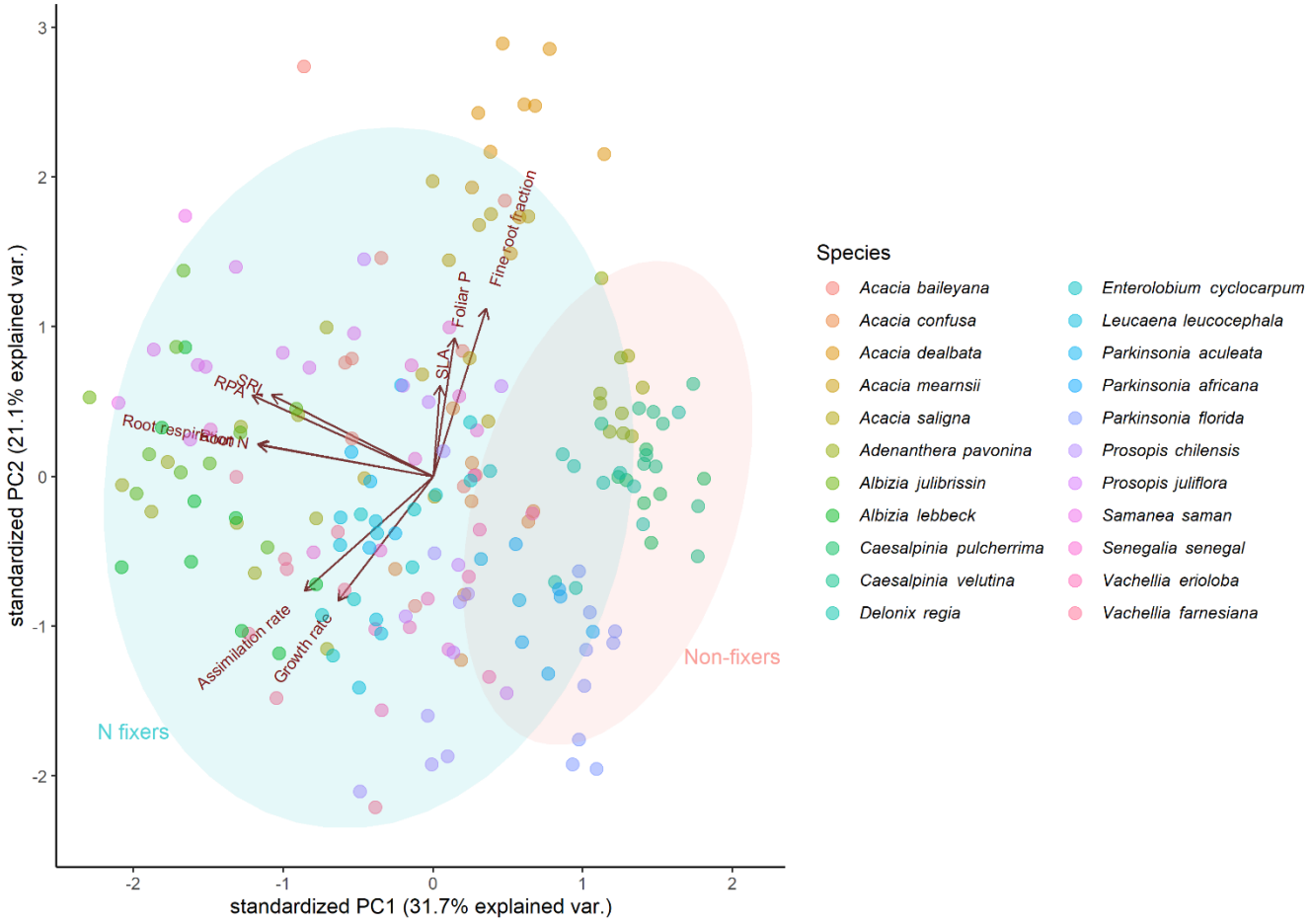

Figure S4. Principal Components Analysis of nine traits related to growth and nutrient acquisition for 22 Fabaceae species, including both N fixers (light blue) and non-fixers (light red). 95% confidence ellipses shown in light red and blue. PC1 and PC2 explain over 50% of the variation in the data. Abbreviations: specific leaf area (SLA); specific root length (SRL); root phosphatase activity (RPA).

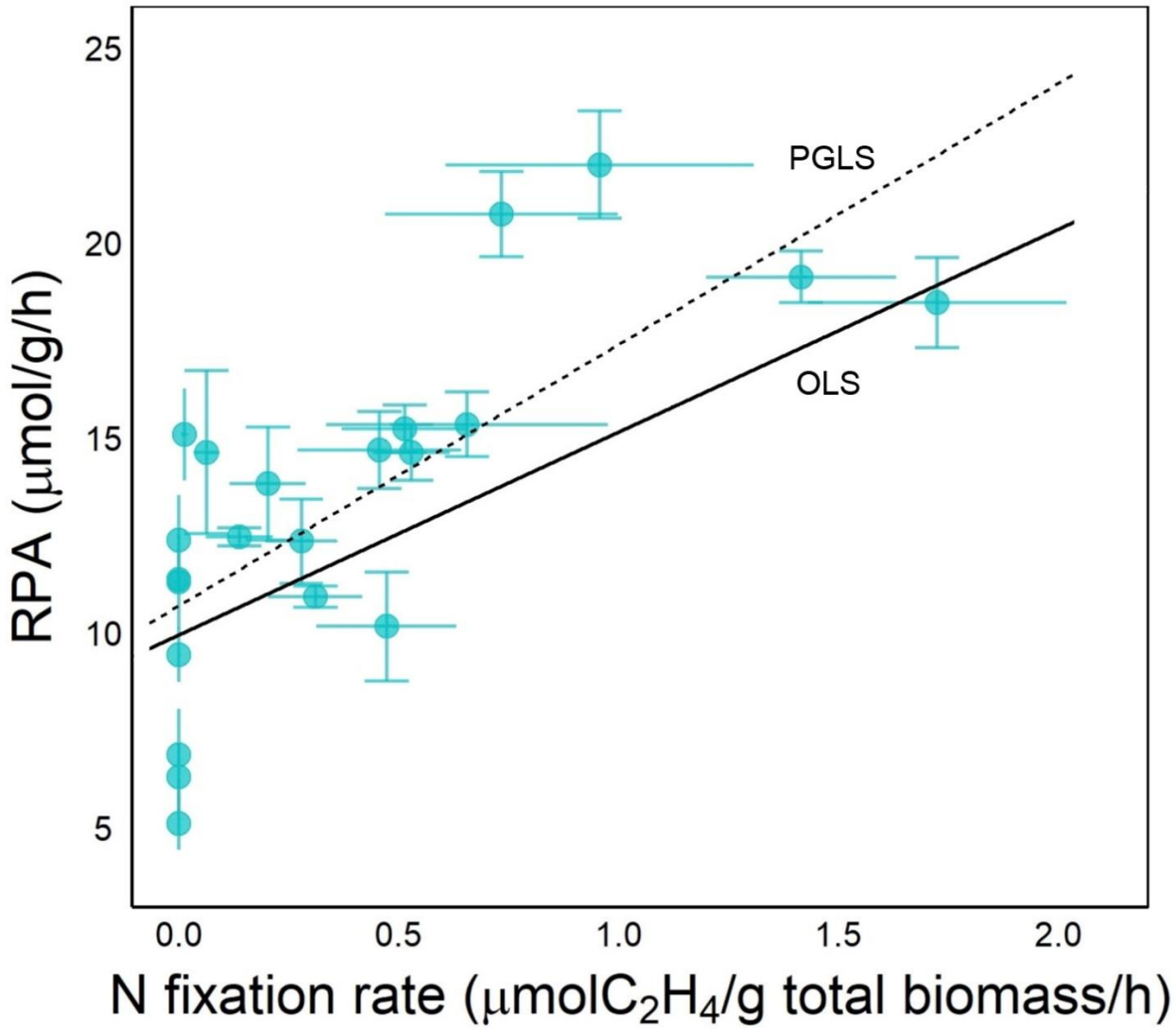

Figure S5. The relationship between mean root phosphatase activity (µmol g^{-1} h^{-1}) and mean N fixation rate (µmol C_2H_4 g total plant $biomass^{-1}$ h^{-1}) for 22 Fabaceae species. ± S.E. bars shown for each trait. Phylogenetic generalized least squares (PGLS) and ordinary least squares (OLS) regression lines shown as solid and dashed lines, respectively. For the PGLS, the maximum-likelihood lambda = 0.42, slope = 5.2, intercept = 9.9, adjusted R^2: 0.36, $p<0.002$. For the OLS (which does not account for phylogeny), slope = 6.7, intercept = 10.7, adjusted R^2= 0.51, $p<0.001$.

Figure S6. Chamber configuration for the acetylene reduction by cavity ring down laser spectroscopy (ARACAS) system used to measure N fixation in whole seedling root systems.