Metabolism of nutrient exchange in bipartite and tripartite nutritional mutualistic interactions with arbuscule mycorrhizal fungi

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INTRODUCTION & BACKGROUND

Plants, a sessile group of organisms, face distinct challenges in interacting with the world in which they live. These interactions are mediated by unique mechanisms of exchanging information and resources, which have developed across the entire plant kingdom [1] and are predominantly mediated through regulation and specialization of metabolism [2]. One such group of interactions, nutritional mutualisms, is built on the exchange of critical resources in the form of metabolic products between host plants and mutualistic symbionts [3]. The process of attracting symbionts is dependent on early signaling events that leverage metabolite exchange between partners [4]. The prime benefit of these associations is the exchange of essential metabolic resources containing carbon (C), nitrogen (N), and phosphorus (P) [3, 5]. Consequentially, plant-microbe mutualisms have key roles in global nutrient cycles, ecosystem functions, and plant evolution [6, 7]. Developing an improved understanding of how these mutualistic interactions work in isolation and in the presence of additional symbionts will be critical for harnessing their power for future biotechnological applications [8]. This proposal is aimed at addressing questions about the metabolism of plant interactions with nutritional microbial symbionts in legumes with a special interest in the movement of nitrogen through both bipartite and tripartite symbiotic interactions.

Nutritional mutualists have had a major role in shaping life on earth due to the importance of mineral nutrient exchange [9]. Mineral nutrients such N and P represent two of the most limiting nutrient resources in natural systems [10]. The movement of N through ecosystems is of primary importance to the net productivity of a given system [11]. Although abundant on earth, most N is in a form, atmospheric dinitrogen (N_2), which is inaccessible to the vast majority of living organisms. Nitrogen fixation, the process of breaking the triple covalent bond (an energetically expensive task) of N_2 to form the bioavailable nitrogenous compound ammonium (NH_4^+), has been cited as a major contributor to global N cycling [11]. The microbes responsible for this task are collectively known as diazotrophs and are critical study organisms for research aimed at improving sustainable agriculture via implementation of biofertilizers [8]. However, diazotrophs are not the only organisms involved in N movement and transfer in natural systems. Due to the complexity of the N cycle, organisms of many types (i.e. denitrifiers and saprophytes) are also involved in the movement through and out of ecosystems, both via transfer of N back to the atmosphere and mobilization in soil environments [12].

Nutritional mutualists, such as the symbiotic diazotrophic rhizobia and the symbiotic arbuscular mycorrhizal fungi (AMF), have been implicated in the movement of N [13, 14]. These mutualists are colonize root systems and are predominately known for trading biologically available N and P for photosynthetically fixed C [15, 14]. Rhizobia, the most extensively studied nutritional mutualists, have attracted significant interest due to their symbiotic nitrogen fixing abilities [14]. Rhizobia are a diverse group of α-proteobacteria and exclusively associate with plants from the family Fabaceae, the third largest group of angiosperms [14]. Nutrient exchange occurs in plant derived structures known as nodules where rhizobia are supplied photosynthetically fixed carbon in the form of dicarboxylic acids for fixed N [14]. The relative contribution of non-diazotrophic microbial organisms on the global N cycle is not well understood and the extent to which various organisms move N is worth further investigation.

Although undoubtedly important, rhizobia have relatively few functions in comparison to a group of mutualistic fungi known collectively as mycorrhizae. Mycorrhizal fungi are numerous in type and generally engage in mutualistic associations [15]. Of the mycorrhizae, the three most ecologically influential forms are ecto-, ericoid and arbuscular [16]. Ectomycorrhizal fungi have earned their fame as nutritional symbionts due to their ability to mobilize mineral nutrients from both organic and inorganic sources. Specifically, ectomycorrhizae are able to breakdown proteins in soil organic matter to make N from organic sources available to host plants. Furthermore, ectomycorrhizae excrete organic acids such as intermediates from the citric acid

cycle to aid in the extraction of mineral nutrients from rock materials and soil organic matter [17]. Ericoid mycorrhizal fungi, a type that associates with plants of the family Ericaceae, aid in nutrient movement in acidic or nutrient poor conditions and appear to help host plants deal with toxic metal compounds in soil environments [18]. Finally, AMF are most notable for their ability to move inorganic P from the soil environment to their host plants [15].

AMF are an early diverging clade of fungi credited with aiding in the evolution of plants from aquatic ecosystems to terrestrial environments over 400 million years ago [19]. Symbiotic interactions with AMF increase the surface area and physical reach of root systems resulting in improved water uptake and communication with other organisms [15]. AMF are considered the most ecologically influential plant mutualists due to their ubiquity and net benefit to hosts [19]. More than 80% of all land plant species interact with AMF and interactions occur in nearly all ecosystems [15, 19]. Recent studies have focused on defining pathways dedicated to nutrient movement and elucidating additional resources transported via AMF; the uptake and transfer of N to host plants is especially intriguing due to its overlapping niche with rhizobia. In contrast to rhizobia, AMF are able to take in and translocate N in the forms of nitrate (NO_3^-) and ammonium (NH_4^+) directly from the soil environment [20]. However, the specifics of the N transfer process are unclear [19, 20] and important questions about nutrient pathways, such as N transfer, remain to be answered [20].

Despite the fact that the legume-rhizobia symbiosis rarely occurs in the absence of an AMF association, studies focused on understanding the tripartite or multipartite state of this symbiosis have been severely limited. Legumes are important for ecosystem functioning and the global N and also for sustainable agricultural practices as they are less reliant on exogenous fertilizers [21]. Considerable interest exists in engineering the rhizobial symbiotic pathway into non-legume crop plants such as wheat and maize [8]. This task is particularly daunting due to a number of issues including the involvement of plant immune systems in symbiosis and the significant overlap between mutualism pathways of rhizobia and AMF [8, 22]. Implementing biotechnological solutions will require fine details of these systems to be manipulated without compromising key components of plant defenses. Additionally, many questions remain unanswered with regards to N and C partitioning between multiple symbionts in legumes, the effects of resource limitations on C partitioning, and interdependence of the two mutualists [23].

Addressing these outlined knowledge gaps will not only aid in the development of sustainable agriculture but also shed light on evolutionary theory questions about the stability of mutualisms [23–25]. Proper understanding of the metabolic regulation that underpins associations with nutritional mutualists is necessary.

OBJECTIVES

The goal of this work is to test hypotheses concerning regulation of macronutrient exchange between plants and their microbial symbionts. Understanding these physiological processes is important to testing hypotheses about the nutritional, ecological, and evolutionary functional roles of plant-microbe mutualisms. To address resource partitioning between plants and nitrogen uptake from rhizobial and AMF symbionts, I will use isotopically labelled N substrates to track allocation from various symbionts. Investigation of P allocation to rhizobia via AMF will employ labelled P substrates to track P uptake and partitioning in plant tissues and their symbionts. To improve our understanding of the pathways that underlie N transfer from AMF to host plants, I will use labelled N and C to trace the fates of acquired NO_3^- and metabolic products involved in AMF N metabolism. Finally, I will apply a constraint-based modelling approach to investigate the metabolic costs of multiple N transport pathways via AMF to host plants. Specifically, I propose the following:

Aim 1: Characterizing nutrient exchange in the legume-AMF-rhizobia tripartite symbiosis

A. What proportion of nitrogen is supplied to leguminous plants by AMF versus rhizobia under non-limiting phosphorus conditions?
B. How is the ratio of nitrogen supplied by rhizobia and AMF altered by phosphorus availability?
C. What proportion of phosphorus supplied to nodules during symbiosis is from AMF?

Aim 2: Characterizing nitrogen uptake and transfer mechanisms in the AMF symbiosis
A. Is nitrate chemically modified during transport by AMF from the soil to plants?
B. What is the fate of ornithine produced in the nitrogen transfer process?
C. What are the implications of different N transfer pathways for symbiosis costs?

AIM 1. CHARACTERIZATING NUTRIENT EXCHANGE IN THE LEGUME-AMF-RHIZOBIA TRIPARTITE SYMBIOSIS

The symbiosis between legumes, AMF, and rhizobia serves as a fascinating system to study tripartite associations due to its exchange of common resources [20, 26, 27]. Past work on this tripartite association has focused on assessing carbon sink strength and effects of altered P supply to the system [28]. Other work has employed different –omics techniques to study regulatory changes in response to one [27] or both symbionts, or focused on assessing functional diversity in ecological experiments [26]. These experiments share the common goal of identifying overlap between mutualism pathways and host responses to microbial nutritional mutualists. However, very little is known about the nutrient exchange fluxes and their environmental regulations.

Significance: Understanding nutrient cycling in ecosystems as well as making progress in developing low-input agriculture legume systems require a better understanding of nutrient exchanges in this tripartite association. It is important that we understand the impact of AMF on the legume-rhizobia symbiosis and resource exchange at all levels, particularly the molecular level. Future work should focus on defining how the presence of AMF affects both host resource allocation and preference for nutrient assimilation. To address this, I propose to determine relative amounts of N supplied to hosts by each symbiont, assess how this relationship is altered by P-limiting conditions, and determine the proportion of P in nodules that is supplied by AMF. Investigating these questions will contribute to a scarce body of work, clarifying mechanisms underlying nutritional symbioses and global nutrient cycling.

1A. What proportion of nitrogen is supplied to leguminous plants by AMF versus rhizobia under non-limiting phosphorus conditions?

Previous studies have demonstrated that AMF appear to have significant roles in N movement to host leguminous plants and can dramatically reduce N stress symptoms (figure 1) [29]. Furthermore, work in the non-leguminous species, *Panicum virgatum*, showed that AMF significantly contribute to organic N acquisition and translocation to host plants [30]. Investigation of N contribution by AMF in tripartite symbiotic interactions between legumes-rhizobia-AMF will be done using ^{15}N tracer experiments. To determine the relative proportion of N transferred from rhizobia and AMF, $^{15}NH_4{}^{15}NO_3$ will be provided to the fungus or roots. The amount of ^{15}N incorporated into plant biomass will be measured. The relative proportions of ^{15}N to ^{14}N will be used to determine N contribution of each symbiont. I hypothesize that under non-limiting nutrient conditions rhizobia will supply a greater amount of nitrogen to host plants relative to their AMF counterparts.

Approach: *Ensifer medicae* WSM 419 will serve as the rhizobial partner and *Rhizophagus irregularis* will serve as the fungal partner. Both species have relatively well-annotated genomes and available genomic resources [19, 31]. *Medicago truncatula* A17 will serve as the host

legume as it associates with both rhizobia and AMF, is privileged with a plethora of available genomic resources, and has a relatively short lifespan [32]. To assess N movement through the tripartite interaction I will grow *M. truncatula* semi- sterilely in a two-compartment system using concentric conetainer pots. The inner conetainer will be spatially separated from the outer conetainer by an air gap and double-layered mesh. This will allow for AMF hyphae to access N that host roots cannot. *M. truncatula* will be inoculated with both *E. medicae* and *R. irregularis*. N movement will be assessed by tracking the movement of labelled ^{15}N through the system. Percent of total N gained over the labelling period will be determined by measuring biomass increase and N contents. In total, 32 plants will be grown for ^{15}N data and an additional 64 will be grown for corresponding biomass data (Table 1, labelling treatments not shown). More specifically, there will be 2 compartment labelling applications and 4 mutualist treatments (AMF only, rhizobia only, neither, both). For the ^{15}N label quantification, 4 replicates per treatment are planned. For biomass measurements, samples will need to be collected before and after treatment application. An additional 8 replicates per treatment will be grown for these measurements.

To grow host plants in conditions not limited by N, application of $^{15}NH_4{}^{15}NO_3^-$ to the fungal compartment and to the root compartment will be analyzed in separate treatments. Application to the fungal compartment will allow the total amount of labelled N per unit biomass of AMF to be calculated. Assuming that AMF uptake and transfer of N is the same per hyphal length in both compartments, we can calculate the amount of the unlabelled N in the host plant is received from AMF in the root compartment. Then, labelling in the root compartment only will allow determination of N provided by rhizobia (by determining total unlabelled N gain). These results will also provide an estimate of total N taken up directly by roots.

Measurements will be made at two time points during linear vegetative growth and will include: nodule number, size, and root percent colonization by AMF as well as fungal biomass in the soil [20, 33, 29]; total plant biomass; and root and shoot tissue mass and N contents and ^{15}N incorporation. Percent N and the ratio of N isotopes will be analyzed by the Stable Isotope Lab at Utah State University using a Europa Scientific SL-2020 system. Atmospheric N is predominantly ^{14}N [34] and enables effective comparison of total N composition. N supplied by AMF will be labelled compared to atmospheric N fixed by rhizobia. Higher rates of ^{14}N relative to ^{15}N would indicate a greater proportion of N was fixed by rhizobia and transferred to host tissues. Collected data will be analyzed using a two-way ANOVA.

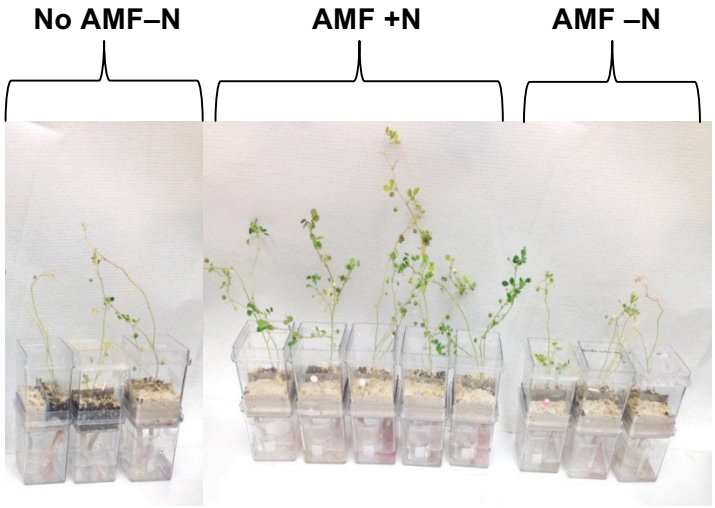


Figure 1: 7 weeks old *M. truncatula*. In order, plants grown without AMF, plants grown with both AMF and addition of KNO_3, and plants grown with AMF and no N source. 2 weeks post treatment (derived from Al-Deeb 2015).

1B. How is the ratio of nitrogen supplied by rhizobia and AMF altered by phosphorus availability?

I will add an additional experimental factor in the tripartite treatment (aim 2A) under conditions of high, medium, and low P supply to the root compartment, to determine changes in N supply plants are more dependent on AMF as a result of P limitation. I hypothesize that under P limitation, hosts will receive a greater proportion of N from AMF.

Approach: In addition to the experimental treatments proposed in aim 2A, P will be included as a factor to test the effect of P limitation on N

uptake. P levels in the form of KH_2PO_4 (low: 1 μM, medium: 500 μM, high: 2 mM) [35] will be included. This requires an additional 64 plants for the labelling experiments and an additional 128 plants for biomass data collection (table 1). Analysis will proceed as described in aim 2A. C allocation will be estimated using biomass, and potentially by $^{14}CO_2$ labelling of the shoots.

1C. What proportion of phosphorus supplied to nodules during symbiosis is from AMF?

As a component of the approach listed in aim 2B, P in the fungal compartment will be labelled with ^{32}P to determine the contribution of AMF to both plant and nodule P nutrition. Previous work has shown that nodules containing N-fixing rhizobia have higher P requirements than surrounding tissues by demonstrating increased nodule biomass and improved rhizobia N-fixation in P-sufficient conditions compared to P-deficient conditions [36]. While work has investigated the total concentrations of P that accumulate in nodule tissue, no focus has been given to where that P originates. I hypothesize that nodules serve as a P sink from AMF and will accumulate a greater proportion of ^{32}P than unlabelled P due to their high P requirements and the reliance of host plants on AMF for P supply.

Approach: Contribution of AMF to nodule P levels will be quantified using ^{32}P in the form of $KH_2{}^{32}PO_4$ in the P treatments listed in Table 1. P treatments will be applied at low (1 μM) and medium (500 μM) concentrations in the root compartment. The remaining P substrate required for the high concentration (2 mM) will be applied to the fungal compartment as to not inhibit the growth and continued colonization of AMF as a consequence of high P [37]. Nodule tissue from AMF and rhizobia dual inoculation experiments will be collected and relative abundance of ^{32}P/ ^{31}P analyzed by the Stable Isotope Lab at Utah State University using a Europa Scientific SL-2020 system. Plants with both AMF and rhizobia will be compared to rhizobia only plants to determine differences in total P content. Collected data will be analyzed with a two-way ANOVA.

Expected results: The collected data will shed light on an important yet understudied ecological question: how do legumes successfully coordinate resource partitioning to two symbionts simultaneously? Previous work has made progress on the intensity of the C sink [38] from heightened respiration demands under varying phosphorus supply conditions. No experiments have investigated the transfer of N from both partners under similar conditions. If N is preferentially taken up via rhizobia under all nutrient conditions, it would provide evidence that hosts prioritize fixed N from rhizobia. Alternatively, if N acquisition strategies vary based on P levels (i.e. if plants obtain more N from AMF when P is limiting) it would suggest that host uptake preference is determined by total cost:benefit ratios.

Phosphorus treatment	AMF inoculum	Rhizobia inoculum
Low (1 μM)	*R. irregularis*	*E. meliloti*
Medium (500 μM)	*R. irregularis*	*E. meliloti*
High (2 mM)	*R. irregularis*	*E. meliloti*
Low (1 μM)	*R. irregularis*	Sterile buffer
Medium (500 μM)	*R. irregularis*	Sterile buffer
High (2 mM)	*R. irregularis*	Sterile buffer
Low (1 μM)	Sterile buffer	*E. meliloti*
Medium (500 μM)	Sterile buffer	*E. meliloti*
High (2 mM)	Sterile buffer	*E. meliloti*
Low (1 μM)	Sterile buffer	Sterile buffer
Medium (500 μM)	Sterile buffer	Sterile buffer
High (2 mM)	Sterile buffer	Sterile buffer

Table 1: Summary of phosphorus and inoculation treatments for *M. truncatula* plants. All inoculations and treatments will be prepared in sterile buffer. Plants treated medium phosphorus levels will serve as normal nutrient treatments for aim 2A. n = 4 for all labelling plants and n = 8 for all biomass plants.

AIM 2. CHARACTERIZATION OF NITROGEN UPTAKE AND TRANSFER MECHANISMS IN THE AMF SYMBIOSIS

Nutrient transfer to host plants via AMF has historically focused on the uptake and transfer of P [15, 39]. Contemporary research has shown that AMF are also able to uptake N from the soil and subsequently transfer it to its host [20, 13]. Nutrients are taken up from soil via the extraradical mycelium (ERM) and translocated to the intraradical mycelium (IRM) inside of the plant for transfer to host [40]. An understanding of the molecular mechanisms and biochemistry that initiate and sustain this association is still incomplete and the regulation of its activity is similarly not well understood [20]. Currently, the accepted framework for N transfer assumes that all N is transferred across the interfacial apoplast (where the host plant and AMF interface for nutrient exchange) in the form of NH_4^+ [20, 13]. Preliminary work by Al-Deeb and Shachar-Hill (*unpublished*) [29] (figure 2) shows that NO_3^- may be transferred across the interfacial apoplast as well. Furthermore, the fate of ornithine (a byproduct of the transfer pathway) in the IRM is unknown.

I propose to test and extend this work using ^{15}N- and ^{18}O-nitrate labeling to determine if soil NO_3^- is transferred from fungi to plants as NO_3^- or metabolized to another nitrogenous compound before being transported. I will use flux balance analysis (FBA) to determine if N obtained as nitrate is a significant contributor to total plant N when AMF have access to both NO_3^- and NH_4^+ pools. To assess the fate of ornithine in the N transfer process, labelled ^{13}C,^{15}N -arginine will be traced through the system.

Significance: Currently, the N transfer pathway from AMF to host plants is not fully understood. Due to our inability to sufficiently manipulate AMF in the lab, there has been relatively little work in the research community to understand the regulatory and nutrient transfer mechanisms in AMF symbiosis [19]. Constraint-based modelling allows us to overcome these limitations by performing *in silico* simulations that are not dependent on molecular or genetic manipulations of the system. Improving our understanding leads to the implementation of these extremely important fungi in biofertilizers and other sustainable agricultural practices.

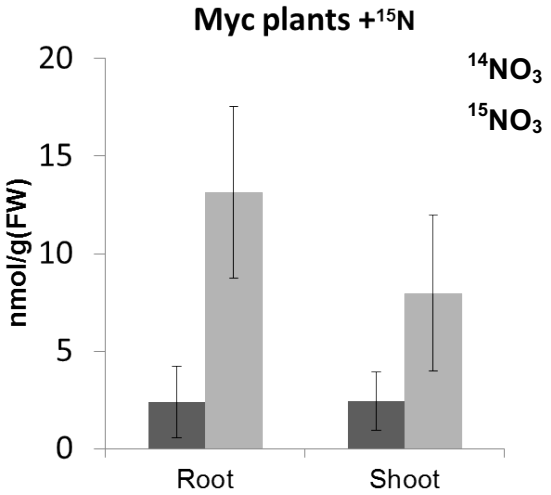


Figure 2: $^{15}NO_3$ recovery in transformed carrot (*Daucus carrota*) roots post-colonization by *G. intraradices* after supplying 4 mM $K^{15}NO_3$ to the fungal ERM compartment in divided petri dishes. Standard errors and means are shown (derived from Al-Deeb 2015)

2A. Is nitrate chemically modified during transport by AMF from the soil to plants?

Previous results found significant accumulation of $^{15}NO_3^-$ in leaf tissue of mycorrhizal plants when associated AMF had access to pools of ^{15}N labelled NO_3^- [29]. The accumulation of ^{15}N label strongly suggests that N is transferred NO_3^-. However, it remains possible that AMF

reduce NO_3^-, instead transferring N as NH_4^+ across the interfacial apoplast before converting it back to NO_3^-. I hypothesize that NO_3^- obtained by AMF remains chemically unmodified for transfer to host and serves as an additional N transport pathway to the arginine pathway previously described (figure 3).

Approach: To test if NO_3^- is transferred intact, dual labelling experiments will be performed using $K^{15}N^{18}O_3$. $K^{15}N^{18}O_3$ will be synthesized from $^{15}NO_3^-$ and $H_2{}^{18}O$ (reactions 1 and 2). *M. truncatula* A17 will be grown sterilely in the Magenta two-compartment system [29] and inoculated with *R. irregularis* DAOM 197198, 48 hours post-germination. *M. truncatula* will be fed Hoagland's solution [35] via a linen-cotton rope wick for the first 5 weeks. At 5 weeks of age, host plants will be deprived of N for 4 days. After, AMF will be supplied with 10 mM $K^{15}N^{18}O_3$ in the fungal compartment. Host plants will be fed $K^{15}N^{18}O_3$ for 2 weeks via the fungal compartment before harvesting. Corresponding controls lacking AMF will be run in tandem. Isolation of N-containing metabolites will occur by harvesting, washing, and grinding plant roots and shoots. Extractions will be performed with Chloroform/Methanol/Water. Metabolites will be separated by charge on a cation exchange column [29] and analyzed using liquid chromatography-mass spectrometry (LC-MS). Synthesis of $K^{15}N^{18}O_3$ from $^{15}NO_3^-$ and $H_2{}^{18}O$ follows the following reactions:

Reaction 1:
$H_2SO_4 + {}^{15}NO_3^-$ (in ^{18}O labelled water) → $H^{15}N^{18}O_3 + HSO_4^-$

Reaction 2:
$H^{15}N^{18}O_3 + KOH + Ba(OH)_2 + SO_4^{2-} \rightarrow K^{15}N^{18}O_3 + H_2O + BaSO_{4(s)} + K_2SO_4 + 2OH^-$

Expected results: If both the ^{15}N and ^{18}O labels are maintained at all positions of recovered NO_3^- then it can be assumed that NO_3^- remains unmodified between AMF acquisition and transfer across the interfacial apoplast from fungi to plant. If two of the three ^{18}O atoms are retained on the extracted NO_3^- then it is likely that NO_3^- is reduced to NO_2^- either before or after crossing the interfacial apoplast. NO_2^- would then oxidize back to NO_3^- in plant tissue. Based on preliminary gene expression analysis [20, 29] this is unlikely. Finally, if only the ^{15}N label is retained it can be ascertained that NO_3^- is likely fully reduced before transfer to the plant with subsequent oxidation to NO_3^- in host tissues.

2B. What is the fate of ornithine produced in the nitrogen transfer process?

In the N transfer pathway described previously, [20, 13] ornithine and urea are produced in the IRM by the breakdown of arginine. Urea is broken down to release NH_4^+ and is transferred to the host. However, the fate of the ornithine is not known. Determining the fate of ornithine will shed light on the total number of N atoms transferred per arginine molecule, hence establishing the efficiency of N transfer. I hypothesize that ornithine produced from the breakdown of arginine is converted to glutamate and subsequently to α-ketoglutarate, sequentially releasing two

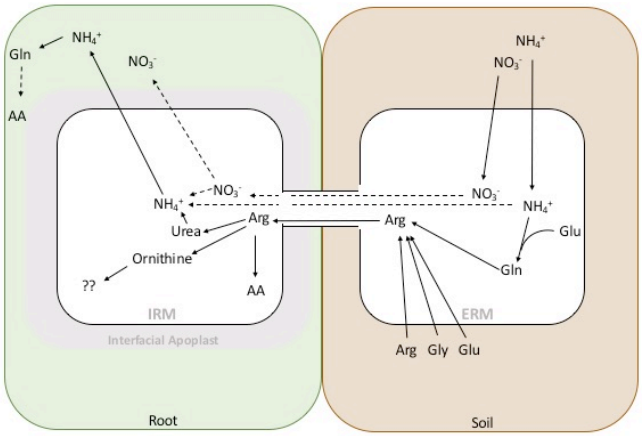


Figure 3: Proposed pathways for N uptake and metabolism. Solid lines represent known pathways, dashed represent hypothesized pathways.

NH_4^+ ions for transfer to the plant.

Approach: This can be assessed by feeding ^{13}C and ^{15}N labelled arginine to AMF (as amino acid carbon atoms have shown to not be transferred to the plant roots) and assessing the movement of carbon through the ERM and IRM by conducting a time course experiment. Alternative fates of ornithine (conversion to polyamines, translocation to the ERM for re-use in arginine synthesis, or breakdown via glutamate and α-ketoglutarate) will be tested by analysis of the labelling time course using LC-MS. The time course will take place over 72 hours to allow sufficient processing of the amino acid input and turnover of the C backbone. Samples will be taken at 0, 2, 4, 6, 8, 12, 24, 48, 72 hours post application of labelled substrate.

Additionally, gene expression analysis conducted with qRT-PCR on root and fungal tissues at 0, 2, 4, 6, 8, 12, 24, 48, 72 hours post application of labelled substrate will allow further verification of possible pathway activities. To assess the possibility of ornithine breaking down into α-ketoglutarate, the genes for ornithine amino transferase 1 and 2 (OAT1, OAT2) will be assayed. Additionally, to test the possibility that ornithine is broken down to polyamines, gene expression analysis will be conducted on ornithine decarboxylase (ODC).

Expected results: The expected outcome of these experiments is the complete transfer of N from arginine resulting in the transfer of N equating to 4 N transferred for every arginine translocated. The first possibility is that ornithine is broken down to α-ketoglutarate. This would be detected by the appearance of ^{13}C labelled TCA cycle intermediates throughout the AMF after the 12 hr time point. The second possible outcome of these experiments is that ornithine is recycled back to ERM from IRM. This would be detected by discovery of ^{13}C ornithine with 2 ^{15}N labels attached on the 24 hr time point. Finally, the third possibility is that ornithine is broken down into polyamines which would result in rapid accumulation of ^{13}C and ^{15}N labelled polyamines in the IRM after the 12 hr time point. All of these results would be further validated by gene expression analysis.

2C. Implications of different N transfer pathways for symbiosis costs

Flux balance analysis (FBA) is a computational method which explores the possible flux patterns that are consistent with the known rates of overall processes, such as growth and the synthesis of metabolic products. Genome-scale reconstructions of metabolic processes are used to define the metabolic reactions of which an organism is capable [41, 42]. This method allows steady-state fluxes in a metabolic network to be predicted by using a constrained solution space informed by biological data and stoichiometric ratios [42]. FBA provides a range of acceptable solutions that enable optimization of a specific function such as growth rate [42]. FBA can be employed to answer questions of function and pathway activity and are especially attractive when the ability to perform molecular genetic manipulations of a system is limited, as the case for AMF.

Previous work has resulted in the development of FBA models for individual organisms that have been beneficial for understanding metabolic fluxes and biomass production capabilities for systems such as *Chlamydomonas reinhardtii* [43]. Although primarily used on single organisms to predict growth under variable conditions, FBA has also been employed to assess costs of symbiotic functions in rhizobial species [44]. One such model was developed for *Rhizobium etli* by simulating microaerobic environments and adding necessary metabolites and reactions to account for N fixation and N export. Models were able to generate preliminary results comparable to empirical data found in the literature [44]. This model was limited in scope due to the inclusion of metabolic processes that are not ubiquitous to all rhizobial species during symbiotic nitrogen fixation [14]. A later expansion of this model used the model rhizobia *Sinorhizobium meliloti* Rm 1021 and modified the metabolic inputs and outputs to be more representative of symbiotic nitrogen fixation by rhizobia as a whole [45]. This model

demonstrates the potential of linking multiple FBA models together to assess optimal pathway fluxes during symbiosis. Using FBA, analysis of the respective activities and costs of multiple N transfer pathways can be analyzed and predictions concerning optimal resource partitioning can be generated.

Approach: First, due to the high conservation of central metabolism within biological kingdoms [46], existing FBA models representing the central metabolism of a mycorrhizal plant and the central metabolism of fungi will be adopted and modified to generate the plant and fungi models, respectively. These two existing models will provide helpful starting material (i.e. reaction lists inclusive of critical metabolic processes such as the tricarboxylic acid cycle, nucleic acid synthesis, glycolysis, etc.). As a foundation for the plant component, the FBA model presented by Grafarhrend-Belau et al. [47] for barley (*Hordeum vulgare*) metabolism will be used. The *in silico* model will be improved by conducting a survey of the *Medicago truncatula* genome and corresponding literature to ensure that pathways necessary for P and N uptake and transport are well-defined. The corresponding system of mass balance equations will be derived from a network of *n* reaction and *m* metabolites that will ultimately be represented as the stoichiometric matrix *S* (*m* x *n*). Additionally, a flux vector *v* will be used to represent *n* metabolic fluxes. Assuming the system is in steady-state, this results in the system being represented as:

$$S \times v = 0$$

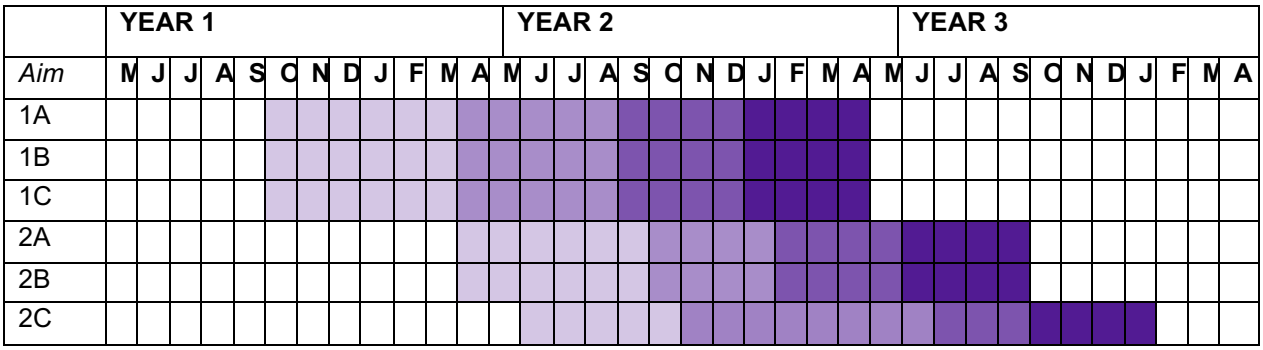
The individual fluxes will each be constrained by lower and upper bounds. FBA uses linear programming to solve the resulting system of equations by searching for solutions within the allowed space to either minimize or maximize the defined objective function. Here, the objective function will be defined by biomass production with strict constraints placed on N to simulate conditions of interest.

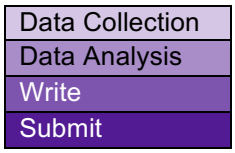
Next, the fungal model with which the theoretical plant will interface will need to be constructed. FBA models of yeast (*Saccharomyces cerevisiae*) have been developed [48] and additional refinements have been published in recent years [49, 50]. AMF belong to an early-diverging monophyletic clade [15] but the genome of the model AM fungus *Rhizophagus irregularis* shows the conservation of all major pathways of central metabolism. The AMF network will be extended based on the genomic annotation provided by Tisserant et al. [19]. Using this annotation, additional components necessary for the two organisms to interface will need to be included such as phosphorus transporters and transmembrane ion transporters. FBA models are improved by the addition of experimental flux measurements to enable more realistic predictions of the capabilities of a system than what can be assumed from genomic and transcriptomic data alone. Incorporation of experimental data from proposed aim 1 (above) will serve as a complement to the limited existing literature data. Once the model is constructed, the objective function will be set as biomass maximization and the system of equations will be constructed as described above. Simulations will be performed in Python using the COBRA toolbox [51, 52].

Expected results: Implementation of an *in silico* model allows investigation of pathway activities that are difficult or impossible in AMF. A genome-scale metabolic reconstruction will allow for a cost: benefit analysis to be conducted for the two proposed paths of N movement from AMF to host plants (via arginine and using nitrate) and shed light on preferred uptake and transfer pathways of N when AMF have access to both NH_4^+ and NO_3^-. Furthermore, manipulation of system constraints can allow for additional investigation of pathway activities and costs to the host plant under various nutrient-limited conditions such as P-limitation. Once implemented, these connected FBA models could further be expanded to model tripartite associations between host legumes, AMF, and rhizobia.

Construction of an *in silico* model has the potential to unveil redundancies and/or deficiencies in the AMF genome. The simulation of metabolism may unveil metabolic deficiencies in AMF that are supplemented by a host plant. The physiological basis of obligate biotrophy in AMF is still unknown and other questions regarding metabolic capabilities are worthy of further investigation. Development and implementation of a model assessing symbiotic costs between AMF and host mycorrhizal plants has the potential to inform future hypotheses and research in the field.

TIMELINE





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