Belowground plant strategies for improved carbon sequestration in diversified agroecosystems

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Abstract

Belowground plant strategies for improved carbon sequestration in diversified agroecosystems
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Diversified agroecosystems supporting greater genetic, structural, and functional diversity improve soil health and ecosystem function. However, there is limited understanding of how multiple forms of diversification, such as mixing cover crop species and adding arbuscular mycorrhizal fungi (AMF), alter belowground carbon supply to soil. In a controlled environment experiment using rhizoboxes, I investigated the belowgound response of cover crops – red clover (*Trifolium pratense*) and barley (*Hordeum vulgare*) – grown in monoculture or mixture, with and without AMF inoculation. Root morphological and mycorrhizal traits that characterize the hypothesized root economics spectrum (RES) were integrated with novel sampling of dissoved organic carbon fluxes and easily extractable glomlin in rhizosphere soil. Results revealed species-specific shifts on the RES suggesting that diversification through species mixing and AMF additions can alter belowground carbon allocation pathways, with potential implacations for plant performance and soil carbon stabilization in agroecosystems.

Keywords: root traits, root economic spectrum, arbuscular mycorrhizal fungi, belowground strategies, carbon sequestration, agroecocsytems, diversified agroecosystems, rhizoboxes, cover crops, easily extractable glomalin, microdialysis

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List of Abbreviations

AMF – Arbuscular Mycorrhizal Fungi

C:N – Carbon to nitrogen ratio

D – Average diameter

DOC – Dissolved organic carbon

EEG – Easily extractable glomalin

N- Nitrogen

RES – Root economic spectrum

RTD – Root tissue density

SOC- Soil organic carbon

SRL – Specific root length

SRTA – Specific root tip abundance

TG – Total glomalin

1.0 Introduction

Agricultural soils provide a tremendous opportunity for global climate change mitigation efforts. Agricultural soils store more carbon than vegetation and the atmosphere combined (Georgiou et al., 2022), but intensive practices of monocultural production have depleted soil organic carbon (SOC) stocks in agroecosystems (Emilia Hannula & Morriën, 2022). Monocultural production relies on excessive use of external inputs, like synthetic fertilizers and pesticides, and disruptive soil management practices, like intensive tillage, which accelerate SOC decomposition and loss to the atmosphere. Depletion of SOC in agroecosystems indicates a decline in soil health and results in large amounts of carbon released into the atmosphere via microbial decomposition and erosion (Guzman et al., 2021). Soil health is the capacity of the soil to function as a living ecosystem which can sustain flora and fauna – this is done by supporting productivity, regulating water and nutrient cycles, and maintaining biodiversity (Lehmann et al., 2020). These soils depleted of SOC provide opportunities for agroecosystems to act as global carbon sinks. If managed properly, agricultural soils offer a pathway to enhanced soil health and assist in climate change mitigation efforts (Emilia Hannula & Morriën, 2022; Treseder & Allen, 2000).

Transitioning from extractive to regenerative agricultural practices requires agroecological diversification – the strategic integration of various plant species, varieties, and functional types in order to promote more genetic diversity, structural complexity, and enhanced ecosystem services (Hufnagel et al., 2020). Compared to monocultural ecosystems, diversified agroecosystems demonstrate superior soil health characterized by greater biodiversity, improved soil structure, and an increase in organic matter (Tamburini et al., 2020). One increasingly common practice to diversify croplands is through the use of cover crops.

Cover crops are plants grown specifically for soil health improvements rather than as a cash crop. Cover crops can contribute carbon directly through release of organic compounds from active roots and through decomposition of roots and aboveground biomass, with additional benefits of improved soil structure, a reduction in erosion, enhanced water retention, and the improved capacity to increase soil nitrogen for subsequent (Chapagain et al., 2020; Crotty et al., 2016). Recent research has highlighted their substantial potential for carbon sequestration, making them a key tool in regenerative and diversified agriculture (Chahal et al., 2020; Zhu et al., 2024).

In addition to cover crops providing direct plant benefits, they can also increase biodiversity and abundance of soil organisms (Kim et al., 2020), including arbuscular mycorrhizal fungi (AMF), compared to monocultural systems without cover crops. AMF are fungi that form mutualistic relationships with plants – AMF hyphae penetrate the root systems to facilitate nutrient exchange (Hawkins et al., 2023). Not only do these fungi enhance nutrient acquisition for their host plant, but they contribute directly to soil carbon through their extensive belowground hyphal networks and production of carbon compounds (Wang et al., 2016).

Recent research on cover crops has mainly focuses on crop yields, soil physical properties, and nutrient management. To understand how cover crops and AMF communities build soil carbon, the focus must shift towards belowground processes that drive carbon inputs. Belowground processes, not aboveground litter inputs, are the main drivers for soil organic carbon inputs (Berhongaray et al., 2019; Dijkstra et al., 2021). Living roots contribute to soil carbon via exudation and respiratory processes, whilst decomposing root materials directly release carbon compounds through enzymatic and microbial decomposition processes that break down organic matter into simpler carbon forms (Kumar et al., 2006). These belowground carbon inputs are mediated by plant root traits – measurable functional attributes that are standardized across species and environments and serve as indicators of how plants allocate carbon belowground (Violle et al., 2007).

The "root economic spectrum" is a framework that provides a lens to understand how plants are allocating their resources (de la Riva et al., 2021). The framework outlines two hypothesized gradients in plant root strategies: the conservation gradient and the collaboration gradient. The *conservation gradient* highlights a trade-off between expedited resource acquisition and resource conservation – from "fast" acquisition strategies focused on rapid growth, and "slow" strategies prioritizing longevity and greater defenses (Bergmann et al., 2020). The *collaboration gradient* reflects a trade-off between "do-it-yourself" strategy that relies on extensive root systems to acquire resources, to "outsourcing" strategies with microbial associations (Freschet et al., 2021). When grown in diversified ecosystems, plant communities exhibit significant phenotypic plasticity in root trait expression when compared to monoculture systems (Levine et al., 2025). This phenotypic plasticity is a reflection of a plant's ability to adjust their acquisition strategy along the root economic spectrum, including both the conservation and collaboration gradients, in response to environmental gradients. Whilst the root economics spectrum is founded on data of interspecific variation, more attention is focused on intraspecific variation, including for agroecological applications (Herrick & Blesh, 2021). Intraspecific plasticity has important implications for agroecological management strategies, particularly in diversified systems where plant-plant and plant-soil interactions are complex. Understanding links between root traits and ecosystem services can inform more sustainable management of agroecosystems. Enhanced understanding of intraspecific trait variation could improve management of agroecosystems, especially through the strategic use of cover crops (Herrick & Blesh, 2021). For example, understanding how cover crop species vary along the economic spectrum can provide practitioners/farmers with the knowledge required to select species with complementary root strategies to enhance resource use efficiency and carbon sequestration (Finney & Kaye, 2017).

Whilst past research has demonstrated that diversification can shift plant strategies along the conservation gradient of the root economic spectrum, much less is known about how agroecosystem diversification influences plant strategies along the collaboration gradient. Critical gaps have recently been identified in understanding how AMF associations withing cropping systems influence plant resources strategies and the ecological processes that govern AMF community dynamics (Conti et al., 2025). Because AMF play a central role in belowground carbon inputs and glomalin production, this knowledge gap has important implications for soil carbon dynamics. At the same time, it remains unclear how cover crop diversification alters root traits, and how root trait expression may shift jointly along both axes of the root economic spectrum when AMF associations change. Clarifying these linkages is important because root and mycorrhizal strategies influence the magnitude and form of belowground carbon contributions—for example through root exudation, AMF hyphal networks, or decomposition processes—yet direct evidence for such mechanisms in agroecosystems is still limited beyond root respiration measures (Borden et al., 2021).

1.1 Study objectives and hypotheses

I established a controlled environment study that simultaneously investigated two forms of agroecological diversification: integration of cover crops and addition of AMF inoculation. Two species of cover crops (barley [*Hordeum vulgare*] and red clover [*Trifolium pratense*]) were grown with a conspecific neighbour (i.e., cover crop monoculture) or a heterospecific neighbour plant (i.e., cover crop mixture), and either without or with AMF inoculation. The aim of my MSc research was to assess how agricultural diversification influences cover crop root trait strategies related to belowground carbon allocation. Specific objectives were to: (1) evaluate the effects of plant species mixtures versus monocultures on root trait expressions; and (2) to determine how AMF inoculation alters root trait strategies and carbon input pathways. I hypothesized that:

1. Plant species diversification increases competitive interactions, leading to shifts in root trait strategies toward acquisitive traits.
2. AMF inoculation reduces plant reliance on direct resource acquisition by providing access to spatially distant nutrients via fungal networks, leading to shifts toward more conservative root strategies.
3. Shifts in belowground plant strategies alter pathways of root-driven carbon inputs to soil through changes in root-microbe interactions.

I predicted that:

1. Barley and red clover grown in mixtures will exhibit traits indicative of acquisitive strategies – plant roots characterized by, as examples, greater specific root length (SRL) and lower root tissue density (RTD) – when compared to monoculture plants.
2. Plants inoculated with AMF will exhibit traits indicative of conservative strategies, characterized by lower SRL and higher RTD.

3. Changes in root trait strategies will correspond to measurable differences in soil carbon indicators, such as glomalin content.

1.2 Literature review

1.2.1 Cover crops

Cover crops are non-harvested plant species that are integrated into cropping systems to provide ecosystem services rather than direct economic revenue from harvest. Unlike cash crops, cover crops are grown specifically to be left unharvested, with their residues returned to the soil to build long term soil health and improve yields of subsequent cash crops. Cover crops function as temporary cover in agroecosystems during fallow periods when fields are more susceptible to leaching of nutrients via groundwater, representing a fundamental shift from simple monocultural systems to more functionally diverse agroecosystems (Drost et al., 2020). The implementation of cover crops increases plant species richness and functional diversity within agroecosystems, therefore plants play a crucial role in shaping multitrophic interactions (Beaumelle et al., 2021). Additionally, agroecosystem diversity through cover cropping creates complex plant-soil-microbe interactions that enhance system resilience to environmental stressors, including drought, temperature fluctuations, and pathogen pressures (Adetunji et al., 2020; Beaumelle et al., 2021).

Cover crops function as biological nutrient capture and recycling systems, reducing environmental losses in the soil while improving nutrient use efficiency in agroecosystems. Cover crops do so by taking up mobile nutrients, such as nitrogen, during periods where cash crops are absent. They store the nutrients in plant biomass and then gradually release them as their residues decompose. Their effectiveness at nutrient retention can vary with species function traits, biomass production, and C:N ratios (Koudahe et al., 2022). Grass cover crops, such as barley, are used for reducing soil erosion and improving soil structure, which can be attributed to their fine and fibrous root structures (Koudahe et al., 2022). Grass cover crops have the ability to absorb substantial amounts of nitrogen from the soil, thus decreasing the risk of nitrate leaching (Koudahe et al., 2022). Additionally, grass cover crops offer a myriad of improvements to soil properties, namely lowering bulk density, enhancing soil stability, and increasing water infiltration and storage (Koudahe et al., 2022). Notably, they are highly effective at preventing soil erosion due to wind and water (Koudahe et al., 2022).

Leguminous cover crops contribute significant quantities of biologically fixed nitrogen through symbiosis with bacteria in root nodules (Koudahe et al., 2022). Through symbiotic relationships with rhizobacteria, leguminous cover crops fix atmospheric nitrogen and incorporate it into their tissues. Upon termination and incorporation into the soil via tillage, crimping or mowing, these leguminous cover crops release fixed nitrogen through decomposition, thus directly supplying nitrogen for the following planted crop (Koudahe et al., 2022). Agroecosystems which have gone through decades of crop production often lead to soils low in nitrogen – leguminous cover crops provide a natural means of sequestering nitrogen, thus highlighting leguminous cover crops as a possible important addition to sustainable agriculture (Koudahe et al., 2022).

Cover crops grown in mixture, rather than monoculture, can provide multiple ecosystem services by mixing complementary functional traits together. Notably, legumes are often combined with grasses to lower the C:N ratio of cover crop residues, which promotes quicker decomposition and minimizes the risk of nutrient immobilisation (Koudahe et al., 2022). A mixture of legumes and grasses can also facilitate interactions between the two functional types, with the legume fixing nitrogen and making it available to the grass (Koudahe et al., 2022).

1.2.2 AMF in agroecosystems

Arbuscular mycorrhizal fungi (AMF) are a group of fungi from the phylum *Glomeromycota* that form symbiotic relationships with approximately 80% of terrestrial plants (Lee et al., 2013). Whilst AMF are proven to be mutualistic with a myriad of plant species, AMF themselves have a low diversity, with only about 332 species currently described (Větrovský et al., 2023). AMF are known to be obligate symbionts, meaning that the totality of their life cycle is dependent on their host plant (Jones & Smith, 2004). Indeed, studies have demonstrated that in the absence of a host plant, AMF germ tubes barely extend from the spores (Bécard & Piché, 1989; Jones & Smith, 2004), but when attached to a host plant, AMF produce mycelia at a much higher rate. Additionally, fruiting bodies are only produced when attached to a photosynthetically active host plant (Jones & Smith, 2004). However, despite apparent generalist nature, different AMF species exhibit varying degrees of host specificity (d'Entremont & Kivlin, 2023). Some AMF species form preferential association with certain functional plant groups, while others are more adapted to specific soil conditions (d'Entremont & Kivlin, 2023). This functional variation highlights why promoting AMF diversity in agroecosystems can be beneficial; a wider range of associations improves crop responsiveness, resilience to soil variability, and ecosystem services such as nutrient cycling and soil stability (Shukla et al., 2025).

AMF hyphae move through the soil toward plant roots via chemical signalling. The hyphae penetrate the root epidermis and enter root cells where they form arbuscles – highly branched structures that serve as the primary site of nutrient exchange (Hawkins et al., 2023). In return for receiving nutrients such as nitrogen and phosphorus, the host plant provide approximately 20% of their photosynthate to support AMF growth (Courty et al., 2015; Lee et al., 2013; Zhai et al., 2021). This symbiotic relationship creates a beneficial cycle of exchange between plant and AMF networks that enhances resource acquisition for both partners.

Beyond the root interface, AMF also extend extensive hyphal networks into the soil, increasing the plant's root surface are. These external hyphae can penetrate into deep soil layers that plant roots may not reach, which provides a continuous supply of nutrients to the hose plant (Lee et al., 2013). Nutrients that are typically beyond the plant's root zone, such as inorganic phosphate, can be more effectively accessed by the AMF hyphal network (Chen et al., 2022).

The enhanced nutrient uptake improves plant nutrient acquisition when soil nutrient availability is low, thus increasing productivity in low-nutrient agricultural systems (Sosa-Hernández et al., 2019). AMF can provide plants with up to 90% of required phosphorus and 60% of nitrogen requirements, while also enhancing their uptake of sulfur, potassium, iron, copper, manganese, and zinc (Khan et al., 2022; Lee et al., 2013). In return, host plants allocate up to 20% of their photosynthetically fixed carbon to support AMF growth, hyphal extension, and reproduction (Parniske, 2008). This carbon cost is generally outweighed by the nutritional and physiological benefits of AMF symbiosis, making the association advantageous for plant growth, especially in soils with limited resources.

This continuous mutualistic relationship may reduce soil nutrient losses from agricultural fields by expanding the area from which nutrients can be accessed by plants. For mobile nutrients like nitrogen that are prone to leaching, AMF networks can capture nutrients from a larger soil volume and retain them during periods where crops are not actively growing. For immobile nutrients like phosphorus, AMF hyphae can access phosphorus pools beyond the root depletion zone. In cash crop systems, this enhanced nutrient capture and retention could potentially reduce fertilizer application rates as soils retain more nutrients over time (Sosa-Hernández et al., 2019). Increased carbon exchange from plant to fungi can also occur with AMF colonization. Mycelia represent the hypothesized pathway through which carbon moves from the plant into the soil (Hawkins et al., 2023). AMF obtain all of their required carbon from the host plant, storing a large portion of this organic carbon in the soil as chitin or glomalin related proteins (Parihar et al., 2020). Through this mutualistic exchange of nutrients and carbon, AMF increases soil organic carbon (Erdel et al., 2023).

Few studies have examined the interaction and symbiosis between cover crops and AMF, with limited research focusing on plant species-specific associations and their effects on subsequent crops. Ortas and Yucel (2020) examined the impact of mycorrhizae on cover crop biomass grown in both single-species and mixed cover crop treatments, using either sterile or non-sterile soils. Their study revealed significant effects of AMF inoculation on both single and mixed cover crop rotations. AMF colonization increased cover crop biomass in both sterile and non-sterile soils, with higher colonization rates observed in sterile soils, likely due to reduced competition from native AMF spores and other soil microorganisms (Ortas & Yucel, 2020).

1.2.3 Belowground inputs of carbon in soil

1.2.3.1 Root inputs

Our understanding of belowground processes as the primary driver for soil organic carbon accumulation has fundamentally transformed our comprehension of carbon cycling in terrestrial ecosystems (Dijkstra et al., 2021a). Root-derived carbon has greater permanence in soil than aboveground litter inputs due to its close association with soil minerals and microbial communities, which reduces CO_2 loss compared to the rapid breakdown and CO_2 release characteristic of aboveground inputs (Cotrufo et al., 2013).

Root systems contribute to soil organic carbon through multiple pathways. Living roots actively release carbon through rhizodeposition, which includes root exudation, cell sloughing, and mucilage production (Omelyanchuk et al., 2024). Root exudates, including organic acids, amino acids, and simple sugars, can account for 20-40% of a plant's photosynthetically fixed carbon investments (Canarini et al., 2019). These exudates not only provide carbon inputs, but assist in nutrient mobilization, pH modification, and microbial community regulation in the rhizosphere (Haichar et al., 2014).

Another pathway for belowground carbon input is that of root decomposition. Unlike aboveground litter, root litter is in direct contact with soil as it decomposes - this causes the formation of mineral associated organic matter, which is a considered relatively stable form of SOC compared to particulate organic matter (Fulton-Smith et al., 2024). Root mortality and decomposition rates have significant variance between species and functional types, but fine roots tend to have higher turnover rates than their coarse counterparts (McCormack et al., 2015). Thus, in more diversified agroecosystems possessing a mixture of different roots, decomposition of roots will contribute to both slow and fast carbon pools (McCormack et al., 2015).

1.2.3.2 AMF Inputs

AMF represents a critical but often under analyzed component of belowground carbon cycling. As obligate symbionts, AMF receive up to 20% of the host plants photosynthetically fixed carbon in exchange for their nutrient acquisition services, though this allocation varies depending on the host plant and its growing environment (Treseder & Allen, 2000). Additionally, AMF contributes to soil carbon in other ways which extend beyond carbon exchange with their host plant.

AMF form extensive hyphal networks belowground which produce substantial amounts of carbon inputs to the soil. Hyphae radiate outward from the host plant, forming extensive networks that can extend from tens of centimetres to over a meter in the soil, creating a vast network that can account for 15-30% of soil microbial biomass (Allen, 2007; Kakouridis et al., 2024). These hyphae contribute to soil carbon inputs through multiple mechanisms: direct hyphal turnover, exudation of organic compounds, and the production of specialized proteins and glycoproteins (Rillig & Mummey, 2006).

Glomalin related proteins are produced exclusively by AMF, representing an important pathway for long-term carbon storage. AMF are the only known producers of glomalin, a unique glycoprotein that is released by the spores and hyphae that significantly improves soil structural stability (Emilia Hannula & Morriën, 2022). Glomalin is a hydrophobic glycoprotein contain a significant amount of carbon (30-40%) and nitrogen (3-5%) (Emilia Hannula & Morriën, 2022). Its recalcitrant molecular structure makes it difficult for decomposer organisms and enzymes to access, giving it high resistance to decomposition (Masebo et al., 2025). This enables glomalin to act as a stable contributor to soil organic matter (Masebo et al., 2025). There are two distinct types of glomalin which can be measured: easily extractable glomalin (EEG) and total glomalin (TG). EEG can be defined as recently deposited glomalin compounds that can be extracted using a milder extraction process, while TG encompasses the totality of extractable glomalin. Both recently deposited and older more recalcitrant forms which require harsher extraction methods (Irving et al., 2021).

There is great potential for glomalin as a potential soil reservoir for SOC. In undisturbed soils, glomalin can contribute up to 25% of SOC stocks, highlighting its substantial contributions to soil carbon pools (He et al., 2020; Singh et al., 2024). However, agricultural tillage practices influence glomalin accumulation, as the practice of tilling disrupts and destroys AMF hyphal networks, leading to lower glomalin content (Nthebere et al., 2025). Research by Gałązka et al. (2017) demonstrated that glomalin content was positively associated with enhanced microbial enzyme activities and improved physical soil properties, such as greater aggregate stability and water holding capacity. Agroecosystems that employ regenerative practices – agricultural approaches focused on rebuilding soil health and enhancing biodiversity – show varying glomalin content dependent on management intensity. Systems under reduced or no-tillage management generally show higher glomalin content when compared t o conventionally tilled soils, as the undisturbed hyphal networks continue to promote increased soil microbial activity and enzymatic activities (Gałązka et al, 2017).

1.2.4 Trait-based research

1.2.4.1 Root economic spectrum

A trait-based approach is a method that focuses on measurable characteristics of plants. It can be used to conceptualize allocation of a plant's resources, highlighting trade-offs between survival and expedited growth of the plant. This trade-off shapes whether plants invest in traits that prioritize rapid resource acquisition and growth, or in traits that enhance persistence and longevity (Weemstra et al., 2016). For example, the leaf economic spectrum conceptualizes trade-offs between short lived leaves, which tend to have faster nutrient investments, and long-lived leaves, which prioritize defence and longevity over expedited investments (Reich, 2014).

The root economic spectrum represents differences in plant resource allocation and ways in which plants attain nutrients, whether it be through roots or via fungal associations. Recent studies conceptualise the root economic spectrum by breaking it down between the conservation gradient and the collaboration gradient.

The conservation gradient describes trade-offs in root trait strategies, ranging from fast to slow resource investment (Bergmann et al., 2020). Root systems with fast resources investment strategy are characterized by high metabolic activity and acquisitive traits: they tend to have lower root tissue density (RTD) and higher root nitrogen content, enabling rapid resource uptake but with shorter tissue lifespans (Rutten & Allan, 2023; Weigelt et al., 2021). At the opposite end of the conservation gradient are roots with a slow investment strategy. Roots on this end of the gradient emphasize persistence and greater defense, showing a high RTD, lower root nitrogen content, and longer tissue lifespan – this reflects a strategy that is focused on conservation rather than rapid resource acquisition (Bergmann et al., 2020; Weigelt et al., 2021).

The collaboration gradient is a more recently identified dimension in plant ecology (Bergmann et al., 2020). The collaboration gradient ranges from "do-it-yourself" to outsourcing. Plants on the "do-it-yourself" end of the spectrum invest in roots that explore soil directly, typically characterized by high specific root length (SRL), thin diameters, and extensive branching that enables efficient nutrient foraging without microbial assistance (Weigelt et al., 2021). In contrast, "outsourcing" plants rely more heavily on mutualistic associations with AMF. The plants on the "outsourcing" end of the gradient produce thicker roots with a larger cortical area to accommodate AMF hyphae, along with lower SRL and reduced branching (Bergmann et al., 2020). In this strategy, nutrient acquisition is delegated to the fungal network, which can explore the soil beyond the roots surface to forage for nutrients (Weigelt et al., 2021). The collaboration gradient highlights how root morphology reflects different nutrient acquisition strategies which are independent of aboveground plant traits.

1.2.4.2 Root traits that define the root economic spectrum and carbon allocation

There are many root traits that are measured as indicators of plant processes summarized in several review papers in the plant trait literature (Freschet, Pagès, et al., 2021; Freschet, Roumet, et al., 2021; Weemstra et al., 2016). For my MSc research, I selected a suite of traits commonly integrated into the RES and are associated with carbon allocation patterns. I also integrated two novel “rhizosphere traits” that can serve as indicator of direct influence of roots on carbon dynamics in soil. Traits measured are summarized in Table 1.

1.2.4.3 Diversification effects on trait expression

Plasticity reflects a plants' ability to adjust their resource acquisition strategies along the root economic in response to competitive growing environments and collaborative opportunities observed in grass-legume cover intercropping systems (Bukovsky-Reyes et al., 2019; Massa et al., 2025). Cover crops exhibit significant phenotypic plasticity in root trait expression when they are grown in diversified agroecosystems versus in monoculture systems (Wendling et al., 2017). However, belowground responses tend to vary between functional groups whilst additionally being influenced by AMF associations (Li et al., 2017; Darch et al., 2022).

Arbuscular mycorrhizal fungi alter cover crop root trait expression through direct physiological effects and by mediating competitive environments in mixed plantings (Chen et al., 2021). The magnitude and direction of these effects will vary with soil conditions, plant functional groups, and the intensity of mycorrhizal colonization.

AMF colonization alters carbon allocation patterns in the host plants, typically resulting in increased root biomass investment and a shift toward more collaborative root architecture strategies (Wang et al., 2023). Mycorrhizal plants often exhibit distinct morphological traits when compared to non-mycorrhizal counterparts, such as lower SRL, reduced branching, and sometimes smaller diameter, reflecting a shift in resource allocation due to the metabolic cost and benefit of maintaining fungal partnerships (Chen et al., 2021; Liese et al., 2017).

The magnitude of AMF effects on root trait expression varies between plant functional groups. Nitrogen-fixing legumes show particularly strong responses to mycorrhizal inoculation, often significantly increasing root biomass when AMF partnerships have been established (Gorgia & Tsikou, 2025; Wahab et al., 2023). This response reflects the high phosphorus demands of the nitrogen fixation processes whereby mycorrhizal phosphorus acquisition becomes critical for nodulation and fixation efficiency (Scheublin et al., 2004)

Grass crops also respond to AMF inoculation, particularly in phosphorus-limited soils, though their responses are typically less pronounced than those observed in legumes (Sui et al., 2022). AMF associations in grasses typically reduce the need for extensive fine root proliferation and root tip abundance when compared to non-mycorrhizal plants, as the hyphae extend the effective foraging range for nutrients; this especially helps with the acquisition of phosphorus in nutrient poor soils. (Campos et al., 2018).

Table 1. Summary of plant functional traits sampled, including units, descriptions, and significance.

Trait	Abbreviation	Units	Description	Significance
WHOLE PLANT TRAITS				
Root dry mass	—	g	Total dry weight of roots	Reflects belowground biomass allocation and resource investment
Root:Shoot	–	Unitless	Ratio of root to shoot biomass	Indicates resource allocation strategy
MORPHOLOGICAL TRAITS				
Specific root tip abundance	SRTA	tips g^{-1}	Number of root tips per unit dry mass	Related to AMF colonization and nutrient acquisition
Average root diameter	D	mm	Mean diameter of root length	Indicates root function and mycorrhizal relationships
Specific root length	SRL	g^{-1}	Fine root length per unit dry mass	Indicates root lifespan, soil exploration, and resource uptake
Root tissue density	RTD	mg cm^{-3}	Root dry mass per unit volume	Associated with longevity, stress tolerance, and resource strategy
MYCORRHIZAL ASSOCIATION TRAITS				
Mycorrhizal colonization count	—	Count	AMF colonization of roots	Higher values indicate greater symbiosis, water and nutrient uptake
RHIZOSPHERE TRAITS				
Dissolved organic carbon fluxes in rhizosphere soil	DOC flux	g C m^{-2} hr^{-1}	Flux of dissolved carbon-containing compounds in rhizosphere soil solution	Indicates microbial signaling, nutrient acquisition, and root activity
Easily extractable glomalin	EEG	µg g^{-1}	Easily extractable glomalin per unit of dry weight soil	Represents glomalin soil proteins recently deposited into the rhizosphere. Binds particles together to form stable soil aggregates, promoting greater soil stability and water infiltration. Directly adds to soil carbon storage.

Intraspecific root trait variation has been observed in plants grown in diversified versus monoculture agroecosystems, with plants in diversified systems often expressing altered root morphology and nutrient acquisition strategies (Garnier & Navas, 2012; Isaac & Borden, 2019). However, there is limited research on how layered diversification, such as the addition of AMF with cover cropping mixtures, influences expressed root trait strategies and their contributions to nutrient foraging efficiency.

2.0 Methods

2.1 Experimental design

To evaluate the effect of different forms of diversification on belowground plant trait strategies, I established a rhizobox experiment to investigate three ‘cover crop diversification’ treatments and their interactions: I) two cover crop species (“species”) that are functionally distinct; II): the cover crops species with conspecific (i.e. monoculture) or heterospecific (i.e. mixture) neighbours (“MixORMono”); and III): with or without the addition of arbuscular mycorrhizal fungi (“AMF”). Each treatment combination and the control had three replicates (Figure 1.).

I selected the cover crops species barley *(Hordeum vulgare)* and red clover *(Trifolium pratense)*. These two species are commonly used cover crops of distinct functional types, with barley being a grass and the clover a legume, and both species have been shown to associate with AMF (García-González et al., 2016). These species were selected has they are commonly used in Ontario, both in monoculture and in mixture, and because of the aforementioned distinction between the two functional types.

2.2 Rhizobox set up

Cover crops were grown in custom built rhizoboxes (30 cm × 18 cm × 2.5 cm; transparent acrylic) (Figure 2); in each box, two plants grown with either the same species (conspecific neighbour) or the other cover crop species (heterospecific neighbour). The control rhizoboxes had no cover crops planted. Plants were grown from seed in plugs using soil collected from Trent Farm (Appendix Table 1) before being transplanted into rhizoboxes and planted 5 cm from each side of the rhizobox.

Soil used in the experiment was collected from Trent Experimental Farm (44.3627° N, 78.2819° W), Peterborough, Ontario, Canada, was passed through 2 mm sieves, homogenized, and gravimetric soil moisture content was estimated on a subsample through oven drying at 105 ˚C for 48 hours. Soil was added to each rhizobox at a consistent bulk density of 1.32 g cm^{-3}, by incrementally adding soil to set depths in the rhizoboxes until reaching final volume and a total of 1.78 kg soil, on a calculated dry weight basis. Soil was composite sampled and sent for a suite of baseline analyses (A&L Laboratories). At the start of the experiment, soil had 3.5 % organic matter, 11 ppm phosphorus (B-P1), and potassium 78 ppm (see Appendix Table 1).

For rhizoboxes with AMF, plants were inoculated with AMF mixture of fungi from the order Glomerales (ARBUSCULAR MYCORRHIZAL FUNGI, Optimize Organics, Kelowna, BC). 70 g of inoculant was applied to the top of the soil (Qin et al., 2022). When seedlings were transplanted into the rhizoboxes, roots were planted within this layer (Martin et al., 2012). Previous studies show Glomaceae are the most abundant and adaptable fungi in their symbiosis of cover crops and associate specifically with red clover and barley (Blackwell & Spatafora, 2004: Ducousso-Détrez et al. 2022).

Plants were grown in growth chambers at Trent University in Peterborough, Ontario (Figure 3). Plants were exposed to a cycle of 16 hours of light and 8 hours of darkness (Kumar et al., 2020, 2023). Daytime temperature reached a high of 26 ˚C with nighttime temperatures of 18 ˚C: night temperature gradually rose throughout the day, with 26 ˚C being reached at 13:00, remaining there for three hours before gradually decreasing to night temperatures. The relative humidity was set to 50 % (Newton, 1993; Saadat et al., 2023). Growth chambers parameters may have ± 1 ˚C variation. Rhizoboxes were positioned at a 45˚ angle to ensure plant growth along the front panel of the rhizobox (Bodner et al., 2019) and covered with opaque wrap to prevent light exposure to the sides (Figure 3). Growth chamber light intensity was measured which revealed some variation. To mediate this variation of intensity, rhizoboxes where randomly shifted to new positions every seven days. Planting and subsequent sampling was staggered over three weeks (weeks of September 17th, September 24th, and October 1st, 2024) to allow for detailed sampling at specific growth stages.

2.3 Plant trait sampling

I sampled a suite of traits above and belowground that relate to key plant functions and impacts on the environment, such as nutrient and carbon cycling (Table 1). I sampled plants at peak biomass, which is when the plant has the largest biomass and begins to flower; for *Hordeum vulgare* and *Trifolium pratense* this is 80-90 days after emergence (Malhi et al., 2006; Manderscheid et al., 1997). The details summarized of how I sampled this suite of traits can be found in Table 1.

2.3.1 Whole plant traits

Aboveground mass, belowground mass, and total biomass was measured on a per plant basis. Wet weights were taken, followed by dry weights after roots and aboveground mass was oven dried for 3 days at 60 °C. Dry weights were added together to calculate total biomass. Separate weights of fine roots (orders 1-3) and excluded root portions were taken. The fine roots, orders 1-3, can be defined as absorptive roots which are associated with resource. 1st order roots are unbranched segments which are the finest branches in the root system (Freschet, Pagès, et al., 2021; McCormack et al., 2015). Second order roots are the parent-order roots in which the 1st order roots branch – these two orders are the primary absorptive roots (Freschet, Pagès, et al., 2021; McCormack et al., 2015). Third order roots begin to show transitional structures between

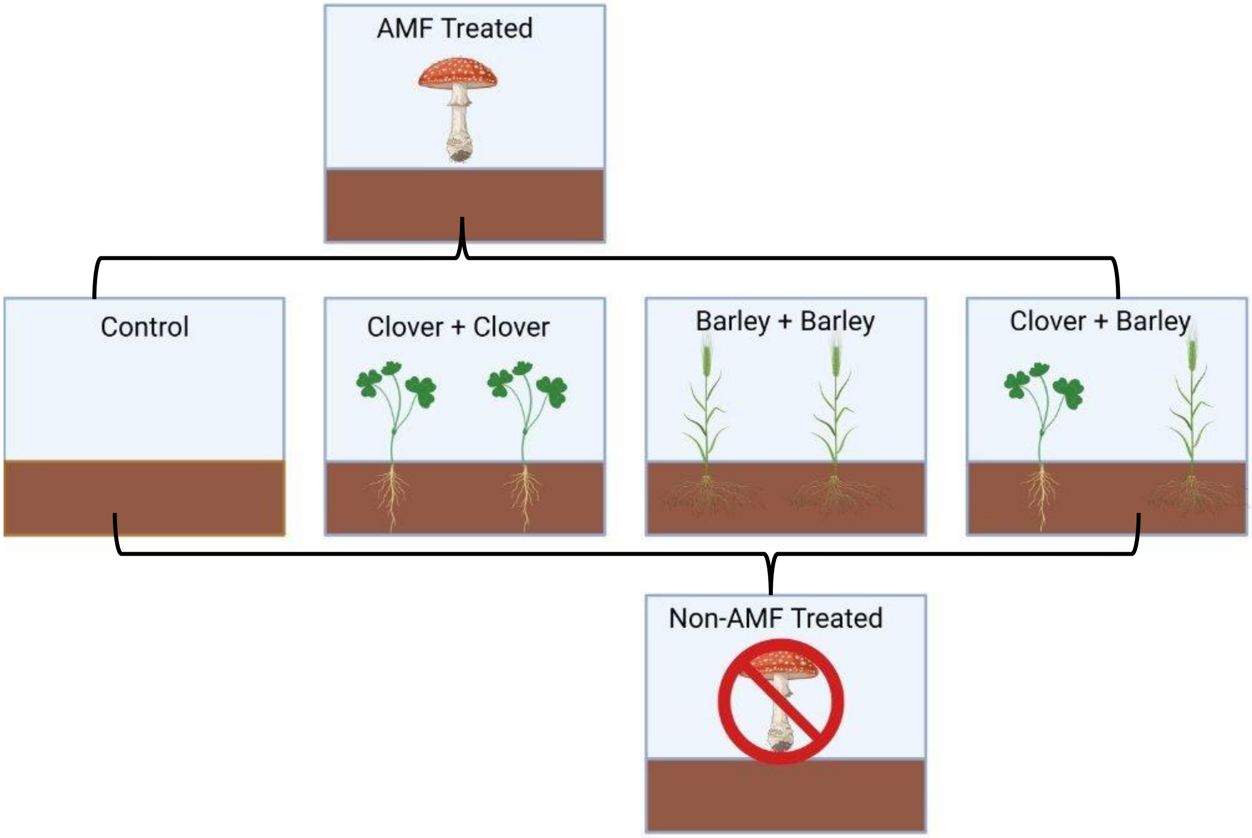

Figure 1. Treatment breakdown replicated three times. n=3 for plants in mixture, n=6 for plants in monoculture.

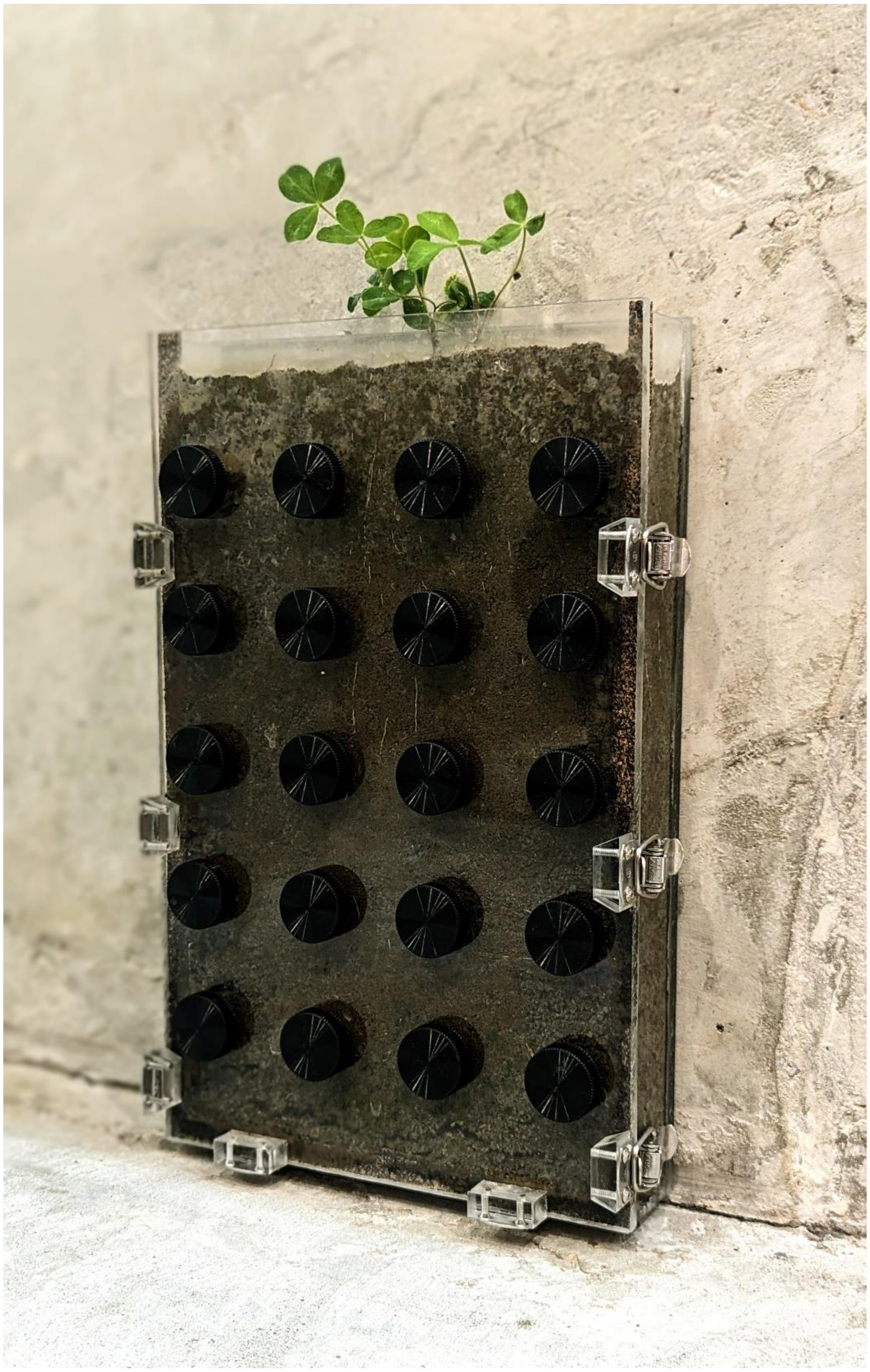

Figure 2. Rhizobox back panel with sampling access ports.

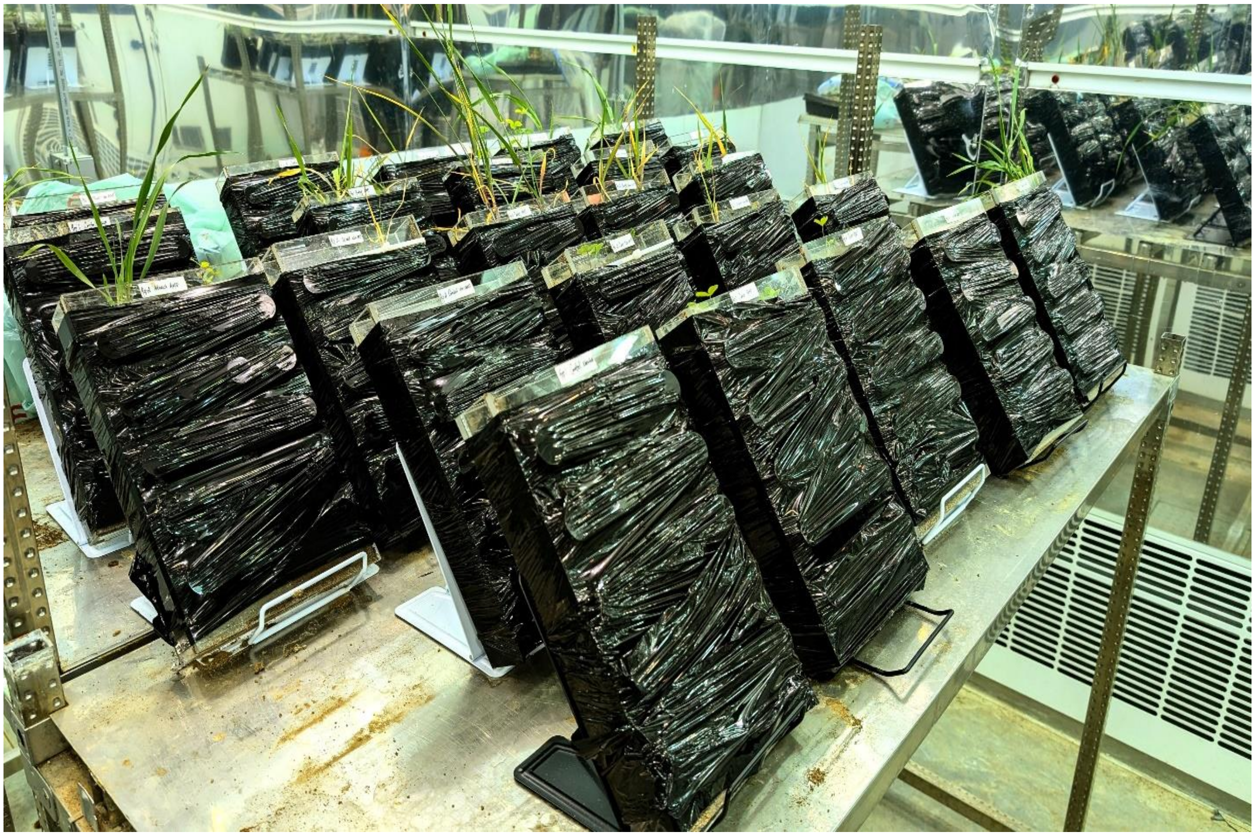

Figure 3. Rhizobox setup in experimental growth chamber at Trent University. Rhizobox placement was randomly shifted every week to account for any variation in light intensity.

fine absorptive roots and transport roots (Freschet, Pagès, et al., 2021; McCormack et al., 2015).

2.3.2 Morphological traits

At peak biomass, the front acrylic panels of the rhizoboxes were removed and the two plants in each rhizobox were carefully removed (labelled as plant A and B along with the rhizobox number). Roots were gently washed and scanned at 3200 dpi. Fine roots of orders 1 through 3 were analyzed in order to target the absorptive roots. Absorptive fine roots were isolated using a scalpel before scanning. Images were analyzed for total fine root length, average root diameter, root tips counts, root volume and surface area (WinRhizo Pro, 2022, Regent Instruments Inc.). The roots were then oven dried at 70 ˚C for 48 hours and weighed to calculate a suite of root traits including biomass per plant, as well as morphological traits: specific root length (SRL), specific root area (SRA), specific root tip abundance (SRTA), and root tissue density (RTD). Wet weight of biomass was measured to calculate moisture content of roots – this ratio allowed for the dry weight of the roots used for the hyphal counts to be calculated and included in total root biomass measurements.

2.3.2 AMF associations

Fresh weight of 2.0 g (approximately 1-2-cm long segments) of fine roots per plant were used for staining and determining the level of AMF colonization. Roots were washed with reverse osmosis (RO) water to remove any particulates. Roots tissue was cleared for staining and microscope analysis using 10% KOH for 30 minutes at 60 °C (X.-G. Sun & Tang, 2012). Roots were then rinsed and placed in a 1% HCl solution for one hour in order to neutralize the pH (Corona Ramírez et al., 2023). Roots were then stained with 0.04% trypan blue (Kumar et al., 2008) by covering the root segments with the dye and leaving for 10 minutes. All roots were examined within three days. Roots that were not immediately processed under a microscope were kept in a lacto-glycerol solution (1:1:1 lactic acid:glycerol:water) and placed in a fridge to ensure the stain remained fresh.

After staining, roots were placed in 50 % glycerol to remove excess stain and then mounted onto a microscope slide. A compound microscope was used to examine stained roots for vesicles, arbuscules, and hyphae at a magnification of 20× (Sharma & Buyer, 2015). For each rhizobox, 15 random stained root segments of approximately 2 cm in length were selected from each plant. Photographs were taken with a Google Pixel 8 phone attached to a microscope mount. Once all samples were photographed, the images were examined for presence of AMF. In areas with AMF presence, vesicles, arbuscles, and hyphae were counted. In order to minimize this component, all root slides were evaluated using a standard procedure performed by a single observer. Images were all counted twice, with the 10 random images selected and recounted to determine the amount of variation. Despite this, some subjectivity in AMF colonization count may still be present.

2.4 Rhizosphere soil sampling

2.4.1 Dissolved organic carbon and total nitrogen fluxes

The back panel of each rhizobox had a total of twenty access ports, arranged in horizontal rows of four, spaced every 5cm vertically down, allowing for soil sampling at different depths (Figure 1). CMA 4004 Infusion pump was equipped with four 5-mL syringes that were connected to the microdialysis probe (CMA 20; 20 kDa, 30 mm membrane) through collection tubes, which were kept on icepacks through the collection process (Buckley et al., 2022). A perfusate (ultrapure water) was injected behind the membrane, creating a concentration gradient and a diffusive flux (DF) of soluble soil chemicals (Buckley et al., 2016). The size-selective microdialysis membrane limits the study of microbial activities that involve larger molecules in samples (Buckley et al., 2020).

Microdialysis probes were immersed in ultrapure water before use. To ensure the dead volume in the microdialysis probe had been flushed, the probes were operated for ten minutes in ultrapure water prior to dialysate collection and after dialysate collection (McKay Fletcher et al., 2021). Previous studies show indicate that a soul moisture level of 70 % water holding capacity optimizes the recovery of soil solute during microdialysis sampling in soil (Buckley et al., 2022). Therefore, before microdialysis sampling, 70 % water holding capacity was achieved by weighing the rhizoboxes with a portable scale and adding water until 70 % water holding capacity was achieved. Four probes were inserted through the sampling ports and positioned next to the plants' roots. For boxes with two different plants, two probes targeted each plant. Control boxes were sampled at similar root depths, but taken from the bulk soil as no roots were present. Samples were collected at a flow rate of 1 µl min^{-1} for 5 hours resulting in a collection of 300 µl per sample (Buckley et al., 2016, 2022; Müller et al., 2023). Dissolved organic carbon (DOC) and total N was analyzed using a total organic carbon analyzer (Shizmadzu) After each collection, dialysates were frozen at -20 °C until they were ready to be processed (Buckley et al., 2022). Collection was done during peak biomass. Results were converted to flux using equation 1:

Equation 1:

$$D = \frac{C \times V}{A_m \times \mathrm{t}}$$

where D is DOC flux, C is the concentration of the solute, V is the dialysate volume, A_m is the surface area of the membrane, and t is time.

2.4.2 Easily extractable glomalin

Easily extractable glomalin (EEG) was targeted for this study. EEG extraction was completed following the widely accepted protocol (Irving et al., 2021; Janos et al., 2008; L. Sun et al., 2018; Wright et al., 2006) written by Wright & Upadhyaya (1998): 1g of fresh soil was added to a centrifuge tube with 8 mL of 20 mM sodium citrate (pH 7.0). The samples were then autoclaved at 121°C for 30 minutes and then immediately centrifuged 5000 xg for 15 minutes, allowing for the supernatant to be removed and stored at 4 °C until use. Protein concentration was determined using the Quick Start™ Bradford Protein Assay (Bio-Rad) with bovine serum albumin (BSA) as the standard, following manufacturer instructions (Wright & Upadhyaya, 1998).

Absorbance was measured at 595 nm using a spectrophotometer after a five-minute reaction period. A standard curve was constructed by plotting BSA standard concentrations (mg/mL) against corresponding absorbance values. Unknown sample concentrations were determined by interpolation from this standard curve (Bio-Rad, 2018). Preliminary tests showed some samples below the standard calibration range of the Bio-Rad assay, so a known concentration of the BSA standard was added to all extracts (Redmile-Gordon et al., 2013). The added spiked BSA was subtracted from the measured amounts. Seventeen of the 48 measurements were below the MDL, with 13 of those being non-AMF plants. It is important to note that accurately quantifying EEG via the Bradford assay remains a challenge due to matrix interferences. A study by Rosier et al. (2006) reported that 41-84% of spiked BSA was recovered in soil extracts, which underscores the suppression effects of co-extracted humic substances on Bradford colour development. Similarly, Jorge-Araújo et al. (2015) showed the Bradford calibration can underestimate EEG by 2-6-fold due to the interference of humic matter. For this study, the interest was not on the absolute concentrations of EEG but rather the relative differences amongst treatment groups. Because all soils were homogenized and processed under identical extraction and assay conditions, it was assumed that matrix interferences (e.g. humic matter) would be comparable across samples. Concentrations were then standardized to soil weight on a per gram basis, with soil weight calculated by correcting for soil moisture content by oven drying samples to obtain dry weight measurements. Results with a concentration that was below the minimum detection limit of 0.0257 µg g^{-1} were set at half, for 0.013 µg g^{-1} (Helsel, 2010).

2.5 Statistical analysis

All statistical analyses were run in R Statistical Computing (version 4.5.1). Linear mixed-effects models (LMMs) were used to analyze treatment effects on plant biomass, AMF colonization, root functional traits, and soil carbon dynamics. Within these LMMs, BoxID and planting batch were included as random effects to account for: (1) pseudo-replication arising from multiple plants sampled within individual rhizoboxes (BoxID) and (2) temporal variation among the three sequential planting batches of rhizoboxes. Fixed effects included plant species (clover or barley), neighbour plant (monoculture vs. mixture), AMF inoculation status (inoculated vs. non-inoculated), and their interactions.

For glomalin analysis, two complementary approaches were used in order to determine which was a better fit. First, a hierarchical LMM examining AMF inoculation status and plant presence effects with BoxID as a random intercept (rhizobox batch was excluded as it had no effect). Second, a full treatment LMM incorporating all treatment combinations. Two different models were run in order to select the model with the best fit. Between the two models, the hierarchical model fit best. Model selection was based on likelihood ratio tests, and post-hoc pairwise comparisons were conducted using estimated marginal means with Tukey adjustment for the multiple comparisons.

Principal component analysis (PCA) was performed separately for each plant species using root functional traits (SRL, SRTA, RTD, and D), along with AMF count, EEG, and DOC flux, Variables that were skewed were $\log_{10}$ transformed to make distributions to meet parametric assumptions. Missing data were imputed by duplicating available measurements from replicate boxes with the same treatment combination. To assess the robustness of my PCA analysis given the low sample size, I applied a nonparametric bootstrap procedure (1000 resamples with replacement). For each resample, the PCA was recalculated and the proportion of variance explained by PC1 and PC2, along with the trait loadings, was recalculated. The provides confidence intervals, which allow for the stability of the PCA to be quantified. PC axis scores were used as indicators of belowground plant strategies (Dawson et al., 2024; Wang et al., 2018) and were tested for treatment effects using LMMs with BoxID and rhizobox batch as a random effect, as described in the biomass analyses. Statistical significance threshold was 0.05.

3.0 Results

3.1 AMF associations

AMF inoculation significantly increased fungal colonization across all plant-soil combinations tested. In barley monoculture, AMF counts per ~30 cm of root length per plant were significantly higher in inoculated plots (114.8 ± 5.4) compared to non-inoculated controls (10.5 ± 5.4; $p < 0.001$) (Figure 4; Table 2). Similarly, barley grown with clover (heterospecific competition) showed elevated AMF counts with inoculation (204.7 ± 38.4) relative to the control counterparts (5.7 ± 38.4), but this difference was not significant ($p = 0.061$).

Clover plants exhibited the largest responses to AMF inoculation. In clover monoculture, inoculated plots had substantially higher fungal counts (223.2 ± 65.6) than non-inoculated rhizoboxes (15.8 ± 65.6), with a marginally significant treatment effect ($p = 0.089$). Clover in mixture showed a significant positive response to inoculation, with AMF counts increasing from 5.7 ± 36.4 in controls to 211.3 ± 36.4 in inoculated plots ($p = 0.016$) (Figure 4; Table 2).

Across all treatment combinations, AMF inoculation resulted in 11- to 37-fold increases in fungal colonization relative to non-inoculated controls, demonstrating the consistent effectiveness of the inoculation treatment in enhancing mycorrhizal establishment.

3.2 Diversification effects on plant biomass

Clover produced substantially more biomass than barley across all measured components (Figure 5; Table 3). Clover showed approximately four to five times greater root dry mass compared to barley plants ($p < 0.001$), two to three times greater aboveground dry mass ($p = 0.008$), and three to four times greater total dry mass ($p = 0.001$).

Monoculture vs mixture did not have significant main effects on any biomass measure (all *p* > 0.3; Table 2). AMF inoculation significantly affected root biomass production (*p* = 0.006), which was a species-specific effect, as shown by a significant Species × AMF interaction (*p* = 0.002) (Table 2). In clover plants, AMF inoculation increased root dry mass by approximately 150 % in monoculture (from ~2.2 to ~5.5 g plant^{-1}) and 135 % in mixture (from ~2.1 to ~4.9 g plant^{-1}). Alternatively, barley showed very little response to AMF inoculation, with its root biomass remaining relatively constant (~0.1 to 0.2 g plant^{-1}) regardless of the AMF treatment.

AMF inoculation had no significant effects on aboveground biomass (*p* = 0.664) or total biomass (*p* = 0.359) for either of the two species. Marginally significant Species × Monoculture vs Mixture interactions were observed for both aboveground (*p* = 0.074) and total biomass (*p* = 0.076) (Table 2).

3.3 Diversification effects on root morphology

Linear mixed models revealed none of the root traits in barley were significantly influenced by the interaction between AMF inoculation and neighbour plant (monoculture vs mixture; all p > 0.28, F < 1.28, df = 1, 28). However, a significant main effect of neighbour plant (MixORMono) was found for SRL (F = 5.89, p = 0.040, df = 1, 28), with higher SRL in plants grown in mixture. No significant effects were found for average root diameter (p = 0.417, F = 0.77, df = 1, 28), SRTA (p = 0.157, F = 2.71, df = 1, 28), or RTD (p = 0.340, F = 1.05, df = 1, 28).

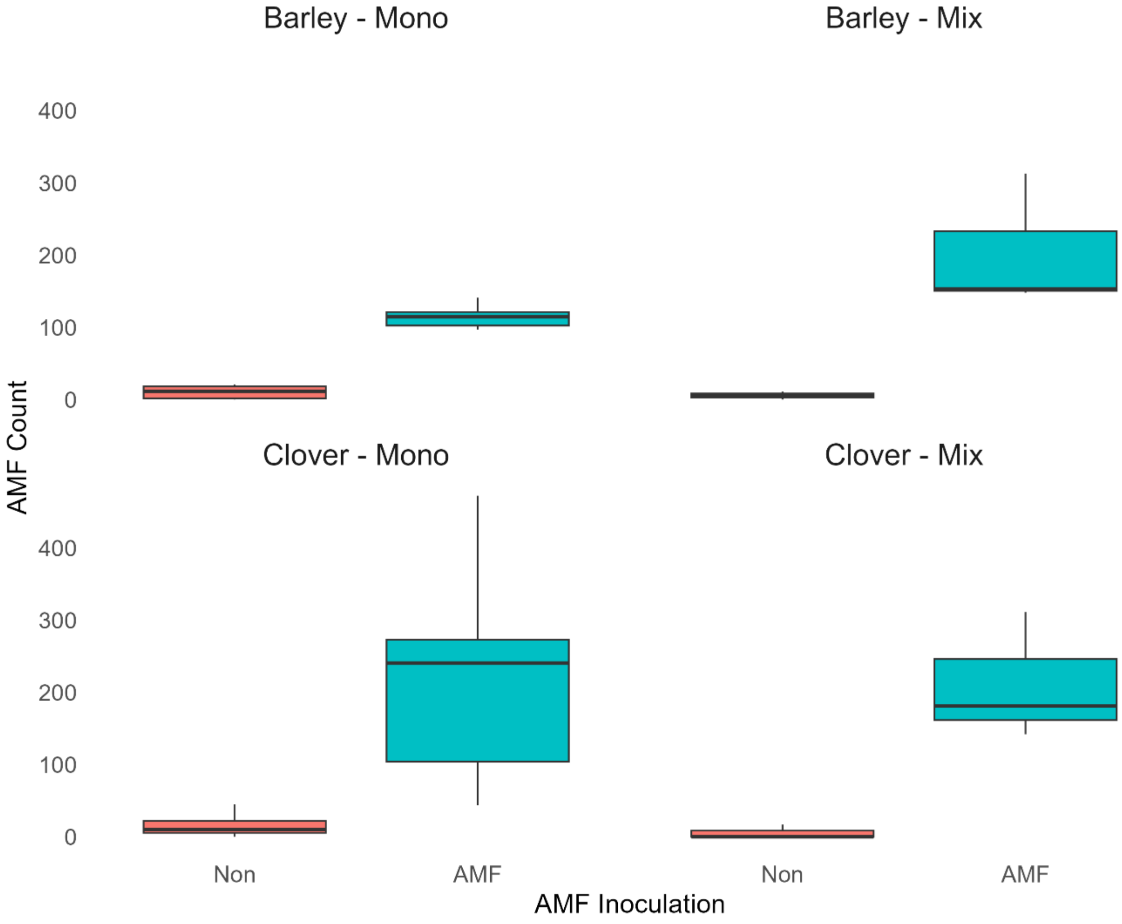

Figure 4. Boxplots comparing AMF counts between inoculated and non-inoculated clover and barley plants, and in mixture and monoculture systems (n = 3 for plants in mixture; n = 6 for plants in monoculture).

Table 2. Mean (± standard error) of AMF inoculation of fungal counts with reported pair-wise comparisons within species and mixormono treatment combinations.

Treatment	Non-inoculated (mean ± SE)	AMF inoculated (mean ± SE)	Difference	p-value	Significance
Barley Monoculture	10.5 ± 5.4	114.8 ± 5.4	+104.3	< 0.001	***
Barley Mixed	5.7 ± 38.4	204.7 ± 38.4	+199.0	0.061	·
Clover Monoculture	15.8 ± 65.6	223.2 ± 65.6	+207.3	0.089	·
Clover Mixed	5.7 ± 36.4	211.3 ± 36.4	+205.7	0.016	**

Significance codes: *** $p < 0.001$, ** $p < 0.01$, · $p < 0.1$

For clover, no significant main or interaction effects were found for any root traits, including average diameter (p = 0.067, F = 5.15, df = 1, 28), SRTA (p = 0.796, F = 0.07, df = 1, 28), SRL (p = 0.730, F = 0.13, df = 1, 28), and RTD (p = 0.364, F = 0.96, df = 1, 28). Mean trait values (± standard error) support this trend (Table 4; Table 5), with barley grown in mixture inoculated with AMF having higher SRL (9.42 ± 3.61 cm mg^{-1}) and SRTA (158.50 ± 87.3 tips mg^{-1}) than in barley grown in monoculture (SRL: 3.95 ± 1.70 cm mg^{-1}, SRTA 35.50 ± 10.86 tips mg^{-1}). Mixture without AMF still had higher SRL (4.63 ± 0.89 cm mg^{-1}) compared to monoculture, but still less than when inoculated with AMF. Root tissue density remained consistent across treatments, while average diameter varied only slightly (0.24 to 0.27 mm).

Clover in monoculture with AMF inoculation exhibited the highest average diameter (1.30 mm), whilst mixture with AMF had the lowest (0.30 ± 0.01 mm). Specific root length values were the highest when in mixture without AMF (15.23 ± 4.21 cm mg^{-1}). In contrast, when grown in mixture with AMF inoculation, clover had the lowest SRL (6.24 ± 2.82 cm mg^{-1}). Much like barley, RTD remained stable across the treatments.

3.4 Carbon dynamics in rhizosphere soil

3.4.1 Dissolved organic carbon and total nitrogen fluxes

Dissolved organic carbon (DOC) fluxes ranged from 0.139 to 0.839 g C m^{-2} hr^{-1} across all treatments, while total nitrogen (TN) fluxes varied from 0 to 0.462 g N m^{-2} hr^{-1} (Table 6). Control treatments without plants in the rhizoboxes taken on bulk soil showed intermediate DOC flux values (0.139 to 0.262 g C m^{-2} hr^{-1}), with AMF-inoculated controls generally exhibiting higher

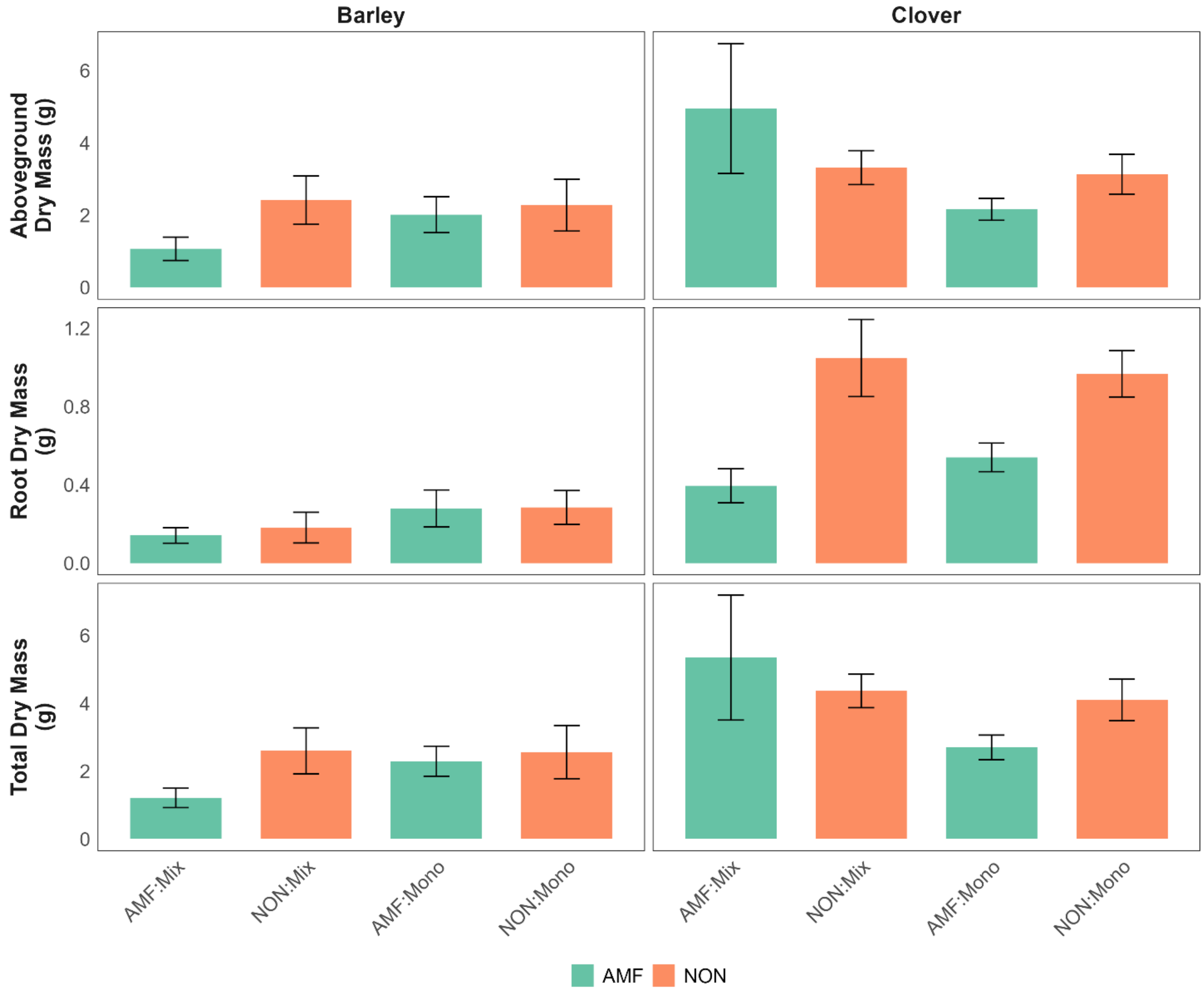

Figure 5. Total, aboveground, and belowground mean biomass, (± standard error) of barley and clover plants grown either in mixture (Mix: teal bars) or monoculture (Mono: orange bars), and with AMF inoculation (AMF) or without (NON) (n= 6 for plants in monoculture; n = 3 for plants in mixture. LMM used to account for box ID and batch as random variables.

Table 3. ANOVA of fixed effects from linear mixed model testing the effects of species identity, mixture treatment, and AMF inoculation on biomass traits.

Effect	df	Root dry mass (F)	p-value	Aboveground dry mass (F)	p-value	Total dry mass (F)	p-value
Species	1,28	45.32	< 0.001 ***	5.47	0.027 *	10.38	0.003 **
Mix/Mono	1,28	0.93	0.342	1.12	0.298	0.76	0.392
AMF	1,28	12.14	0.002 **	0.57	0.456	1.52	0.228
Species × Mix/Mono	1,28	0.31	0.580	3.39	0.076 ·	3.39	0.076 ·
Species × AMF	1,28	10.64	0.003 **	0.29	0.594	0.00	0.972
Mix/Mono × AMF	1,28	0.68	0.417	0.55	0.464	0.35	0.561
Species × Mix/Mono × AMF	1,28	0.37	0.550	3.24	0.083 ·	2.65	0.115

*Significance codes: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, · $p < 0.1$*

fluxes than non-AMF controls. TN fluxes in controls ranged from 0.067 to 0.462 g N m^{-2} hr^{-1}, with higher values observed in some AMF treatments.

Amongst the rhizoboxes with plants, clover monocultures displayed the highest variability in DOC flux, ranging from 0.352 to 0.839 g C m^{-2} hr^{-1}, with AMF-inoculated treatments showing both the lowest (0.364 g C m^{-2} hr^{-1}) and highest (0.839 g C m^{2} hr^{-1}) values. Barley monocultures exhibited more consistent DOC fluxes (0.183 to 0.452 g C m^{-2} hr^{-1}), with AMF treatments generally showing higher fluxes than non-AMF treatments.

Mixtures (clover-barley) showed intermediate DOC flux values when compared to monocultures, with fluxes ranging from 0.200 to 0.456 g C m^{-2} hr^{-1}. Interestingly, TN fluxes were predominantly zero across most planted treatments, with exceptions in specific AMF-inoculated barley monocultures (0.315 g N m^{-2} hr^{-1}) and clover monocultures (0.003 to 0.007 g N m^{-2} hr^{-1}).

3.4.2 Easily extractable glomalin

In the hierarchical model, EEG was significantly affected by both AMF inoculation status ($p = 0.0004$) and the presence of plants ($p = 0.004$), but not by their interaction ($p = 0.187$) (Table 7). Specifically, AMF inoculated rhizosphere soil showed significantly higher EEG values than non-AMF-inoculated soil ($p = 0.002$). Rhizosphere soil also had significantly higher EEG compared to soils without plants ($p = 0.010$) (Figure 6; Table 7).

A second model incorporating full treatment combinations (plant species × neighbour plant × AMF inoculation × control) also supported significant treatment effects ($\chi^2 = 23.39$, df =

Table 4. Mean (± standard error) of root morphological traits for red clover grown in monoculture or mixture with and without AMF.

Treatment	SRL (cm mg^{-1})	SRTA (tips mg^{-1})	RTD (mg cm^{-3})	Diameter (mm)
Non-AMF Mono	9.27 ± 0.71	21.42 ± 2.00	1.77 ± 0.11	0.28 ± 0.01
Non-AMF Mix	15.23 ± 4.21	45.97 ± 7.63	1.21 ± 0.22	0.28 ± 0.01
AMF Mono	7.96 ± 0.40	24.99 ± 4.95	1.79 ± 0.08	0.30 ± 0.01
AMF Mix	6.24 ± 2.82	19.69 ± 9.41	11.86 ± 9.71	0.26 ± 0.02

Table 5. Root morphological traits (mean +- SE) barley grown in monoculture or mixture with and without AMF

Treatment	SRL (cm mg^{-1})	SRTA (tips mg^{-1})	RTD (mg cm^{-3})	Diameter (mm)
Non-AMF Mono	3.23 ± 0.52	36.52 ± 10.32	7.01 ± 2.01	0.27 ± 0.01
Non-AMF Mix	4.63 ± 0.89	48.19 ± 16.96	6.77 ± 3.39	0.24 ± 0.05
AMF Mono	3.95 ± 1.70	35.49 ± 10.86	8.56 ± 2.56	0.27 ± 0.01
AMF Mix	9.42 ± 3.61	158.50 ± 87.33	3.02 ± 1.15	0.26 ± 0.03

9, *p* = 0.005). Estimated marginal means showed that EEG was generally highest in AMF inoculated plant treatments and lowest in control soils without AMF.Post-hoc testing indicated that the overall positive effect of AMF across all treatments was not statistically significant after correction (p = 0.095). There were no statistically significant differences in mean EEG between control vs planted treatments, mixture vs monoculture treatments, or barley vs clover after correction. However, trends were observed, with AMF-inoculated soils generally showing higher EEG values and non-AMF controls being lowest across treatments.

3.5 Effects of diversification on belowground plant strategies

Principal component analysis revealed distinct patterns in root functional trait responses to diversity and mycorrhizal inoculation treatments in both clover (Figure 7) and barley (Figure 8). The first two principal components explained on average 71.9% of variation in clover (PC1: 48%, 95% CI: 35.6-67.0%; PC2: 23.7%, 95% CI: 15.4-31.4%) and 68.4% in barley (PC1: 42.9%, 95% CI: 34.5-52.6%; PC2: 25.5%, 95% CI: 18.6-32.6. The bootstrapped loadings showed relatively narrow confidence intervals, indicating that the contributions of individual traits to PC1 and PC2 were generally consistent across resamples. This suggests that the contributions of individual traits to each axis (Appendix Table 4.) were generally consistent across resamples. Overall, the robustness checks provide confidence that the main PCA patterns reflect underlying belowground root strategies rather than noise from the sample size limitations.

In clover, PC1 represented the root economic spectrum conservation gradient, with negative values indicating more acquisitive strategies, characterized by higher specific root length (SRL) and specific root tip abundance (SRTA), and positive values representing more

Table 6. Mean ± SE of DOC and TN fluxes.

AMF	Species	MixORMono	DOC flux (Mean ± SE); g C m^{-2} hr^{-1}	TN flux (Mean ± SE); g N m^2 hr^{-1}
AMF	Barley	Monoculture	0.452 ± 0.000	0.315 ± 0.000
AMF	Clover	Monoculture	0.549 ± 0.124	0.005 ± 0.004
AMF	Mixed	Mixture	0.200 ± 0.000	BDL
Non-AMF	Barley	Monoculture	0.241 ± 0.038	BDL
Non-AMF	Clover	Monoculture	0.352 ± 0.000	0.003 ± 0.000
Non-AMF	Mixed	Mixture	0.456 ± 0.000	BDL

BDL = below detection limit

conservative strategies (characterized by larger root tissue density (RTD)). PC2 was driven primarily by larger average root diameter and higher DOC flux, with AMF colonization also contributing positively.

Mycorrhizal inoculation marginally affected clover plants' root trait profiles on PC1 ($p = 0.069$), shifting plants toward more conservative root strategies in inoculated treatments. The interaction between diversity and inoculation also showed a marginal trend ($p = 0.084$) on PC1 scores. Diversity significantly influenced clover PC2 scores ($p = 0.010$), with clover in mixtures generally showing higher PC2 values regardless of inoculation status. There was also a significant interaction effect between diversity and inoculation on PC2 ($p = 0.019$), suggesting that the combination of planting diversity and mycorrhizal inoculation drives distinct functional trait combinations.

In barley, PC1 also reflected the conservation-acquisitive trade-off, with SRL and SRTA loading negatively and RTD loading positively. PC2 was dominated by AMF count, DOC flux, and EEG, reflecting treatment-driven differences in belowground strategies. No significant main effects were observed for barley on PC1. For PC2, AMF inoculation had a strong effect ($p = 0.003$), with inoculated plants showing distinctly different trait profiles compared to non-inoculated plants. No significant effects of diversity ($p = 0.805$) or diversity x inoculation interaction ($p = 0.141$) were found for barley on PC2.

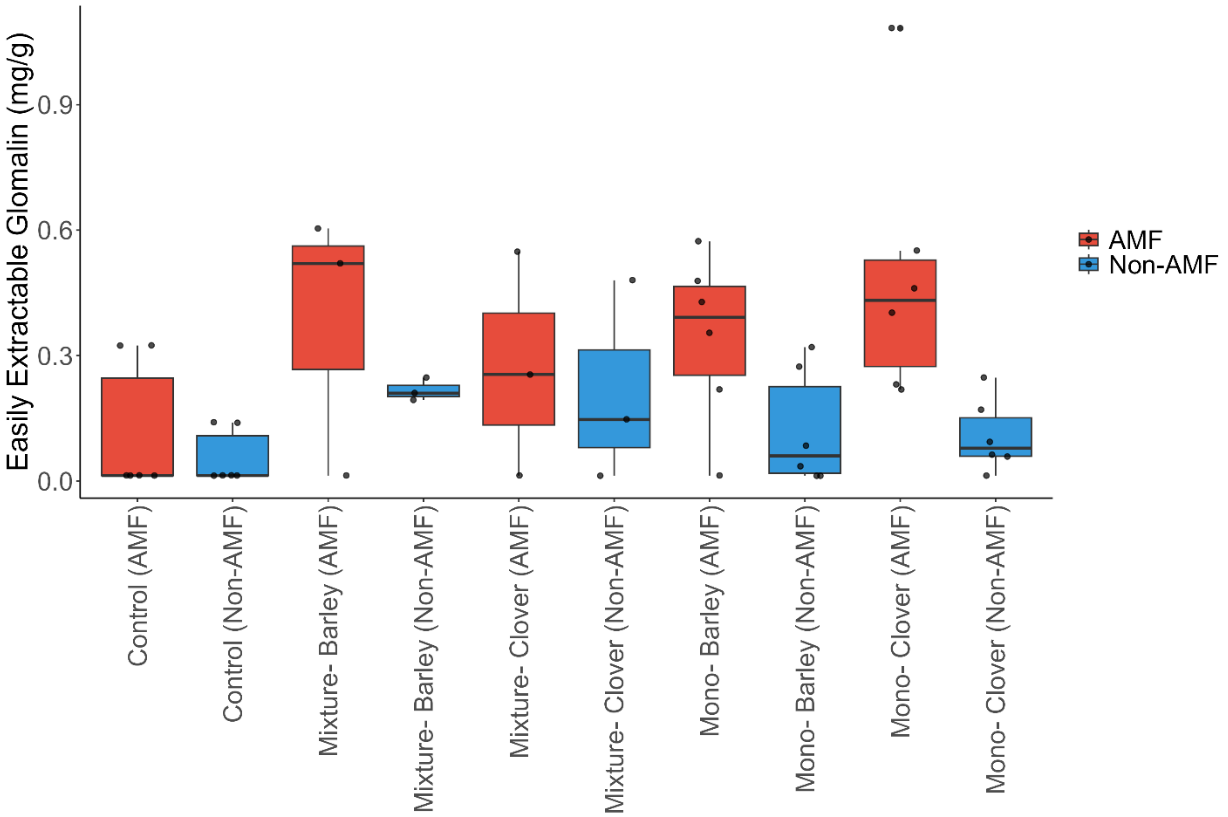

Figure 6. Boxplots showing easily extractable glomalin (mg g soil^{-1}) for plants inoculated with AMF versus non-AMF treatments across the two cover crop plant species and their combinations. AMF inoculated soils tended to have higher EEG values than non-AMF soils, with the lowest concentrations observed in control treatments.

Table 7. AMF inoculation and plant presence cause an increase in glomalin concentrations (EEG) when compared to non inoculated plants: Results from mixed-effects models and targeted contrasts.

Model	Effect / Contrast	Estimate	SE	df	p-value	Significance
Hierarchical Model						
	Inoculated (Non-AMF vs. AMF)	-0.2384	0.0671	20.0	0.0020	**
	HasPlants (No Plants vs. Plants)	-0.2704	0.0949	20.0	0.0099	**
	Inoculated × HasPlants (Interaction)	0.1771	0.1342	20.0	0.202	ns
Full Treatment Model						
	AMF vs. Non-AMF (Overall)	0.1774	0.0625	16.0	0.0953	ns
	AMF vs. Non-AMF (Controls only)	0.1620	0.1610	35.9	1.0000	ns
	Control vs. Plants (AMF)	0.0729	0.1200	37.5	1.0000	ns
	Control vs. Plants (Non-AMF)	0.0922	0.1200	37.5	1.0000	ns
	Mix vs. Mono (Barley, AMF)	0.0723	0.1410	28.7	1.0000	ns
	Mix vs. Mono (Barley, Non-AMF)	-0.0902	0.1410	28.7	1.0000	ns
	Barley vs. Clover (Mix, AMF)	-0.1470	0.1180	16.0	1.0000	ns
	Barley vs. Clover (Mix, Non-AMF)	0.0157	0.1180	16.0	1.0000	ns

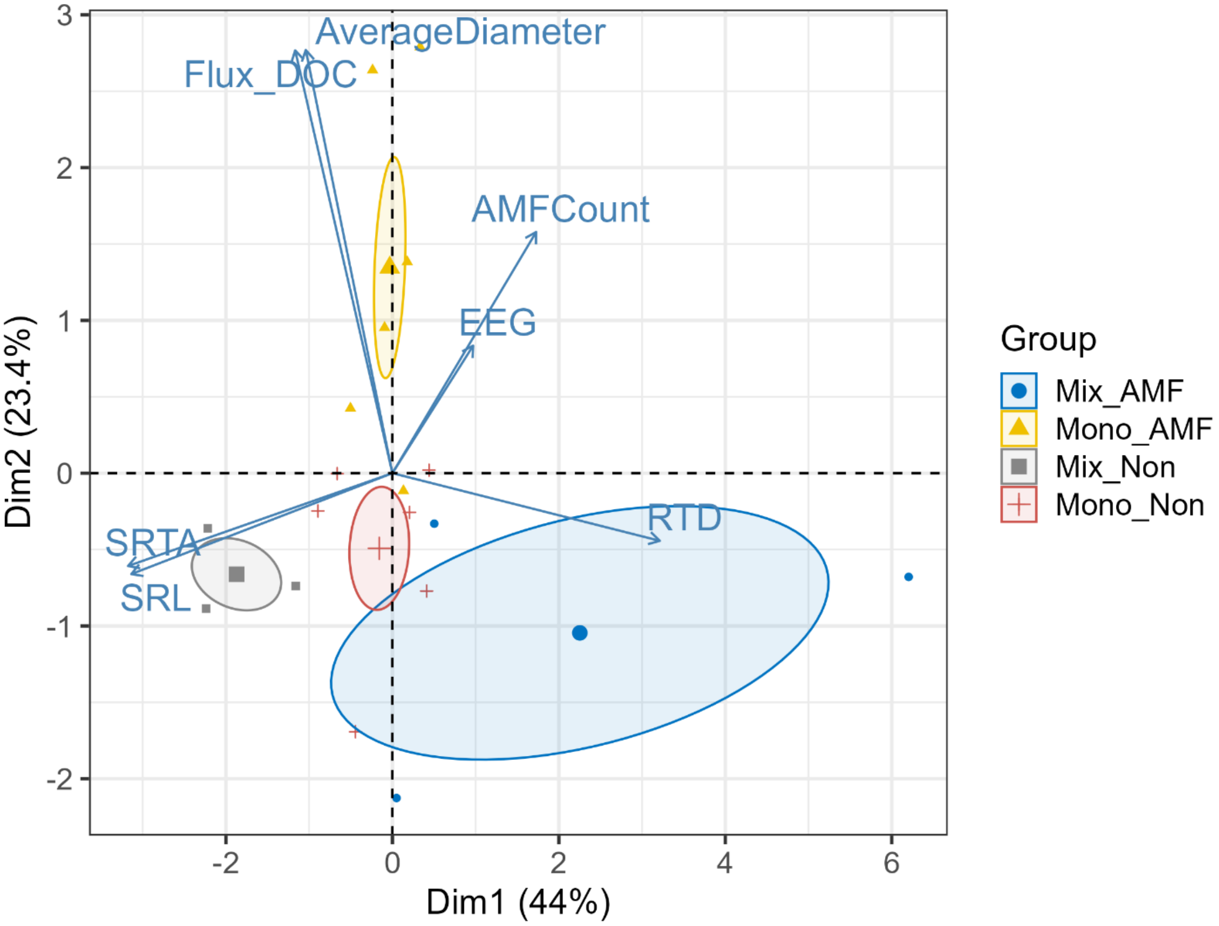

Figure 7. PCA biplot of clover responses to AMF inoculation and mixture vs monoculture treatments. Symbols represent Mix_AMF (blue circles), Mono_AMF (yellow triangles), Mix_Non (gray squares), and Mono_Non (red crosses). Ellipses show 95% confidence intervals. Dim1 represents PC1 and Dim2 represents PC2.

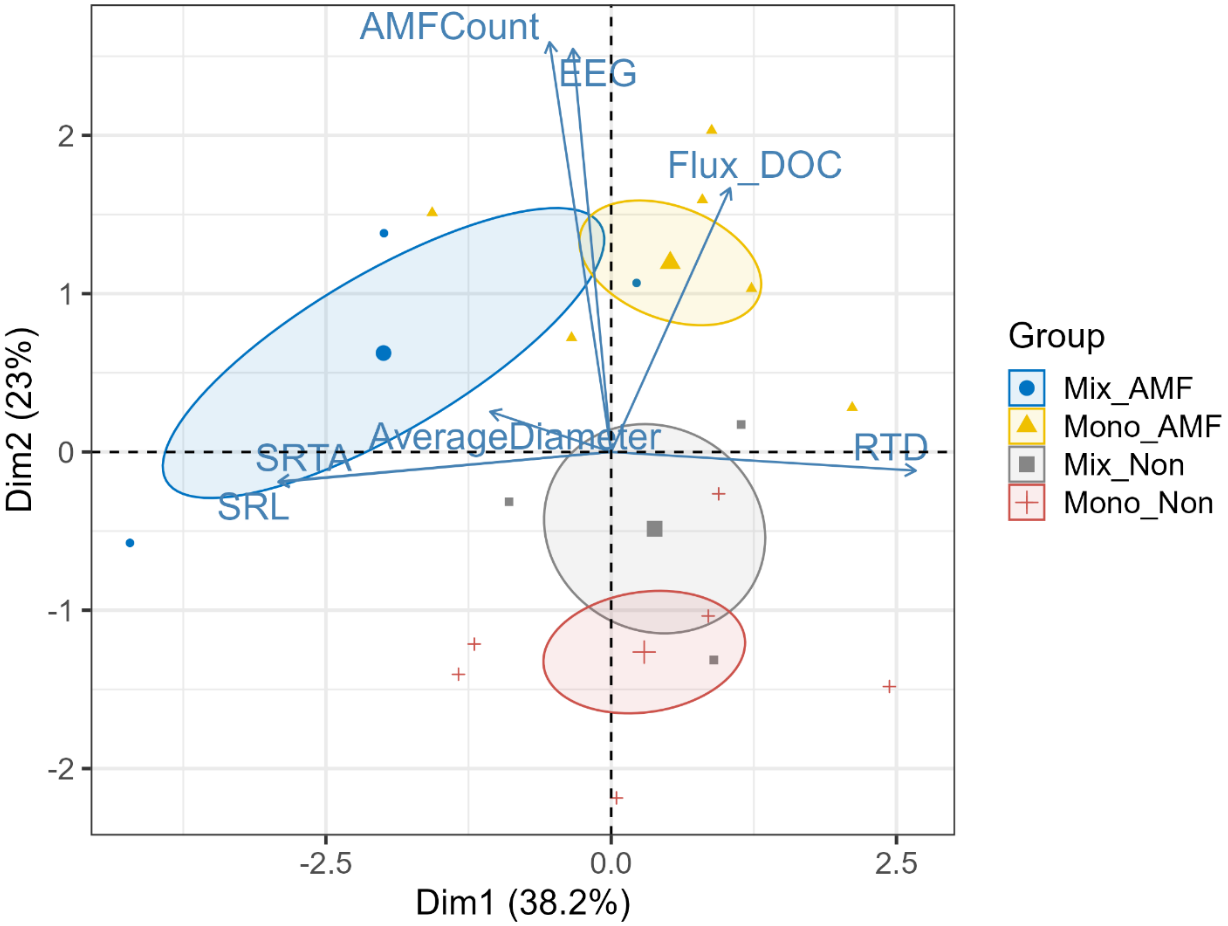

Figure 8. PCA biplot of barley responses to AMF inoculation and mixture vs monoculture treatments. Symbols represent Mix_AMF (blue circles), Mono_AMF (yellow triangles), Mix_Non (gray squares), and Mono_Non (red crosses). Ellipses show 95% confidence interval. Dim1 represents PC1 and Dim2 PC2.

Table 8. Linear mixed-effects model (LMM) results testing treatment effects on PCA-derived root trait axes (PC1 and PC2) for clover and barley. Denominator degrees of freedom (df) are from Satterthwaite approximation.

Species	PC	Effect	df (Den)	F-value	p-value	Sig.
Clover	PC1	MixORMono	7.82	0.079	0.785	
		Inoculated	7.82	4.440	0.069	.
		MixORMono × Inoculated	7.82	3.931	0.084	.
	PC2	MixORMono	6.07	13.488	0.010	*
		Inoculated	6.07	4.349	0.082	.
		MixORMono × Inoculated	6.07	10.147	0.019	*
Barley	PC1	MixORMono	7.11	3.288	0.112	
		Inoculated	7.11	2.587	0.151	
		MixORMono × Inoculated	7.11	3.801	0.092	.
	PC2	MixORMono	7.56	0.065	0.805	
		Inoculated	7.56	18.931	0.003	**
		MixORMono × Inoculated	7.56	2.705	0.141	

Table 9. Trait loadings on the first two principal components (PC1 and PC2) for clover and barley. Stronger absolute values indicate stronger contributions of traits to each axis.

Trait	Clover PC1	Clover PC2	Barley PC1	Barley PC2
SRL	-0.518	-0.150	-0.564	-0.048
SRTA	-0.525	-0.137	-0.564	-0.047
RTD	0.531	-0.100	0.516	-0.029
Average Diameter	-0.172	0.627	-0.205	0.063
AMF Count	0.285	0.357	-0.105	0.645
DOC Flux	-0.193	0.626	0.202	0.415
EEG	0.160	0.189	-0.065	0.634

4.0 Discussion

4.1 Collaboration potential of AMF and cover crops

There is strong collaboration potential of AMF and cover crops in this experiment, with all treatment combinations in AMF inoculation showing a distinct increase in AMF associations within the roots. The magnitude of these increases (11- to 37-fold) demonstrates the receptiveness of both barley and red clover to mycorrhizal partnerships. Soils used for this experiment were low in organic matter with a history of intensive grain management, thus the strong association response may be attributed to these depleted conditions. Similar responses to AMF inoculation have been documented in other studies examining cover crops in degraded agricultural soils, where mycorrhizal partnerships become increasingly crucial for plant establishment and performance (Thirkell et al., 2017).

Clover's root biomass increased substantially in AMF inoculated rhizoboxes when compared to non-inoculated treatments (135 to 150 % increases). This reflects altered allocation patterns that prioritize belowground investment when AMF partnerships are available. This response aligns with the phosphorus-demanding nature of nitrogen-fixing legumes, where AMF associations become particularly valuable for accessing this limiting nutrient (Houlton et al., 2008). Whilst there were no observed significant overall differences in aboveground and total biomass at the time of harvesting, I observed some striking differences in the aboveground biomass of the plants in the early stages of the experiment, although I did not measure this quantitatively (Figure 9). A potential AMF 'boost' to total plant biomass did not persist by the harvest end, which suggests that mycorrhizal benefits may be most pronounced during establishment phases.

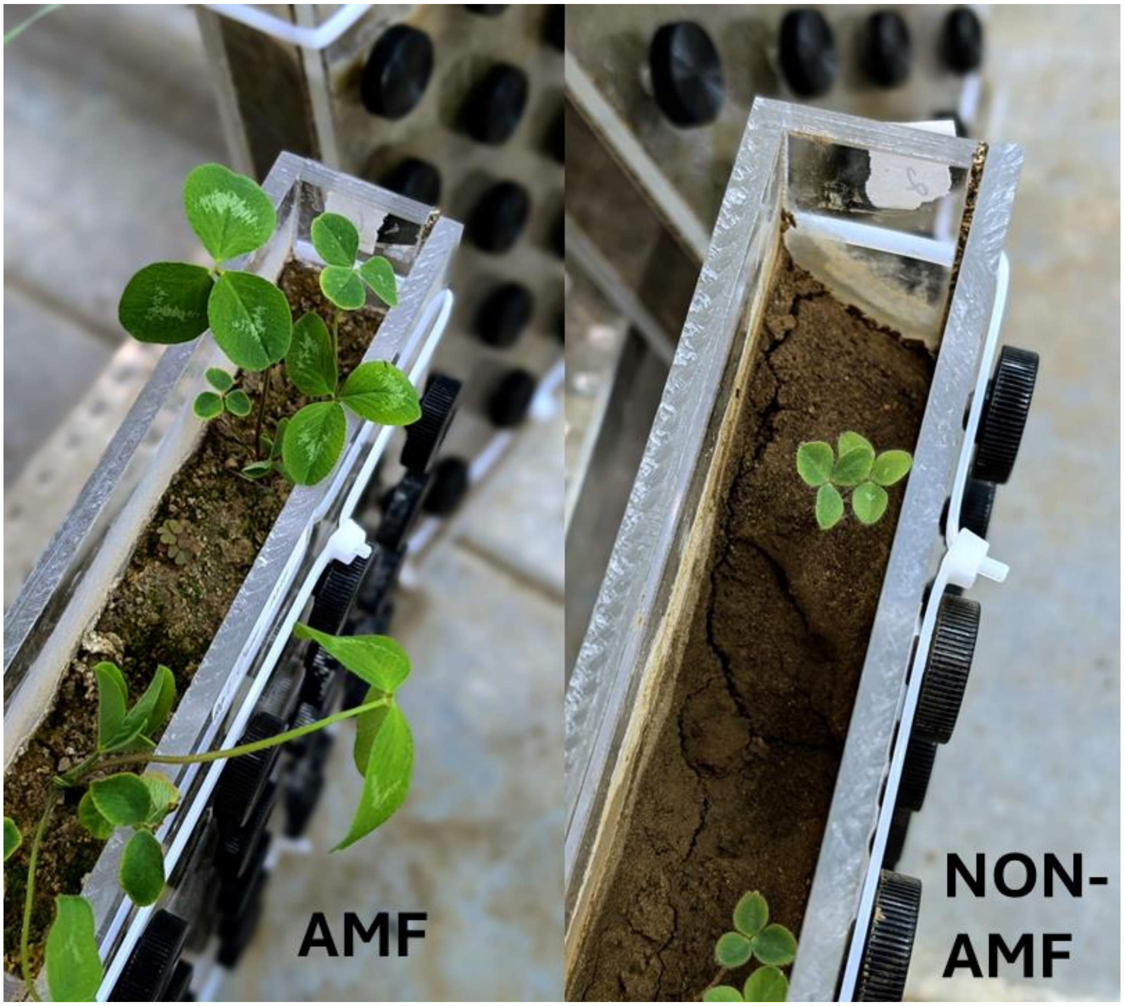

Figure 9. Seedlings at beginning of growth period showing striking differences in the aboveground biomass in the early stages of the experiment – AMF on the left, non-AMF on the right.

Additionally, I saw that red clover and barley did not reach flowering in non-inoculated soils by the end of the experiment, whereas plants in AMF inoculated soils did, providing anecdotal evidence of the importance of AMF for plant performance and reproductive success. Mycorrhizal associations are not merely growth-enhancing but may be essential for completing life cycles under resource-limited conditions. Although not quantitatively measured, observed differences in early growth and in flowering point to a compelling direction for future studies.

Constrained conditions of the rhizoboxes may have also impacted plant growth, where artificially limited soil volumes and box surfaces may influence root architecture or root trait expression compared to field conditions (Poorter et al., 2012). The constrained environment may have dampened the full expression of mycorrhizal benefits that would be more apparent under field conditions, which would have greater soil volume and spatial heterogeneity.

AMF inoculation generally showed a greater effect than species combination on cover crop plants' belowground strategies (see section 4.2). This pattern supports the stress gradient hypothesis, where plants are more likely to seek facilitative interactions rather than competitive ones when growing under suboptimal conditions (García-Cervigón et al., 2013; Sarneel et al., 2022). The low organic matter in the soils used and intensive management history likely created sufficiently stressful conditions that mycorrhizal facilitation outweighed any competitive effects from species mixing. The importance of these associations is further outlined by the minimal baseline AMF counts in non-inoculated soils (5.7-15.8 spores), despite soils not undergoing sterilization. This could suggest that the years of intensive management have mostly eliminated native mycorrhizal communities. Depleted AMF soils likely provided ideal conditions for detecting the inoculation effects, as the low native AMF levels reduced interference from existing mycorrhizal communities. The minimal baseline levels of AMF highlight the receptiveness of the study plants to re-establish and form partnerships with introduced fungi, demonstrating the soils of poorer quality benefit from AMF re-introduction.

4.2 Does diversification modify cover crop belowground function?

Under highly controlled environmental conditions, I observed intraspecific responses to diversification treatments on root economic spectra reflecting different carbon allocation strategies of barley and clover. While the root economic spectrum framework is typically based on interspecific variation observed at large geographical scales, this approach demonstrates significant variation within species grown in the same soil and environmental conditions, with some of the variation being explained by the diversification treatments. This finding supports growing evidence that the hypothesized root economic spectrum framework can be applied at finer scales to evaluate effects of the environment on plant function (Bergmann et al., 2020; Borden et al., 2020; Borden & Isaac, 2019; Weemstra et al., 2016).

The root economic spectrum, conceptualized as a trade-off between conservative strategies and acquisitive resource strategies, shapes plant-soil-microbe interactions in diversified agroecosystems, with distinct root trait responses observed in barley and clover (Freschet et al., 2021; Weemstra et al., 2016). In both species, the root economic spectrum manifested as a gradient from acquisitive traits (e.g., high SRL and SRTA) to conservative traits (high RTD). Clover tended to shift toward more conservative strategies in response to mycorrhizal inoculation and showed distinct trait combinations when grown in mixture. Greater plasticity in clover may reflect evolutionary advantages of legumes, which often rely on both rhizobial and mycorrhizal symbioses for collaborative resource acquisition (Haichar et al., 2014; Lei et al., 2023; Ostonen et al., 2007). Barley showed limited responsiveness to plant diversity, with trait adjustment more strongly linked to AMF inoculation.

Species-specific responses highlight the role of root traits in shaping belowground interactions. Clover exhibited higher responsiveness to both diversity and inoculation, suggesting opportunities to leverage legume plasticity in mixed cover cropping systems. Barley exhibited a stronger reliance on AMF associations, emphasizing the importance of AMF partners for non-legume functional strategies.

As an N_2-fixing species growing in low-P soils, clover's strong response to AMF makes ecological sense, as nitrogen-fixing species require substantial phosphorus for nodulation and fixation processes (Yao et al., 2022). Mycorrhizal partnerships are particularly important for legume performance. The increase in clover’s root biomass with AMF associations could potentially lead to increased competitive pressure with barley roots, which may explain the observed shift toward more acquisitive strategies in barley when grown in mixture, representing a form of niche differentiation. Alternatively, mycorrhizal hyphal networks may create facilitative connections between the plant species, reducing competition through resource sharing (Selosse et al., 2006).

4.3 Impact of diversification practices on soil carbon sequestration

The contrasting responses of barley and clover mixture versus monoculture conditions provide insights into optimizing carbon cycling in agricultural systems. Mixed plantings appear to promote more acquisitive root strategies in grasses like barley, potentially enhancing soil carbon inputs through increased fine root production and turnover rates (Dijkstra et al., 2021).

For legumes like clover, maintaining effective carbon allocation and avoiding over-investment in root infrastructure appears to depend on sustaining sufficient AMF presence when grown in diverse systems (Kakouridis et al., 2024). The significance of these collaborations for long-term carbon storage is further highlighted by AMF's ability to promote carbon transfer to mineral-associated organic matter through glomalin production and hyphal networks (Kakouridis et al., 2024).

Increases in easily extractable glomalin aligned with gradients of AMF associations and plant investment in root systems, suggesting that mycorrhizal partnerships may contribute to soil carbon inputs through glomalin deposition. Glomalin, produced exclusively by AMF, serves as both a carbon storage mechanism and soil aggregation agent (Wright & Upadhyaya, 1998, Rillig et al., 2001). In this study, glomalin levels were significantly higher in AMF-inoculated soils and in soils with plants compared to the control boxes. The lowest concentrations occurred in non-AMF controls. While trait-based studies examining glomalin relationships are limited, this finding supports the growing evidence that mycorrhizal associations represent a key pathway for stable carbon sequestration in agricultural soils (Rillig & Mummey, 2006).

The increases in EEG observed in this study under AMF inoculation and plant presence are consistent with patterns reported in other studies. Wright & Upadhyaya (1998) documented 0.2-0.4 mg g^{-1} increases over a single growing season when looking at cropland soils, which is the equivalent to about 0.03-0.07 mg g^{-1} per month. More recent field and inoculation trials confirm that AMF associations promote glomalin production (Li et al., 2022; Zhang et al., 2020). At the system level, grassland and perennial ecosystems often sustain higher glomalin stocks, whereas heavily fertilized or intensely tilled croplands may show negligible changes of even declines due to supressed AMF activity (Rillig et al., 2001; Rillig & Mummey, 2006). Overall, the magnitude difference observed between non-AMF and AMF rhizosphere soils in the current study align with published ranges, which reinforces the interpretation that AMF-driven glomalin deposition contributes to soil carbon pools in agricultural soils.

The DOC and N flux measurements provide additional insights into how belowground strategies influence rhizosphere carbon and nitrogen dynamics. These fluxes serve as proxies for root influence on carbon and nitrogen cycling in the rhizosphere, inclusive of root exudation and microbial-released compounds. The predominance of zero nitrogen flux values in most planted treatments, with exceptions primarily in AMF-inoculated systems, indicates that mycorrhizal networks may play crucial roles in nitrogen mobilization and transfer. This aligns with findings by Hodge et al. (2000), who demonstrated that AMF enhance DOC and nitrogen uptake and transfer in plant-soil systems, particularly under nutrient-limited conditions.

Variation in DOC fluxes (0.139 to 0.839 g C m^{-2} hr^{-1}) and the patterns of nitrogen flux across the different treatment levels (AMF inoculated, neighbour plant) suggest that the different root strategies may be associated with differences in soil carbon and nitrogen dynamics but in this study was independent from the conservation gradient and the shifts induced from diversification, which is contrary to my third hypothesis and the findings by De Deyn et al. (2008), who linked plant functional traits to soil carbon and nitrogen cycling. However, due to the challenges in collecting multiple reps of microdialysis samples, alignment of rhizosphere carbon fluxes with root economics spectra should be interpreted with caution. A future study with better soils that have better water holding capacity (to maintain ideal conditions for diffusion through the microdialysis membrane) may be more insightful. Additionally, future studies could integrate isotopic tracing methods to provide greater specificity in tracking organic compounds from individual plants and distinguishing plant versus microbial contributions to these fluxes.

The integration of root functional strategies with soil carbon dynamics has important management implications for diversified cover crop systems. Findings demonstrate that clover adjusts root strategies in response to both diversity and AMF inoculation, whilst barley primarily responds to AMF colonization through shifts in carbon fluxes. Species-specific responses indicate that combining legumes and grasses with AMF inoculation may foster complementary belowground functions. Clover exhibited plastic root trait adjustments to diversity and AMF, while barley responded strongly to AMF colonization through carbon flux–related traits. These patterns point to potential benefits for nutrient acquisition and soil carbon processes, although longer-term studies are needed to confirm impacts on soil organic matter stabilization

5.0 Conclusion

This study presents a novel integration of a functional trait approach with soil carbon dynamics in order to understand how diversified agroecosystems and AMF interactions influence belowground processes. By adopting a trait-based framework, specifically the hypothesized root economic spectrum, I tested how intraspecific variation in root strategies relates to soil carbon dynamics. This study provides further support to a growing body of literature that mycorrhizal partnerships are an extension of trait-based ecology in agroecosystems, by demonstrating that single-species root strategies can shift significantly in response to neighbour identity and inoculation status. The treatments represented layered application of agroecological diversification: adding cover crops, mixing cover crop species, and adding AMF inoculant, providing insight unto how agricultural management decisions help shape belowground processes. The species-specific nature of the observed responses—with clover strategies primarily driven by AMF collaboration while barley showed a clear inoculation-driven response which was reflected in higher AMF colonization and altered carbon fluxes—highlights the importance of considering plant functional diversity in agricultural design.

The observed relationships between belowground plant strategies and easily extractable glomalin levels in soil suggest that root functional strategies may play an important role in soil carbon dynamics. However, EEG was associated primarily with trait combinations on the secondary axis (rather than the conservation-acquisitive gradient), which indicated that additional dimensions within the root economic spectrum, in this case the collaboration gradient, also contribute to AMF-related carbon pathways. The links between AMF colonization, EEG, and root functional strategies suggest a potential mechanism by which management can influence soil organic processes.

Future research should look to validate these findings under field conditions, where environmental variability may modify the plant-soil-microbe interactions. Longer-term studies examining how plant-AMF-glomalin relationships persist across a myriad of environmental conditions and multiple growing seasons would be an important next step toward understanding the broader applications of these finding. Additionally, more functional types and cover crop species should be included. Such work could reveal more generalized belowground patterns to inform the design of carbon-enhancing diverse agroecosystems.

The usefulness of integrating carbon measurements with functional trait analysis suggests that the combination of these two methods could be used more widely to improve understanding of carbon dynamics in various agricultural contexts. By linking measurable plant strategies with ecosystem functions, this framework can provide a foundation for evidence-based management practices that aim to optimize agricultural productivity and environmental sustainability.

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7.0 Appendices

Appendix Table 1 Soil baseline analysis results from A&L Laboratories

Property	Value	Units
Organic Matter	3.5	%
Phosphorus (Bray-P1)	11	ppm
Potassium (K)	78	ppm
Magnesium (Mg)	70	ppm
Calcium (Ca)	4650	ppm
Sodium (Na)	24	ppm
pH	7.7	–
Buffer pH	Not reported	–
CEC (Cation Exchange Capacity)	24.1	meq/100g
Base Saturation – K	0.8	%
Base Saturation – Mg	2.4	%
Base Saturation – Ca	96.4	%
Base Saturation – Na	0.4	%
Sulfur (S)	16	ppm
Zinc (Zn)	2.2	ppm
Manganese (Mn)	75	ppm
Iron (Fe)	70	ppm
Copper (Cu)	0.8	ppm
Boron (B)	0.8	ppm
Soluble Salts	1.0	ms/cm
Aluminum (Al)	577	ppm

Property	Value	Units
Aluminum Saturation	0.0	%
Nitrate-Nitrogen (NO_3-N)	0.33	ppm
K/Mg Ratio	Not reported	–
Ca/Mg Ratio	40.2	–
ENR (Estimated N Release)	47	lbs/ac

Appendix Table 2. Results of linear mixed model testing effects of diversity and inoculation on log transformed clover traits.

Trait	**Effect**	**F-value**	**p-value**	**Sig**
Average Diameter	Mix/Mono	5.15	0.066	·
	Inoculated	0.03	0.879	
	Mix × Inoculated	3.63	0.108	
SRTA	Mix/Mono	0.07	0.796	
	Inoculated	2.11	0.186	
	Mix × Inoculated	2.90	0.128	
SRL	Mix/Mono	0.13	0.730	
	Inoculated	3.14	0.127	
	Mix × Inoculated	2.02	0.205	
RTD	Mix/Mono	0.96	0.364	
	Inoculated	1.22	0.312	
	Mix × Inoculated	1.21	0.314	
Root:Shoot	Mix/Mono	3.73	0.074	·
	Inoculated	10.79	0.005	**
	Mix × Inoculated	2.42	0.142	
EEG	Mix/Mono	0.14	0.714	
	Inoculated	3.04	0.116	
	Mix × Inoculated	1.26	0.291	
AMF Count	Mix/Mono	0.84	0.381	
	Inoculated	35.32	<0.001	***
	Mix × Inoculated	1.46	0.255	

Appendix Table 3. Results of linear mixed model testing effects of diversity and inoculation on log transformed barley traits

Trait	Effect	F-value	p-value	Sig
Average Diameter	Mix/Mono	0.77	0.417	
	Inoculated	0.14	0.723	
	Mix × Inoculated	0.18	0.687	
SRTA	Mix/Mono	2.71	0.157	
	Inoculated	1.01	0.357	
	Mix × Inoculated	0.99	0.363	
SRL	Mix/Mono	5.89	0.040	*
	Inoculated	0.74	0.412	
	Mix × Inoculated	1.28	0.289	
RTD	Mix/Mono	1.05	0.340	
	Inoculated	0.16	0.706	
	Mix × Inoculated	0.88	0.379	
Root:Shoot	Mix/Mono	0.89	0.379	
	Inoculated	1.27	0.299	
	Mix × Inoculated	0.006	0.943	
EEG	Mix/Mono	0.37	0.553	
	Inoculated	5.88	0.029	*
	Mix × Inoculated	0.0003	0.986	
AMF Count	Mix/Mono	0.03	0.867	
	Inoculated	49.62	<0.001	***
	Mix × Inoculated	0.85	0.375	

Appendix Table 4. Variance explained by the first two principal components (PC1 and PC2) in red clover and barley, with 95% confidence intervals using 1000 bootstrap samples

Species	PC	Mean variance explained (%)	95% CI (%)
Clover	PC1	48.2	35.6 – 67.0
Clover	PC2	23.7	15.4 – 31.4
Barley	PC1	42.9	34.5 – 52.6
Barley	PC2	25.5	18.6 – 32.6

Appendix Table 5. Summary of trait–principal component relationships for clover and barley. Traits are listed under the axis where they had the strongest loadings (|loading| > 0.3). Signs (+/–) indicate direction of loading, and interpretations link axes to ecological strategies.

Species	Axis	Key Traits (loadings)	Ecological interpretation
Clover	PC1 (48.2%, 95% CI: 35.6–67.0%)	SRL (–), SRTA (–), RTD (+)	Root economics spectrum: acquisitive (–) vs conservative (+)
	PC2 (23.7%, 95% CI: 15.4–31.4%)	Average diameter (+), DOC flux (+), AMF colonization (+)	Structural/carbon flux axis (larger roots, higher DOC release, greater AMF association)
Barley	PC1 (42.9%, 95% CI: 34.5–52.6%)	SRL (–), SRTA (–), RTD (+)	Root economics spectrum: acquisitive (–) vs conservative (+)
	PC2 (25.5%, 95% CI: 18.6–32.6%)	AMF colonization (+), EEG (+), DOC flux (+)	Microbial/exudation axis (greater AMF colonization, root exudation, and carbon release)