Intraspecific variation in mycorrhiza-associated traits predicts plant growth and stress tolerance under resource limitation

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Abstract

While interspecific variation in arbuscular mycorrhizal fungi (AMF) colonization and associated root traits is relatively well documented, intraspecific variation in these traits and their role in plant performance under environmental stress remain poorly understood. To identify AMF root colonization and associated root traits linked to drought and nutrient limitation tolerance in forage legumes, we collected intact mature plants of alfalfa (*Medicago sativa*) and sainfoin (*Onobrychis viciifolia*) and subjected them to control, drought, and low-nutrient treatments.

Drought induced the greatest intraspecific variation in plant performance and AMF-associated traits. In both species, pre-treatment levels of hyphal and arbuscular colonization were strongly associated with subsequent responses to drought stress. However, species-specific patterns emerged following stress. In alfalfa, lower shoot mortality was associated with greater vesicle colonization and increased average root diameter, indicating a shift toward carbon storage and altered root morphology during stress recovery. Although sainfoin exhibited overall higher arbuscular and hyphal abundance, variation in stress tolerance within the species was strongly linked to root tissue density.

These findings highlight that intraspecific variation in the relative abundance of pre-established AMF structures is critical for predicting plant stress tolerance. Furthermore, interactions between AMF colonization patterns and root traits play a key role in shaping plant responses to environmental stress. Our results underscore the importance of incorporating intraspecific variability and trait-based approaches to better predict plant–mycorrhizal dynamics under changing environmental conditions.

Introduction

Arbuscular mycorrhizal fungi (AMF) are important plant endophytes, increasing plant fitness by providing access to limiting nutrient resources in exchange for carbon (van der Heijden et al. 1998; Gianinazzi et al. 2010; Bauer et al. 2012). Consequently, this relationship is critical for plant stress tolerance by allowing plants to maintain physiological functions when resources are limited (Johnson et al. 2010). However, plant-AMF relationships vary greatly with environmental conditions, plant and AMF species (Klironomos 2003; Kiers et al. 2011; Hart et al. 2013; Treseder 2013), making it difficult to predict mycorrhizal interaction outcomes. AMF-associated traits, such as root colonization or root diameter (RD), have been described as indicators of potential variation in AMF benefits among species (Salloum et al. 2016; Wen et al. 2019; Stahlhut et al. 2021). These same traits, however, can vary greatly within species (Ryan et al. 2016), affecting both species interactions and community processes (Stahlhut et al. 2021). Intraspecific variation in AMF-associated traits is largely determined by host genotype (Li et al. 2023). However, these traits also exhibit significant phenotypic plasticity, changing across environmental conditions, due to both plant responses and environmental effects on AMF composition and function (Fort et al. 2015; Kumar et al. 2019). Understanding how the linkages among root traits and AMF benefits vary is crucial for predicting plant community responses to environmental changes.

AMF colonization provides proxies for mycorrhizal performance and fitness (Barceló et al. 2020; Chaudhary et al. 2022). Commonly used AMF metrics, such as total root length AMF colonization, quantify overall AMF abundance within roots but do not distinguish among AMF structures that differ functionally (arbuscules, vesicles, intraradical hyphae) (Treseder 2013). For example, large intraradical mycelium and numerous arbuscules in the root cortex are indicative of a high degree of nutrient flux to the plant, while numerous vesicles and spores indicate higher nutrient retention, especially phosphorus, by the AMF in the root cortex (Fitter 2006; Olsson et al. 2011). Although recent studies acknowledge these structural roles (Sendek et al. 2019; Grünfeld et al. 2022; Caccia et al. 2024), AMF structural composition is rarely evaluated as a predictive trait for plant performance, particularly under stress. Further, it is unclear how structural composition fits within the umbrella of current theory, which focuses on trade-offs between self-sufficient foraging or reliance on AMF for nutrients and a fast–slow nutrient acquisition strategy (Bergmann et al. 2020). Within this context, plants with high specific root length (SRL) and low average root diameter (RD) may rely less on AMF colonization and arbuscule formation, while plants with thicker roots and lower SRL are more likely to invest in AMF-mediated nutrient uptake, expressed through greater intraradical hyphal proliferation and arbuscule abundance. In addition, AMF colonization itself may influence root trait expression by promoting longer root lifespan and greater structural durability associated with high root tissue density (RTD) (Wang et al. 2025). However, which AMF structures (arbuscules/hyphae/vesicles) contribute to this root trait expression is relatively unknown. Integrating AMF structural colonization with root trait spectra, therefore, offers a more mechanistic framework to predict plant performance under environmental changes.

Limited soil resource availability, particularly water and nutrients, is a common stress faced by plants, and plays a critical role in structuring plant–soil interactions by influencing both root functional traits and associations with arbuscular mycorrhizal fungi (AMF) (Legay et al. 2020; de Vries et al. 2021). Under phosphorus limitation, plants often shift from direct nutrient acquisition to greater reliance on AMF-mediated foraging, a strategy commonly associated with increased intraradical colonization and arbuscule formation to enhance nutrient exchange (Kumar et al. 2019). Conversely, excessive nitrogen and phosphorus inputs can lead to lower carbon allocation belowground and declines in AMF colonization intensity and arbuscule abundance (Lin et al. 2020). Under water shortage, AMF hyphae form a bridge across the root–soil interface, and root systems that are extensively colonized by AMF hyphae may be better at maintaining contact with soil water than roots that mainly rely on root hairs (Santander et al. 2017; Püschel et al. 2020). In this regard, plant genotypes with larger root diameter and higher AMF hyphal abundance are likely to coexist as an efficient trait in water fluxes within the root cortex (Abdalla et al. 2023). Similarly, genotypes with higher root tissue density may preferentially support greater AMF colonization, reflecting a coordinated investment in durable roots and symbiotic pathways for resource acquisition (Hushan et al. 2025). Despite these recognized traits–symbiosis linkages, how variation in root structural traits among genotypes or within species regulates AMF colonization under water and nutrient stress remains insufficiently understood.

To explore how intraspecific variation in AMF colonization and associated root traits correlates with perennial legume growth, stress tolerance and shoot N content, we focused on two commonly seeded

perennial forage legumes: alfalfa (*Medicago sativa*) and sainfoin (*Onobrychis viciifolia*). Alfalfa is a significant forage legume for the global livestock industry because of its wide adaptability, high productivity and quality output. AMF can benefit both alfalfa growth and stress tolerance (Guerchi et al. 2023), yet these benefits are cultivar-specific and may depend on the identity of the AMF (Kuper-Psenicnik & Bennett 2024). Sainfoin is another forage legume gaining popularity due to its high palatability and reduced risk of bloat (Liao et al. 2023; Sun et al. 2025). As a more recently domesticated species, it has undergone less intensive breeding and may retain more traits supporting AMF symbioses, yet its mycorrhizal ecology is largely unknown.

To determine how AMF-associated traits, both pre- and post-treatment, affect plant growth and stress tolerance, we excavated intact mature plants of alfalfa and sainfoin and established them in the greenhouse. We subjected them to three different treatments - control, low nutrients, and drought - to evaluate the associations among plant growth, stress tolerance, mycorrhizal colonization, and root traits. We tested the following hypotheses. H1: Arbuscules and hyphae proportions would be more functionally important and predictive of plant performance than total AMF colonization or vesicle colonization; H2: Pre-treatment AMF hyphae and arbuscules would positively predict plant performance under resource limitations because of the persistence and functional legacy of pre-established AMF structures; H3: If plants exhibit a conservative strategy and increased dependency on AMF for nutrient acquisition under stress, higher RD and RTD would be associated with better plant performance and AMF hyphae and arbuscule proportions would be positively correlated to RD.

Materials and methods

Field site and sampling

We obtained intact mature alfalfa and sainfoin plants from breeding nursery field plots located at the Agriculture and Agri-Food Canada field station in Saskatoon, Saskatchewan, Canada. These plants had been maintained in the breeding nursery for 4 years previously and were grown under predominantly natural field conditions without the addition of synthetic fertilizers. The alfalfa nursery contained 5 commercial cultivars of varying ancestry and 25 crossed half-sibling populations, and the sainfoin was a mixture of 6 wild populations coupled with 5 commercial cultivars (see supplementary data for more information). Plants were sampled on June 7–8, 2021, for alfalfa and on June 15, 2021, for sainfoin. We selected approximately 100 plants per species across a range of heights to represent the intraspecific variation in plant size within the nursery (33 cm to 62 cm for sainfoin and 44 to 68 cm for alfalfa), ensuring that each population/cultivar was sampled no more than three times. We excavated the plant and soil from its rooting zone (approximately 12 L), keeping the shoots, crown and root system as intact as possible.

Greenhouse experiments

Further experiments were conducted at the University of Saskatchewan Agriculture Greenhouse in Saskatoon. Before repotting the plants in the greenhouse, approximately 5 g of fine roots were collected from different portions of each plant to obtain a standardized and representative sample. The roots were then washed and stored in 70% ethanol for pre-treatment AMF colonization assessments. Within three days of field sampling, we repotted each plant into its own root-zone soil. Following potting, we watered plants to saturation and fertilized them with one gram of 20-20-20 fertilizer (NPK Plant-Prod AllPurpose) in 50 mL of water immediately after potting to aid in reestablishment. Alfalfa was watered to saturation 3 days per week for 7 weeks, and sainfoin for 9 weeks until established (defined as no further dieback or mortality). As the plants were colonized by the indigenous field microbiome, we applied no additional inoculants of AMF or rhizobia. Once established, plants were cut to 30 cm in height to measure regrowth during the stress treatments. If present, we removed all dead shoot biomass from the plant. We also measured the initial basal area of each plant to account for size variation during statistical analysis by the following formula:

$$\text{Plant initial size} = \frac{\pi \times \text{W1} \times \text{W2}}{4}$$

$$\text{W1} = \text{maximum crown diameter},\ \text{W2} = \text{crown diameter perpendicular to the maximum}$$

For both species, we randomly selected 60 plants and divided them into control, drought and low-nutrient treatments (20 plants per treatment per species). We watered control and low-nutrient plants to saturation every two days, while drought treatments were watered to saturation every six days. We fertilized control and drought plants every three weeks with one gram of 20-20-20 fertilizer (NPK Plant-Prod AllPurpose) in 50 mL of water, whereas for the nutrient limitation treatment, plants received no further fertilizer. We subjected the plants to stress for 4 months before harvesting them (alfalfa from July 28 to November 28, 2021; sainfoin from August 17 to December 17, 2021).

For harvest, each plant was clipped at the soil level, with the live and dead shoot plant biomass separated to assess the degree of shoot mortality over the treatment period. At the same time, we collected fine roots directly from each plant after washing the soil from the root system over a 0.5 mm fine sieve. For the root collection, we collected the fine roots randomly from different parts of the whole root and mixed them to avoid bias. We then allocated two grams of fine roots (fresh weight) for AMF root colonization assessments and three grams for root trait analysis. Live and dead plant biomass were then oven-dried at 56°C for 48 hours and weighed separately. Dried live plant material was then ground with a Wiley mill (standard model 4, Arthur H. Thomas Co., Philadelphia, PA) to pass through a 1000 micron-mesh screen. Crude protein (%) was measured using near-infrared reflectance spectroscopy (NIR; FOSS Analytical, Slangerupgade, Denmark) with models previously calibrated for alfalfa and sainfoin based on the Dumas combustion method (Mihaljev et al. 2015). Nitrogen concentration was then estimated by dividing the crude protein concentration by a conversion factor of 6.25 (Salo-väänänen & Koivistoinen 1996).

AMF colonization and root trait assessment

AMF clearing and staining followed a procedure modified from Wilkes et al. (2020). Roots were placed into permeable cassettes and immersed in 10% KOH for 2 hours at 90°C. Following clearing, we rinsed the roots with water, then acidified them with 2% HCl for 30 minutes to improve stain adherence. We stained the roots with 2.5% sheaffer blue ink in a 1:1:1 solution of glycerol-5% acetic acid-water for 30 minutes at 90°C. We destained roots in 0.1% acetic acid for at least 24 hours at 4°C. After preparing slides, we then quantified five randomly selected 5 cm lengths of roots per plant for percent colonization at 10X using a compound microscope, using 20X and 40X to confirm structure identification as needed. Structures' presence was assessed as the presence of hyphae, vesicles or arbuscules at 50 intersections (McGONIGLE et al. 1990). When usable root tissue was limited due to issues with preparation, however, the number of intersections was reduced. Total AMF colonization was quantified as the proportion of colonized intersections out of the total intersections examined, while individual AMF structure proportions were quantified as the proportion of intersections containing each specific AMF structure (arbuscules/vesicles/hyphae) out of the total intersections examined.

For root trait measurement, 1st and 2nd order roots were scanned on an Epson Perfection V800, ensuring no overlap among root fragments. Scanned images were analyzed using WinRhizo™ (Regent Instruments, Québec, Canada) to obtain estimates of average root diameter (RD), total root length, and total root volume for each sample. We dried and then weighed the scanned root samples to calculate specific root length (SRL; length (cm) /dry mass (g)) and root tissue density (RTD; dry mass (g) /volume (cm3)).

Statistical analyses

All statistical analyses were conducted in R version 4.4.0 (R Core Team 2024). General linear models were fitted using the lm () function in base R. Model assumptions were evaluated by testing homoscedasticity with Levene's test using the *car* package (Fox & Weisberg 2019), and by visually inspecting residual diagnostics generated with the DHARMa package (Hartig 2022). Residual normality was further assessed using Shapiro–Wilk tests in base R.

Treatment effects on the proportion of total root length colonized by AMF, individual AMF structural proportion and root traits were analyzed using linear models (lm) followed by type III ANOVA to obtain F-tests. Estimated marginal means were extracted and visualized using the afex package (Singmann et al. 2021).

To assess the relationships between pre- and post-treatment AMF colonization, root traits, and plant regrowth and stress tolerance, we fitted a series of stepwise linear regression models (forward and backward both) for three response variables: (1) total shoot biomass, calculated as the sum of dried live shoot biomass and dead shoot mass, and serving as an indicator of the total amount of biomass produced even if some eventually died; (2) dead shoot proportion, used as an indicator of drought-

induced tissue mortality and calculated as the ratio of dead shoot mass to total shoot biomass produced after treatment; and (3) shoot nitrogen concentration, used as an indicator of plant nitrogen acquisition. In all cases, we included the initial basal area to rule out the variation caused by initial plant size. To test if structural AMF colonization is a better predictor of plant performance than total AMF colonization, four models were fitted for each response variable, with the following predictor sets interacting with species and treatment: (i) pre-treatment total AMF colonization; (ii) pre-treatment structural AMF colonization; (iii) post-treatment total AMF colonization; (iv) post-treatment structural AMF colonization. To test how the root traits were linked to plant stress tolerance and N concentration, one additional stepwise regression model was fitted for each response variable, including the following predictors: (v) post-treatment root traits (RD, SRL and RTD). Response and predictor variables were log-transformed when needed to meet the assumptions of normality and homoscedasticity. We checked for multicollinearity among the predictor variables using the Variance Inflation Factor (VIF). Whenever a VIF exceeded 5, we dropped the redundant independent variable and retained the variable most relevant to our research question. For instance, RD and SRL were highly negatively correlated across both species; consequently, we dropped SRL from our model and opted for RD and RTD as our primary root traits. Furthermore, because RD and RTD exhibited high collinearity in models predicting dead shoot proportion, these two traits were fitted in separate models to avoid multicollinearity for this particular model. After fitting the model, the predictors' relationship with the response variable was visualized by partial residual plots using the interactions package (Long 2019). We also tested the co-variation among root traits and AMF colonization with PCA (the figure is attached in the supplementary table)

Results

Interspecific variation in AMF colonization and root traits

AMF colonization patterns varied significantly between alfalfa and sainfoin following the treatments (Table S1; Fig. 1). Although total AMF colonization did not differ significantly between species (P>.05) under the control condition, structural colonization varied significantly. Alfalfa roots were predominantly occupied by vesicles (Fig. 1(b)), which was 80% higher in alfalfa control plants compared to sainfoin (0.29 vs. 0.06; Fig. 1b). Conversely, sainfoin roots were heavily colonized by hyphal networks and arbuscules (Fig. 1(c), (d)). Arbuscule proportion was 64% greater in sainfoin than in alfalfa under control conditions (0.31 vs. 0.11; Fig. 1d). In terms of root traits, Alfalfa had significantly lower average RD and significantly higher RTD (P<.0001; Fig S1; Table S1) than sainfoin.

Treatment effects on AMF colonization and root traits

In alfalfa, total AMF colonization was significantly reduced by drought (p = 0.016) compared to the control, but no difference was observed between the control and low nutrient treatments (Fig. 1a; Table S1). A similar trend was observed for vesicle proportion, with drought reducing vesicle proportion and low nutrient treatment increasing vesicle proportion relative to the control (Fig. 1b). In contrast, hyphal

and arbuscule proportions in alfalfa showed no variation among treatments (Fig. 1c, d). In sainfoin, drought slightly reduced total AMF colonization relative to the control, whereas low nutrient treatment slightly increased colonization, but the differences were not significant (Fig. 1a; Table S1). Vesicle proportion remained low across all treatments in sainfoin, with no significant differences found between the control and resource-limited treatments (Fig. 1b). Although low nutrients tended to increase hyphae and arbuscule proportion in sainfoin, these differences were not significant relative to the control (Fig. 1c, d). Regarding root traits, drought marginally reduced RD ($P = 0.07$) but not RTD, in alfalfa; conversely, drought marginally reduced RTD ($P = 0.08$) but not RD, in sainfoin (Fig S1(a, b); Table S1). No differences were found in either RD or RTD between low nutrient and control in either species.

Total AMF colonization vs structural metrics as predictors of plant performance

Pre-stress AMF structural colonization was a stronger predictor of plant performance than total AMF colonization (Fig. 2; Table S2). For all three response variables measured after treatment (total plant mass, dead shoot proportion, and shoot nitrogen), models including pre-treatment structural colonization showed better model fit, as indicated by consistently lower AIC values compared to models using total AMF colonization alone (Table S2). These models also explained a substantial proportion of the variation in total biomass ($R^2 = 0.64$), dead shoot proportion ($R^2 = 0.56$), and shoot nitrogen (%) ($R^2 = 0.34$) (Table S2). However, this pattern did not persist after treatment (Table S2). In post-treatment analyses, models including total AMF colonization showed lower or comparable AIC values relative to those including structural colonization, depending on the response variable (Table S2). Total AMF colonization explained 64%, 56%, and 36% of the variation in shoot biomass, dead shoot proportion, and shoot nitrogen (%), respectively.

Overall, these findings suggest that structural colonization is a more informative predictor of plant performance before stress, whereas after treatment, total AMF colonization provides similar or better model fit and explanatory power.

Pre- vs post-treatment AMF colonization traits

The predictive effects of pre-treatment AMF colonization on total shoot biomass and dead shoot proportion were strongly context-dependent (Tables S3, S4). While pre-treatment vesicle colonization rate was generally associated with larger shoot biomass ($P = 0.016$) regardless of species and treatment effect, hyphal colonization showed a treatment-specific effect. Pre-treatment hyphal colonization was positively correlated to total shoot biomass only under drought conditions (Fig. 2a; Table S3), with a stronger positive relationship for alfalfa.

When dead shoot proportion was used as the response variable, pre-treatment arbuscule colonization was consistently associated with reduced shoot mortality under drought for both species (Fig. 2b; Table S4). In contrast to biomass and survival responses, structural colonization (arbuscules and vesicles) did

not significantly predict shoot nitrogen concentration (Fig. 2c; Table S5). Instead, shoot nitrogen was primarily influenced by species, treatment, and initial plant size, indicating that pre-stress AMF colonization effects on nitrogen content are weaker and less interaction-driven than those observed for biomass and survival responses.

Post-treatment vesicle proportion in alfalfa and sainfoin was positively associated with total shoot biomass under low-nutrient conditions ($P = 0.006$; Fig. 3a; Table S6) and strongly reduced shoot mortality under drought ($P < 0.001$; Fig. 3b; Table S7) for alfalfa, but not for sainfoin. However, for the shoot N, the relationship with AMF colonization was divergent for both species; under low nutrients, sainfoin had higher shoot nitrogen concentration with higher arbuscule colonization, but for alfalfa, the relationship was neutral (Fig. 3c; Table S8). These results suggest the species-specific and context-dependent functional role of AMF structures.

Root traits correlation with AMF colonization and plant performance

Root traits, particularly average root diameter and root tissue density, mediated plant responses to stress in complex ways. When dead shoot proportion was used as the response variable, alfalfa and sainfoin exhibited distinct drought-tolerance strategies. Alfalfa showed reduced shoot mortality with larger root diameter (RD) (Fig. 4a; Table S10). Importantly, larger root diameter was positively associated with higher total AMF colonization and vesicle proportion in alfalfa from PCA (Fig S2), indicating that larger roots with higher vesicle colonization may be important for alfalfa's reduced shoot mortality. In contrast, sainfoin relied on structurally conservative traits, with higher root tissue density (RTD) significantly reducing shoot mortality (Fig. 4b; Table S11). In contrast to alfalfa, sainfoin's RTD or RD variation was unrelated to root colonization (Fig S2). When shoot N concentration was used as the response variable, alfalfa exhibited reduced N concentration with higher root tissue density, while sainfoin showed a weak or neutral relationship between RTD and shoot N concentration ($P = 0.20$; Fig. 4c; Table S12), whereas. These findings suggest species-specific, trait-based strategies for drought tolerance and nutrient acquisition.

Discussion

Understanding intraspecific variation in mycorrhizal colonization and root traits is critical for predicting plant community stability and resilience under a changing climate. Our experiment shows that plant responses to drought and nutrient limitation were shaped not only by the total AMF colonization, but by its structural composition, timing, and interaction with root functional traits. Across both species, the influence of AMF and root traits was most pronounced under drought stress, followed by nutrient limitation, indicating that symbiotic functioning and rootplasticity become particularly important when soil resource availability is constrained. Interestingly, alfalfa and sainfoin carried different drought tolerance strategies, where alfalfa was more dependent on average root diameter, while sainfoin was more dependent on root tissue density.

AMF colonization and root traits variation between species

Species with larger root diameters are typically thought to provide greater space for mycorrhizal colonization (Delpiano et al. 2025; Reinhardt & Miller 1990); however, most studies have not distinguished among the functional structures of AMF occupying this root space. In our study, although sainfoin had a greater root diameter than alfalfa, post-treatment total AMF colonization did not significantly differ between the two species. Instead, we observed pronounced differences in the composition of AMF structures, suggesting species-specific strategies in carbon allocation and nutrient exchange. The higher abundance of arbuscules and hyphae in sainfoin may reflect its evolutionary adaptation to calcareous, high-pH soils where phosphorus availability is limited (Carbonero et al. 2011), leading to a stronger dependence on AMF-mediated nutrient acquisition. Furthermore, sainfoin's relatively limited breeding history may have preserved its mycorrhizal associations (Martín-Robles et al. 2017). In contrast, alfalfa, which has undergone extensive breeding for increased root size and productivity (Li & Brummer 2012; Bucciarelli et al. 2021), may rely less on AMF for nutrient uptake, resulting in a colonization pattern dominated by the carbon-storage structure of AMF, i.e., vesicles. Alternatively, these differences may arise from associations with distinct AMF taxa exhibiting different colonization and functional strategies (Hart & Reader 2002; Camenzind et al. 2014); however, compositional data would be needed to test this hypothesis.

Pre-stress AMF colonization as a predictor of plant performance

Our findings highlight the importance of explicitly considering the structural composition of AMF colonization rather than relying solely on total colonization as a predictor of plant stress tolerance and nitrogen uptake. We show that pre-stress AMF hyphae and arbuscules explain more variance in final plant total biomass and shoot mortality in both species than total AMF colonization. Previous studies have reported direct and indirect mechanisms of AMF-mediated drought stress tolerance (Cheng et al. 2021). While evidence linking the physical abundance of AMF colonization to drought resilience is insufficient, mycorrhizal plants are typically reported to have better access to water and nutrients than non-mycorrhizal plants (Khalvati et al. 2005; Allen 2007; Kakouridis et al. 2022). Importantly, mycorrhiza-mediated water acquisition is largely attributed to the proportion of hyphae in the root and soil (Jansa et al. 2013; Abdalla et al. 2023; Li et al. 2025). Hyphal networks expand soil exploration far beyond the rhizosphere, enhancing access to both water and nutrients when root uptake is constrained (Allen et al. 2003). Additionally, under resource limitations, plants typically allocate more biomass belowground to improve resource acquisition (Munns & Cramer 1996). In our experiment, however, clipping plants' tops before stress likely constrained this response by reducing photosynthetic capacity. Consequently, plants already established with a higher abundance of AMF hyphae and arbuscules are perhaps costly at the initial stage of clipping but ultimately pay off during subsequent stress with better plant fitness and stress tolerance under resource limitations (Jakobsen et al. 1992; Vannette & Hunter 2011; Werner & Kiers 2015). The pre-established AMF structures, however, did not predict post-treatment shoot nitrogen. We acknowledge that because AMF primarily evolved as a mechanism for P acquisition, measuring plant

tissue P concentrations is critical for interpreting symbiotic benefits (Chiu & Paszkowski 2019; Etesami et al. 2021). Future studies should consider the pre-stress AMF structural colonization as a predictive trait to understand plant N and P acquisition and other growth-related benefits.

Post-treatment AMF colonization and root trait association with plant performance

Exposure to stress can influence the plant–fungus symbiotic relationship by changing plant water and nutrient demands, thereby modifying the composition of fungal colonization (Le Pioufle & Declerck 2018; Caccia et al. 2024). We found that, in contrast to pre-stress AMF colonization, post-stress total AMF colonization captured adequate variation for predicting plant performance. While individual AMF structures did not contribute additional predictive power for post-stress AMF colonization, they still provided specific information about the AMF structural role. For instance, higher vesicle colonization was associated with reduced shoot mortality under drought and greater biomass under low-nutrient conditions in alfalfa. The transition from arbuscule- and hyphae-dominated associations before stress to vesicle-dominated associations after stress suggests a dynamic functional shift within the symbiosis in response to declining soil resources (Dumbrell et al. 2011). Because vesicles are not major sites of nutrient exchange, their accumulation likely reflects symbiotic persistence, carbon storage, or fungal survival during prolonged stress (Cabello 1997). Vesicle formation thus represents a conservative phase of the symbiosis in which carbon allocation to fungal tissues continues despite reduced reciprocal nutrient transfer. Furthermore, under resource limitation, excess fixed carbon may drive increased fungal abundance independently of plant nutrient demand, allowing AMF to function as a carbon sink rather than purely as a nutrient exchange partner (Prescott et al. 2020).

Variation in alfalfa post-stress vesicle colonization can be further explained by differences in average root diameter, which was negatively associated with shoot mortality (Table S10) and positively associated with vesicle and total colonization (Fig S2). The linkage between thicker roots and vesicle proportion supports the idea that AMF associations function as part of a conservative stress strategy (Hushan et al. 2025). Under water limitation, plants may redirect carbon towards thicker roots, which not only support growth and maintenance of plants under resource limitations but also provide habitats for carbon-storage structures such as vesicles in root cortex (Hasibeder et al. 2015; Zwicke et al. 2015; Kalra et al. 2024).

By contrast, sainfoin exhibited limited post-stress variation in arbuscule or hyphal abundance in predicting plant biomass and dead shoot proportion, indicating a more uniform symbiotic configuration across the cultivars. Yet, sainfoin's shoot N was positively related to arbuscule proportion under low nutrients condition, indicating greater nutritional benefits from the abundance of arbuscules when additional nutrients were not applied. This supports the idea that plants may benefit from synergistic colonization by higher AMF abundances and rhizobia in perennial legume (Primieri et al. 2021). However, drought tolerance in sainfoin was not associated with root diameter or root colonization. Instead, this was more closely tied to root tissue density (RTD), a conservative trait associated with longer root

lifespan, structural robustness, and reduced turnover, and typically linked to slower nutrient acquisition strategies (Ryser 1996; Lozano et al. 2019). The negative association between RTD and tissue mortality suggests that high-RTD cultivars achieved better drought tolerance. High RTD may enable the maintenance of healthy, long-lived shoots while investing in durable root systems (Dietrich et al. 2025). However, we did not find any association of root tissue density with mycorrhizal colonization. While few studies report a strong positive association between AMF colonization and RTD (Wang et al. 2025; Qiu et al. 2026), the lack of such a relationship in our study aligns with the conservation–collaboration hypothesis (Bergmann et al. 2020), which places RTD on a separate axis from AMF colonization and root diameter.

Overall, our findings demonstrate that plant responses to drought and nutrient limitation cannot be understood solely through total AMF colonization but require consideration of the structural composition of fungal symbiosis and its interaction with root functional traits. The contrasting strategies observed between alfalfa and sainfoin highlight species-specific pathways to stress tolerance: alfalfa relies on a more dynamic, carbon-driven association characterized by vesicle formation and root thickening, whereas sainfoin maintains a more stable, functionally active symbiosis supported by arbuscules and conservative root traits such as high tissue density. Importantly, the predictive value of pre-stress AMF structures underscores the role of early symbiotic establishment in shaping subsequent plant performance under stress. Ours is the first study, to our knowledge, to attempt to consider pre-stress AMF colonization as a predictive trait, where most studies assess the AMF colonization after stress response, especially in inoculated plants, which limits the understanding of natural conditions where plants face abiotic stress with the existing AMF colonized roots. It is important to consider that AMF colonization can vary according to soil nutrient patches and roots' spatial distribution (Grünfeld et al. 2022); therefore, before considering AMF as a predictive trait, standardized root colonization assessments need to represent a whole plant's colonization status. Measuring the extraradical hyphae along with intraradical colonization is also critical for understanding the ecological role of AMF symbiosis in detail, especially in response to environmental variables. Further studies are needed to identify key mycorrhizal and root traits to understand plant resource foraging strategies and community interactions in a changing climate.

Declarations

The authors declare that they have no conflict of interest.

Author Contribution

JB conceived the study and designed the experimental framework. BB contributed to the selection of plant cultivar lines, provided relevant cultivar information, and provided the materials for the experiment. SG conducted post-harvest sample processing, performed data analysis, and wrote the manuscript. JW

and LP conducted the fieldwork and greenhouse experiments. JB edited and revised the manuscript. JW, LP, and BB also contributed to revising and editing the manuscript.

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Data Availability

The datasets generated during the current study are available from the corresponding author upon request.

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Figures

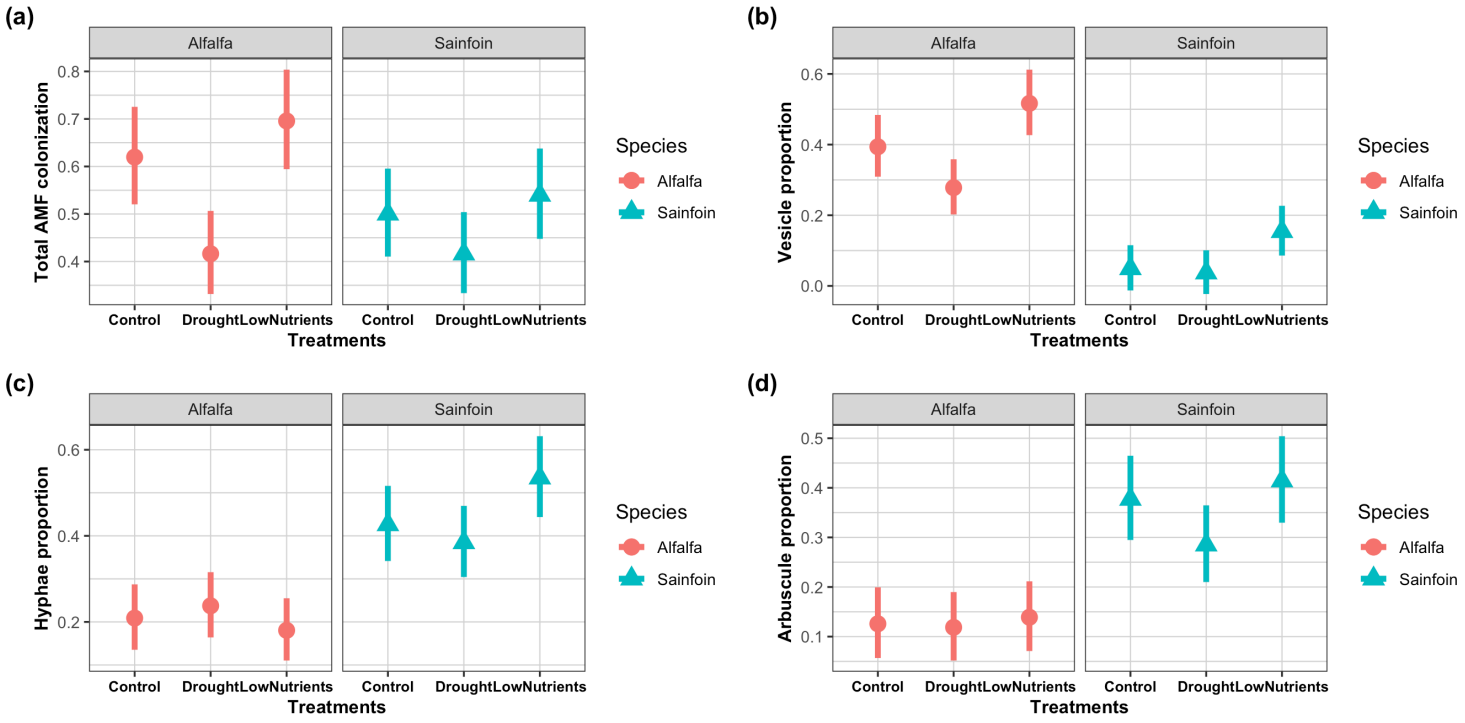

Figure 1

The effects of Drought and low nutrient treatment on total AMF colonization (a) and individual AMF colonization proportion (vesicles, hyphae and arbuscules) (b,c,d). Points and whiskers represent model-estimated means and 95% confidence intervals

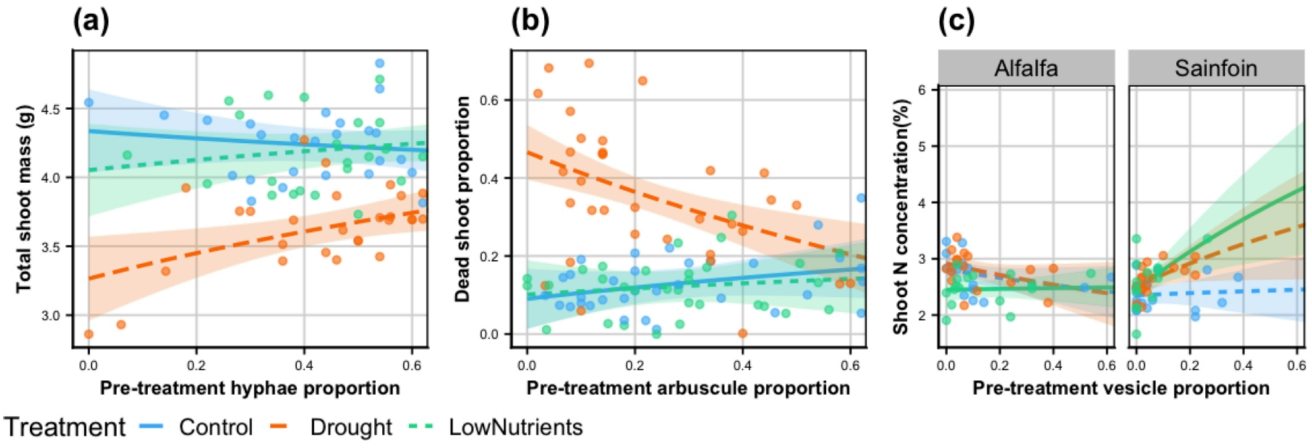

Figure 2

The association of pre-treatment AMF structural colonization with plant performance metrics: total shoot mass(g) (a), dead shoot proportion (b), and shoot N concentration (%) (c). Regression lines show model-estimated relationships and their 95% confidence intervals plotted against the partial residuals

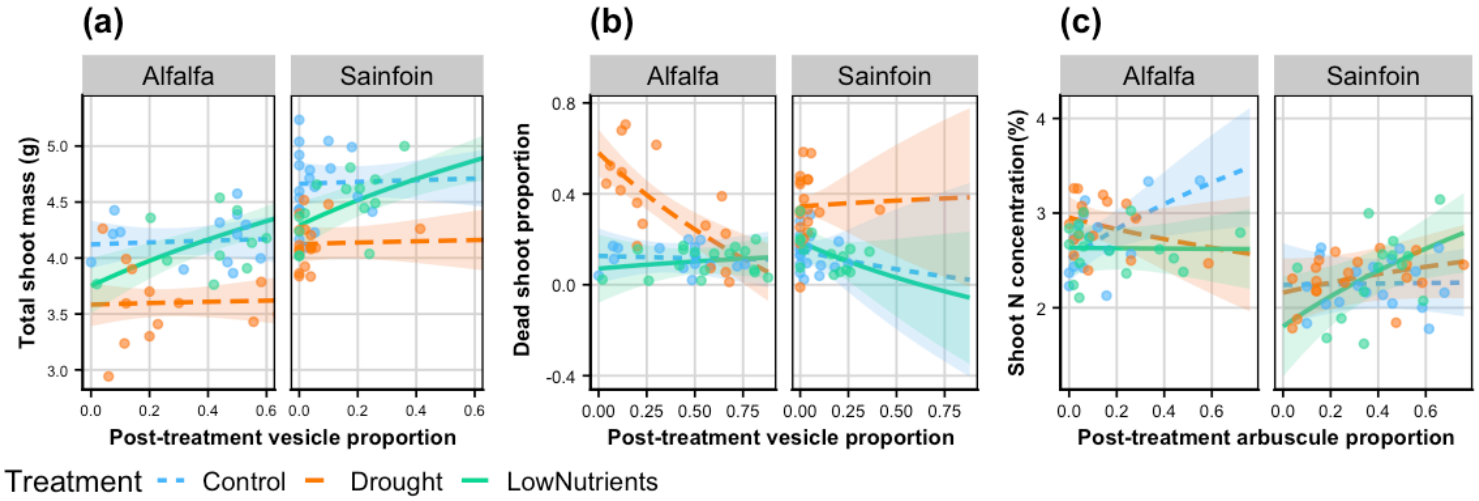

Figure 3

The association of post-treatment AMF structural colonization with plant performance, total shoot mass(g) (a), dead shoot proportion (b) and shoot N concentration (%) (c). Regression lines show model-estimated relationships and their 95% confidence intervals plotted against the partial residuals

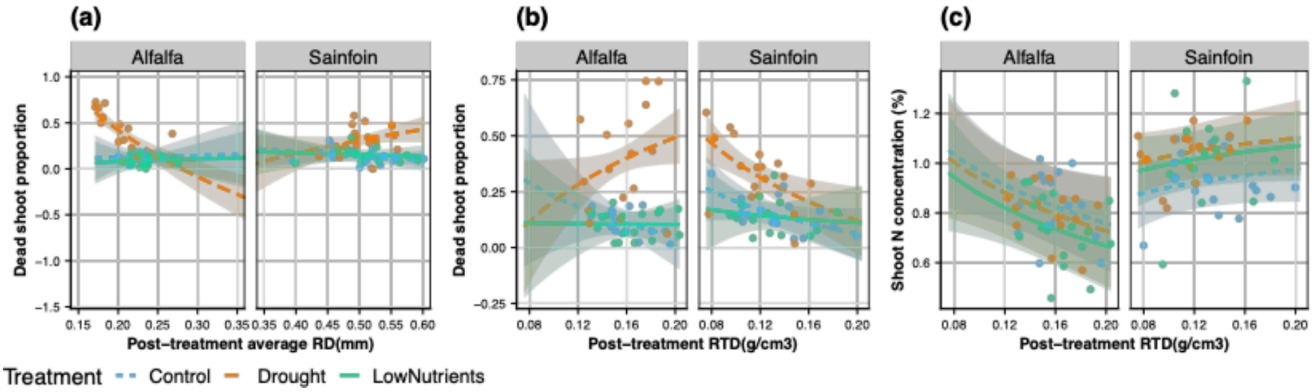

Figure 4

The association of post-treatment AMF structural colonization with plant performance. Dead shoot proportion and average RD (a), dead shoot proportion and RTD (b), and shoot N concentration (%) and RTD (c). Regression lines show model-estimated relationships and their 95% confidence intervals plotted against the partial residuals

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