

Nitrogen-driven plasticity of root architecture and biomass allocation in three functionally diverse pasture species

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Abstract

Background and aims

Multi-species pastures (grass-legume-herb mixes) can improve the seasonal distribution of forage production and reduce reliance on nitrogen (N) fertiliser compared with perennial ryegrass monocultures, but the root architectural traits supporting these benefits remain poorly understood. This study investigated species-specific root characteristics and N-driven plasticity in three pasture species.

Methods

Perennial ryegrass (*Lolium perenne* cv. Base AR37), white clover (*Trifolium repens* cv. Legacy) and chicory (*Cichorium intybus* cv. Puna II) were grown individually in 25-cm-tall sand columns for 7 weeks under low or high N supply, then shoot and root biomass were measured. Washed roots were analysed using WinRhizo, while micro-computed tomography (micro-CT) quantified three-dimensional architecture and depth-specific root distribution in intact columns.

Results

Chicory produced the greatest shoot and root biomass, particularly under high N, and showed greater root length, surface area, branching and fine-root volume than perennial ryegrass and white clover. Micro-CT revealed spatial rooting patterns: chicory had greater root volume both near the soil surface and below 15 cm, perennial ryegrass roots were concentrated in the upper 4.5 cm, and white clover maintained lower root volume across all depths. White clover sustained similar biomass under both N treatments, despite lower root volume, consistent with greater nodulation under low N.

Conclusion

Root spatial distribution and fine-root formation were key traits linking species identity with productivity under contrasting N availability. The contrasting rooting strategies suggest functional complementarity among grasses, legumes and herbs in mixed pastures, while micro-CT provided spatial information not captured by destructive root scanning.

Introduction

Multi-species pastures are increasing in popularity in pasture-based livestock systems because they can enhance productivity, reduce reliance on nitrogen (N) fertiliser inputs (Shackleton et al., 2024; Thomson & Albornoz, 2023), and create a more even seasonal distribution of feed (McNally et al., 2015; Sanderson et al., 2005). This is largely attributed to the functional complementarity among pasture species, particularly between the functional groups (i.e., grass, legume, and herb), which differ in their strategies of resource acquisition and utilisation (Thomson & Albornoz, 2023). While aboveground productivity in multi-species pastures has been widely studied, the belowground traits and interactions that may contribute to these benefits remain poorly understood.

Root systems play a central role in plant-soil interactions by governing the acquisition of water and nutrients and are important for competition and facilitation among neighbouring species (Fitter, 1996). Variations in root architectural traits, including root volume distribution, branching patterns, and the relative biomass allocation to fine and coarse roots, can strongly influence the efficiency and spatial patterns of soil resource capture. Grasses possess a fibrous root system typical of monocotyledons. Perennial ryegrass, one commonly used grass in multispecies pastures and a common monoculture for dairy pastures, has its fibrous roots originating from the plant base and predominantly occupying the top 15 cm of the soil profile (Russell, 1977). In comparison, white clover is a relatively shallow-rooted legume that relies on biological N₂ fixation to meet much of its N demand (Hoekstra et al., 2015). In contrast, forage herbs such as chicory develop a dominant taproot capable of penetrating substantially deeper into the soil profile than white clover (Haughey et al., 2020; Sanderson & Elwinger, 2000). These contrasting rooting strategies are likely to influence the spatial distribution of resource acquisition, as species with deeper or more highly branched root systems may have greater access to soil water and nutrients, thereby enhancing overall productivity (Atkinson et al., 2014).

The availability of N is a key driver of root development and plasticity. Plants often adjust their root architecture in response to N supply. For example, the root diameter reflects the investment of resources into root development. An increased root diameter is often associated with reduced root branches and length, which can constrain soil exploration and ultimately affect forage productivity (Wu et al., 2016). In contrast, traits such as surface area, the number of tips, forks and crossings reflect branching patterns. Greater branching increases soil contact and results in enhanced resource capture (Atkinson et al., 2014). However, these responses are highly species-specific and may reflect contrasting nutrient-acquisition strategies. For example, with increased available N, wheat roots increase in biomass (Yan et al., 2023) while white clover nodulation decreases with no effect on root biomass (Zhang et al., 2019).

Despite the recognition of these root traits, quantitative measurement of root architecture remains challenging due to methodological limitations. Destructive sampling methods that involve WinRhizo-based root scanning are commonly used in the field (Cardinael et al., 2015; Yang et al., 2020) and controlled environment studies (Chen et al., 2011), but they cannot measure the same root system repeatedly or capture its three-dimensional architecture in soil. Advances in imaging approaches, such as X-ray computed tomography (CT), provide new opportunities to capture the spatial distribution of root system structure in a non-destructive and less labour-intensive way (Mooney et al., 2022). Integrating new methods can improve our ability to quantify root traits across spatial and temporal scales, although their relative sensitivity and comparability require further evaluation.

In this study, we investigated the root architectural traits and biomass allocation of three pasture species representing the major functional groups: perennial ryegrass (*Lolium perenne*, grass), white clover (*Trifolium repens*, legume), and chicory (*Cichorium intybus*, herb), under a controlled environment with contrasting N availability. We hypothesised that: 1. Species differ in their root characteristics, reflecting distinct approaches to soil exploration and N acquisition; and 2. N availability induces species-specific changes in root architecture and biomass allocation. We conducted a controlled sand column experiment and quantified root traits using both WinRhizo and micro-CT scanning.

Materials and Methods

Sand columns

The experiment was conducted for 2 months (May to June) in 2024, in a glasshouse with an average temperature of 19°C (12.8–23.3°C) and relative humidity of 76% (37–92%). The experiment was arranged as a 3 × 2 factorial design with 5 replicates per treatment, comprising three plant species: perennial ryegrass (PRG), white clover (WC), and chicory (Chi), at two nitrogen (N) levels (high N and low N). Sand columns were PVC pipes (25 cm height, 6.5 cm inner diameter) with nylon mesh fixed to the bottom using metal hose clamps to contain the sand. Columns were filled with 2 mm sieved, acid-washed river sand, gently tamped down by hand to ensure uniform packing (bulk density 1.48 g/cm³), leaving a 1 cm gap at the top. Columns were maintained upright during growth and allowed to drain freely from the base after irrigation. Plants of perennial ryegrass (*Lolium perenne* cv. Base AR37), white clover (*Trifolium repens* cv. Legacy), and chicory (*Cichorium intybus* 'Puna II') were grown from seed in the sand columns. Six seeds of a single species were sown per column, germinated within the sand column, then thinned to a single plant. White clover was inoculated with Group B inoculant (Peat) before sowing (New Edge Microbials Pty Ltd). All sand columns were maintained under high or low N treatments for 7 weeks to measure root architecture and response to N for the three species. Plants were hand-watered twice per week with nutrient solution (Hoagland's, Sigma-Aldrich), with all macro- and micronutrients supplied to avoid nutrient limitation. The low N (39.32 mg/kg soil) treatment received sufficient N to support plant growth while permitting root nodule formation in the clover, modified from Zhang et al. (2019), whereas the high N (58.97 mg/kg soil) treatment received additional N supplied as urea ammonium nitrate (UAN). This approach ensured that N was the only variable differing between treatments. The nutrient solution also supplied the following elements (mg/kg soil): P (5.80), K (43.90), Ca (30.00), Mg (9.10), S (12.01), B (0.094), Mn (0.094), Zn (0.009), Cu (0.004), Mo (0.002), Fe (0.273), and Cl (0.121).

The amount of nutrient solution received at each watering for each plant increased as plants developed (Fig. 1).

Plants were harvested after 7 weeks, and the shoots were harvested at the soil surface. The below-ground root samples were collected via removal of the entire column of sand and root washing. The roots from each column were treated as one complete

sample. Root traits, including Length (cm), Surface Area (cm²), Average Diameter (mm), Root Volume (cm³), Tips (number (#)), Forks (#), and Crossings (#), were measured using a flatbed scanner at 600 dpi, with subsequent analysis of collected scan images via WinRhizo software (version 2016; Régent Instruments, Quebec, Canada). Collected root and shoot biomass were oven-dried at 75°C for 48 hours after scanning was completed and then weighed for dry matter production.

Micro-CT Scanning and Analysis

Repeated X-ray micro-computed tomography (micro-CT) has been used to monitor root development in living plants without destructive sampling (Zappala, Helliwell, et al., 2013; Zappala, Mairhofer, et al., 2013). Accordingly, micro-CT scans were conducted at weeks 3, 5 and 7 (Fig. 1), with plants returned to the glasshouse after scanning to continue growth. At weeks 3 and 5, one replicate from each species × N-treatment combination was scanned, as the number of replicates at these intermediate time points was limited by scanning costs. At week 7, three replicates from each species × N-treatment combination were scanned before the final harvest, allowing micro-CT-derived root measurements to be compared with destructive measurements.

Scans were performed using a Phoenix Nanotom M (Waygate Technologies), operated using XS Control and phoenix datos|x acquisition software (Waygate Technologies). At week 3, a single scan of the upper 12 cm of the sand column, including the soil-air interface, was captured at a voxel resolution of 65 µm. At weeks 5 and 7, two scans were collected to capture the full sand column height (0–12 cm and 13–25 cm) using a multi-scan feature of the acquisition software at the same voxel resolution. Each scan took 15 minutes using an X-ray tube voltage of 120 kV and tube current of 300 µA, collecting 1200 2D projections through a full 360-degree rotation of the sample. A 0.1 mm copper filter was used on the X-ray source to pre-harden the X-ray. Volume reconstruction (i.e. converting 2D X-ray projections into a 3D model) was performed using the phoenix datos|x reconstruction software, applying an inline median filter, region of interest (ROI) filter and beam hardening filter during reconstruction. Data were exported in a 32-bit format for analysis.

Reconstructed volumes were imported into Avizo3DPro version 2019.3 (Thermo Fisher Scientific) for analysis. Thirty-two (32)-bit images were converted to 16-bit images using the same scaling and offset parameters for further analysis. Roots were segmented using a consistent workflow from sample to sample using the steps outlined below and in Fig. 2:

- 1) Grey values within a defined threshold range (i.e. intensity values used to distinguish the object of interest from background) were selected to create a binary mask of low-density material in the sand columns, including root structures. This initial segmentation also captured areas at the boundaries of high-density sand grains and air-filled pores in the sediment, due to the partial volume effect.
- 2) To remove areas segmented by the partial volume effect in step 1, a selective erosion algorithm was applied to remove thin boundary regions, leaving thicker areas of low-density material segmented.
- 3) The binary image from step 2 was analysed to determine the volume of connected objects in the segmentation. Objects with a volume less than 0.1 mm³ were removed from the segmentation, assuming they represented disconnected and isolated objects of low density, as opposed to connected root structures with a larger volume.
- 4) A selective dilation of the remaining segmentation was conducted using the same parameters as the erosion algorithm in step 2, to dilate the remaining structure back to the original boundaries.
- 5) Further manual processing of each segmentation was applied to remove noise at the soil-air interface generated by artefacts, and any plant material above the soil-air interface. The low-density plant identification labels were also removed from the segmentation.
- 6) The remaining structure was assumed to be root material, and its volume was determined as a function of horizontal cross-sections (1 voxel thick) through the vertical column using Avizo's volume fraction tool. These trends were further processed to determine the total root volume in 3 cm intervals (462 slices) of the sand column height.

Statistical analyses

Statistical analyses were performed using Minitab software version 21.4.2. Data were analysed using general linear models. For biomass, WinRhizo root traits and root volume by diameter class, species, N treatment and the species $\times$ N interaction were fitted as fixed effects. Residuals-versus-fitted plots were inspected to assess homogeneity of variance and potential non-random patterns in the residuals. Where residual diagnostics indicated strong departures from model assumptions, variables were log- or square-root-transformed before analysis. When significant treatment effects were detected, Tukey-adjusted pairwise comparisons were used to compare treatment means. Different letters in tables indicate significant differences among species $\times$ N treatment combinations at $p < 0.05$.

Results

Biomass production and root distribution

Biomass production differed markedly among species (Table 1).

Table 1

Shoot and root biomass and shoot-to-root ratio of perennial ryegrass, white clover and chicory grown under low and high nitrogen (N) supply. Values are means $\pm$ SEM (g DM/column); shoot-to-root ratios are calculated from the mean shoot and root biomass for each treatment. Different letters within a row indicate significant differences among species $\times$ N treatment combinations based on Tukey-adjusted pairwise comparisons ($p < 0.05$). P-values indicate the species $\times$ N interaction from the general linear model.

Biomass (g/column)	Perennial ryegrass		White clover		Chicory		P-value						
	Low N	High N	Low N	High N	Low N	High N							
Shoot	0.18 ± 0.02	c ± 0.02	0.32 ± 0.02	b ± 0.03	0.09 ± 0.03	c ± 0.03	0.12 ± 0.03	c ± 0.05	0.32 ± 0.03	b ± 0.03	0.47 ± 0.03	a ± 0.03	< 0.001
Root	0.18 ± 0.04	bc ± 0.03	0.23 ± 0.03	ab ± 0.02	0.12 ± 0.02	c ± 0.01	0.15 ± 0.01	bc ± 0.03	0.24 ± 0.03	ab ± 0.03	0.35 ± 0.06	a ± 0.06	< 0.001
Shoot-to-root ratio	1		1.4		0.75		0.8		1.33		1.34		N/A

Under high N, shoot biomass followed the order chicory (0.47 g/column) > perennial ryegrass (0.32 g/column) > white clover (0.12 g/column), with significant differences ($p < 0.05$) among all three species. In comparison, chicory (0.32 g/pot) still produced significantly greater ($p < 0.05$) shoot mass than the other species under low N treatment, but the difference between the perennial ryegrass (0.18 g/pot) and white clover (0.09 g/pot) was not significant ($p > 0.05$). The high N treatment significantly ($p < 0.05$) increased the shoot biomass of chicory (by 46%) and perennial ryegrass (by 81%) relative to the low N treatment, while no change was found in the shoot biomass of white clover (Table 1).

Chicory produced significantly ($p < 0.05$) greater root biomass than white clover under both low N (0.24 vs 0.12 g/column) and high N (0.35 vs 0.15 g/column), whereas perennial ryegrass (0.18 and 0.23 g/column under low and high N, respectively) did not differ significantly from either species. Nitrogen supply had no significant effect on root biomass within any of the three species (Table 1).

The shoot-to-root ratio of each species showed changes in biomass allocation in response to available N. Perennial ryegrass showed a significant increase ($p < 0.05$) in shoot biomass without a corresponding increase in root growth with increased N, resulting in a greater increase (40%) in shoot-to-root ratio in the high N treatment (1.4) relative to the low N (1) than observed with white clover and chicory. White clover showed a small increase in shoot-to-root ratio (7%) from low N (0.75) to high N (0.8) treatment, while chicory showed the smallest increase (0.7%) from low N (1.33) to high N (1.34) treatment.

The three-dimensional reconstructions provided a visual representation of the rooting pattern and architecture of the roots over the experiment (Fig. 3).

Root distribution along the sand column also varied among species (Fig. 4).

Chicory developed significantly ($p < 0.05$) greater root volume than both perennial ryegrass and white clover at the soil surface (0–3 cm) and at depth (below 15 cm). In the upper 3 cm, root volume followed the order chicory > perennial ryegrass > white clover, with significant differences among all three species ($p < 0.05$). And to the top 9 cm, white clover tended to have less root volume than the other two species ($p < 0.1$). At intermediate depth (6–15 cm), perennial ryegrass had root volumes similar to chicory. Below 15 cm, perennial ryegrass had significantly ($p < 0.05$) lower root volume than chicory ($p < 0.05$) but did not differ significantly from white clover.

Root architectural responses to nitrogen

Root architectural traits differed strongly among species and responded differently to N availability (Table 2).

Table 2

Root characteristics of perennial ryegrass, white clover and chicory grown under low and high N supply, measured using WinRhizo after destructive harvest at week 7. Values are means $\pm$ SEM. Different letters within a row indicate significant differences among species $\times$ N treatment combinations based on Tukey-adjusted pairwise comparisons ($p < 0.05$). P-values indicate the species $\times$ N interaction from the general linear model.

Measurements	Perennial ryegrass		White clover		Chicory		P-value
	Low N	High N	Low N	High N	Low N	High N	
Length (cm)	1048.75 ± 127	bc ± 196	1162.64 ± 150	bc ± 137	682.34 ± 185	c ± 277	< 0.001
Surface Area (cm ²)	143.54 ± 23.7	bc ± 31.9	176.87 ± 18.0	bc ± 20.2	86.9 ± 27.4	c ± 42.3	< 0.001
Average Diameter (mm)	0.43 ± 0.02	a ± 0.02	0.48 ± 0.01	a ± 0.02	0.41 ± 0.01	a ± 0.01	0.041
Total Root Volume (cm ³)	1.58 ± 0.32	b ± 0.44	2.16 ± 0.17	b ± 0.24	0.88 ± 0.33	b ± 0.57	< 0.001
Tips (#)	3413 ± 376	ab ± 491	3505.8 ± 575	ab ± 325	1621.2 ± 484	b ± 579	< 0.001
Forks (#)	4268.2 ± 920	b ± 862	5086.2 ± 535	b ± 516	2301.6 ± 796	b ± 1345	< 0.001
Crossings (#)	507 ± 90.7	b ± 105	569.2 ± 84.8	b ± 72.2	355.2 ± 99.8	b ± 205	< 0.001

Across both N treatments, chicory had significantly greater ($p < 0.05$) root length and surface area than white clover. In comparison, the root length and surface area of chicory were only significantly greater ($p < 0.05$) than those of perennial ryegrass under high N. The difference between perennial ryegrass and white clover was not significant ($p > 0.05$). Differences in root volume among species were observed only under high N, where chicory had significantly greater ($p < 0.05$) root volume than perennial ryegrass and white clover. The numbers of tips, forks and crossings did not differ significantly among species under low N. Under high N, chicory was found to have a significantly greater ($p < 0.05$) number of forks and crossings than perennial ryegrass and white clover, and a significantly greater ($p < 0.05$) number of tips only compared to white clover. The addition of N resulted in significant increases ($p < 0.05$) in all parameters except the average root diameter of chicory, whereas no changes were found in perennial ryegrass and white clover (Table 2).

Analysis of root volume distribution across diameter classes revealed differences between species and N nutrition (Table 3).

Table 3

Root volume (cm³) distribution across diameter classes for perennial ryegrass, white clover and chicory grown under low and high N supply, measured using WinRhizo after destructive harvest at week 7. Values are means $\pm$ SEM. Different letters within a row indicate significant differences among species $\times$ N treatment combinations based on Tukey-adjusted pairwise comparisons ($p < 0.05$). P-values indicate the species $\times$ N interaction from the general linear model.

Diameter class (mm)	Perennial ryegrass				White clover				Chicory				P-value
	Low N		High N		Low N		High N		Low N		High N		
> 0 and ≤ 0.5	0.39	c	0.41	c	0.31	c	0.37	c	0.84	b	1.61	a	< 0.001
	± 0.05		± 0.07		± 0.06		± 0.06		± 0.08		± 0.10		
> 0.5 and ≤ 1	0.85	bc	1.02	bc	0.37	c	0.44	bc	0.90	b	1.69	a	0.042
	± 0.11		± 0.18		± 0.07		± 0.08		± 0.15		± 0.21		
> 1 and ≤ 1.5	0.40	ab	0.65	a	0.14	b	0.16	b	0.30	ab	0.54	ab	0.481
	± 0.13		± 0.16		± 0.03		± 0.04		± 0.06		± 0.13		
> 1.5	0.49	a	1.06	a	0.39	a	0.59	a	1.07	a	1.71	a	0.844
	± 0.18		± 0.28		± 0.17		± 0.03		± 0.19		± 0.85		

Across species, chicory produced significantly more fine roots (0-0.5 mm) than both perennial ryegrass and white clover ($p < 0.05$) with or without the addition of N. When investigating the effect of additional N on each species, only chicory showed a significant increase in the volume of fine roots in the 0-0.5 mm ($p < 0.05$) and 0.5-1 mm ($p < 0.05$) diameter classes (Table 3).

White clover exhibited a significantly stronger ($p < 0.05$) nodulation response to N, with almost three times the number of nodules (an average of 44.8 ± 5.37 nodules per plant) found under the low N compared to the high N treatment (an average of 15.8 ± 2.87 nodules per plant).

Comparison of Root Measurement Methods

Overall, micro-CT scanning detected significantly less (47%) ($p < 0.05$) root volume compared to the WinRhizo scanning method. When further investigating the root volume detected per species, both methods yielded similar root volume for perennial ryegrass and white clover, whereas chicory had significantly lower ($p < 0.05$) relative root volumes when measured with micro-CT scanning compared to WinRhizo scanning. The WinRhizo scanning method detected significantly higher ($p < 0.05$) root volume in chicory under high N in comparison to the low N treatment, but not in white clover and perennial ryegrass. In comparison, micro-CT scanning did not detect significant N effects for any species (Table 4).

Table 4

Root volume (cm³) and detection ratio estimated by WinRhizo and micro-CT for perennial ryegrass, white clover and chicory grown under low and high N availability at week 7. Values are means $\pm$ SEM (cm³); detection ratios are treatment means calculated as micro-CT/WinRhizo. Different letters within a row indicate significant differences among species $\times$ N treatment combinations within each measurement method based on Tukey-adjusted pairwise comparisons ($p < 0.05$). P-values indicate the species $\times$ N interaction from the general linear model fitted separately for each measurement method.

Measurement methods	Perennial ryegrass				White clover				Chicory				P-value
	Low N		High N		Low N		High N		Low N		High N		
WinRhizo	1.58	b	2.16	b	0.88	b	1.1	b	2.25	b	4.24	a	< 0.001
	± 0.32		± 0.44		± 0.17		± 0.24		± 0.33		± 0.57		
Micro-CT	1.23	ab	0.98	ab	0.57	ab	0.49	b	1.44	ab	1.71	a	0.032
	± 0.35		± 0.09		± 0.09		± 0.08		± 0.17		± 0.46		
Micro-CT/ WinRhizo	0.78		0.45		0.65		0.45		0.64		0.40		N/A
Detection ratio													

Discussion

This study demonstrated that species-level differences in shoot biomass production under contrasting N availability are closely associated with their distinct root architectures. In particular, chicory achieved greater shoot biomass and showed deeper rooting and greater fine-root formation than perennial ryegrass and white clover, indicating a greater capacity to explore the sand column and intercept water and dissolved nutrients moving downward before drainage. Higher N supply further enhanced these advantages. In contrast, white clover showed comparable shoot biomass to perennial ryegrass despite having lower root volume under low N conditions, likely reflecting its ability to acquire N through biological N₂ fixation.

Biomass and root distribution

Chicory produced greater shoot biomass than perennial ryegrass and white clover in this study, indicating a stronger early-growth response. This pattern is consistent with controlled or short-term studies reporting comparable growth responses under similar conditions. For example, Jiang et al. (2016) observed a similar yield in perennial ryegrass as in this study using sand as a growth medium. In addition, McElroy et al. (2017) reported comparable shoot biomass responses in perennial ryegrass as in this study under repeated nutrient-solution application. The lower shoot biomass of white clover compared with perennial ryegrass is also consistent with Chiy & Phillips (1999), who reported that perennial ryegrass yielded 40.4% more shoot biomass than white clover under a relatively low N condition (24 kg N/ha). Although the N supply in the present study was higher (approximately 58 and 87 kg N/ha for the Low N and High N treatments, respectively), both studies showed a similar relative growth advantage of perennial ryegrass over white clover. Similar productivity advantages of chicory over perennial ryegrass have also been reported under field conditions, suggesting that the pattern observed in this controlled experiment is consistent with broader pasture studies (Høgh-Jensen et al., 2006). These consistent biomass patterns across the three species provide a useful context for interpreting differences in root development and spatial root distribution observed in this study.

Chicory produced greater root volume near the surface and at depth than perennial ryegrass and white clover (Fig. 4). In general, for all species, most roots were found close to the soil surface. This finding is consistent with a previous rhizobox study on a taproot plant, alfalfa (Pan et al., 2023) and similar conclusions were also drawn from rhizotron studies on perennial ryegrass (Black et al., 2017). This pattern is also consistent with the tendency for greater root growth in zones of frequent water and nutrient input, particularly near the surface, where irrigation and nutrient solution are applied (Van Oorschot et al., 2024). Because the sand columns in this study allowed free drainage, the applied water and dissolved nutrients would have moved downward through the profile before draining from the base. This means that deeper roots could have intercepted those resources below the surface, although N distribution by depth was not directly measured. The greater root volume of chicory at

depth is likely due to its extensive root system as a deep-rooted herb, producing a taproot that extends deeper into the soil profile than the shallow-roots of grass and clover (Haughey et al., 2020; G. Li & Kemp, 2005). In pasture systems receiving N from multiple sources (fertiliser, urine, decomposing plant material), pastures likely have N access but may face strong inter-plant competition. Greater root placement at depth may therefore provide a performance advantage by increasing access to resources from a larger, deeper soil profile (McNally et al., 2015), and may be beneficial for multi-species pasture systems.

In contrast, perennial ryegrass allocated more roots near the soil surface than white clover. This distribution is consistent with its fibrous root system, which enables rapid exploitation of water and nutrients concentrated in the upper soil layer through a dense network of shallow roots (Sapkota et al., 2012; Wang et al., 2025). Such a strategy is advantageous when nutrients remain available near the soil surface. However, under the freely draining conditions of this experiment, mobile nitrate was likely transported deeper through the sand profile, reducing the relative advantage of shallow rooting and increasing the importance of nutrient acquisition at depth. These contrasting rooting patterns therefore suggest different resource acquisition strategies, with the deeper-rooting habit of chicory likely contributing to its greater biomass production through improved access to nutrients distributed throughout the soil profile.

Root architectural responses to nitrogen

Availability of N influenced species performance through distinct allocation strategies and root trait responses. In perennial ryegrass, increased N primarily increased shoot biomass with little corresponding increase in root characteristic measurements (Table 2), leading to an increased shoot:root ratio under high N supply. This pattern indicates a shoot-focused resource allocation strategy, where additional nutrients are primarily allocated to aboveground growth instead of forming deeper and more extensive roots. This is consistent with previous work showing that the rooting depth of perennial ryegrass did not increase with a large quantity of additional N (from 75 to 100 kg N/ha/year), while the above-ground biomass production was more than doubled with only a roughly 40% increase in total root C (Mortensen et al., 2025).

In contrast, chicory responded to increased N with improvements in both above- and below-ground biomass, indicating no clear shift in resource allocation away from root allocation under higher N supply. This balanced response may reflect its strategy to invest equally in resource capture and above-ground production, which is likely driven by the root architectural changes under increased N availability. Fine root (diameter < 0.5 mm) production by chicory increased with N addition. In taproot systems, ammonium and nitrate N stimulate lateral root growth, increasing fine root prevalence (Gruber et al., 2013; Lima et al., 2010). This effect was observed in our study as an increased volume of fine root formation in chicory under the high N treatment. Similar results have been found in lettuce, a well-understood vegetable crop from the same family as chicory, where increasing UAN concentrations correlated with increased root volume for roots of < 0.5 mm diameter (C. Li et al., 2024). This greater number of fine roots and root branches likely improved the nutrient capture ability of chicory (Atkinson et al., 2014). With an increased volume of fine roots exploring the sand more thoroughly for nutrients and moisture, the observed increase in shoot biomass production was therefore sustained.

For white clover, increased N did not affect either above-ground or below-ground biomass production but did reduce the number of root nodules. The reason that root volume does not respond to the N fertiliser is likely due to biological N₂ fixation within clover root nodules, which compensates for lower soil N availability. Application of high N significantly reduced nodule formation, aligning with previous white clover studies under similar timeframes (Liu et al., 2023) and observations in soybean (Gan et al., 2004; Xia et al., 2017). Increased nitrate concentration in the soil reduces root hair formation, decreasing rhizobium infection and nodule development (Abdel Wahab et al., 1996). However, root growth occurs independently of nodule formation and is promoted by N exposure (Xia et al., 2017). While the plant can access soil N and also fix N₂ from the atmosphere, the total available N that affects root volume cannot be easily determined. A previous study stated that plant N derived from N₂ fixation decreases with increased fertilisation (Gan et al., 2004), suggesting that in this experiment, high N-treated white clover acquired more N from fertiliser, while low N-treated white clover relied more on N₂ fixation, resulting in similar total available N. This is consistent with the similar above- and below-ground biomass of white clover.

Methodological Considerations

Micro-CT underestimated root volume by 47% compared with destructive WinRhizo analysis. This underestimation was likely due to the limited ability of micro-CT to detect fine roots at the voxel resolution used in this study, as suggested by Daly et al. (2018). This explanation is supported by the stronger difference in chicory, particularly under high N, where WinRhizo detected the greatest root volume and the greatest fine-root volume, but micro-CT captured a smaller proportion of total root volume. Similar constraints have been reported in micro-CT studies of complex root-soil systems, where fine roots, soil moisture and root-soil contrast can reduce segmentation accuracy (Flavel et al., 2012; Mairhofer et al., 2015; Zappala, Mairhofer, et al., 2013). The relatively large column size used in this study may also have limited scan resolution compared with studies using smaller samples (Daly et al., 2018; Kurogane et al., 2021). However, this column size was selected to provide sufficient rooting volume and reduce physical restriction of root development during the seven-week growth period. The study therefore involved a trade-off between achieving finer image resolution and examining root development within a larger, more representative rooting volume.

Despite this underestimation, micro-CT captured spatial information that destructive WinRhizo analysis could not provide. In particular, it allowed root distribution to be quantified by depth in intact sand columns and enabled visualisation of three-dimensional rooting patterns. Therefore, the two methods should be considered complementary rather than interchangeable: WinRhizo provided more complete estimates of total washed-root volume, whereas micro-CT captured spatial information on root placement within the soil profile.

Conclusion

This study showed that perennial ryegrass, white clover and chicory differed substantially in root architecture, biomass allocation and response to N availability. These contrasting strategies provide a mechanistic basis for understanding how grasses, legumes and herbs may partition soil resources across depth in multispecies pastures. Combining shallow-rooted grasses, N₂-fixing legumes and deeper-rooted herbs may improve resource-use complementarity and reduce reliance on fertiliser N, although field studies are needed to determine whether these interactions are maintained under more complex environmental conditions. Methodologically, combining WinRHIZO with micro-CT enabled both total root quantification and depth-resolved assessment of root architecture, providing a more complete characterisation of species-specific rooting strategies than either method alone. Together, these findings advance our understanding of how contrasting rooting strategies influence plant responses to N availability and resource acquisition in pasture systems.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Haoran Fan and Jay R. Black. The first draft of the manuscript was written by Haoran Fan, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author.

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Figures

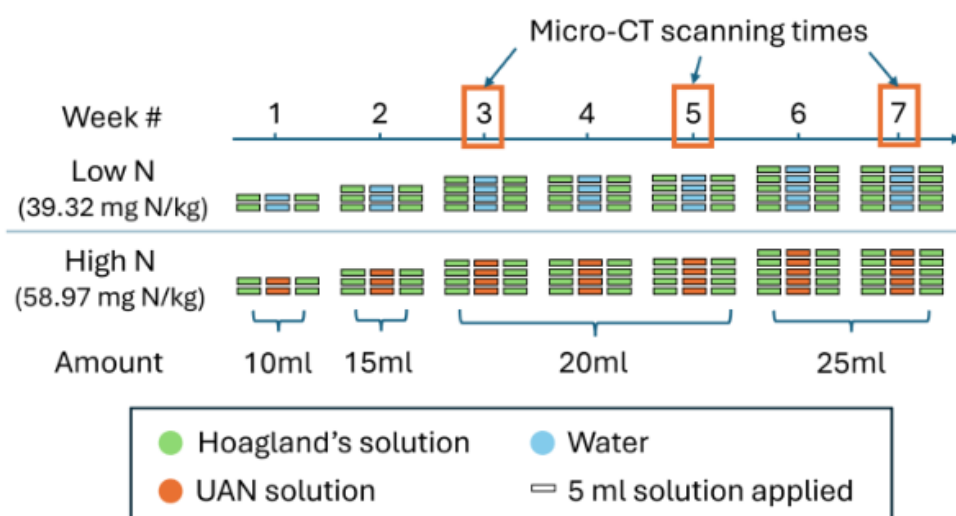


Figure 1

Nutrient maintenance regime over the 7-week sand column experiment. Plants received nutrient solution under low or high nitrogen (N) treatments, with application volume adjusted as plants developed. Within each week the plants received two applications of Hoagland's plus either water (low N) or UAN (high N), with applications of between 2 (week 1) and 5 (week 7) lots of 5 ml. UAN = urea ammonium nitrate.

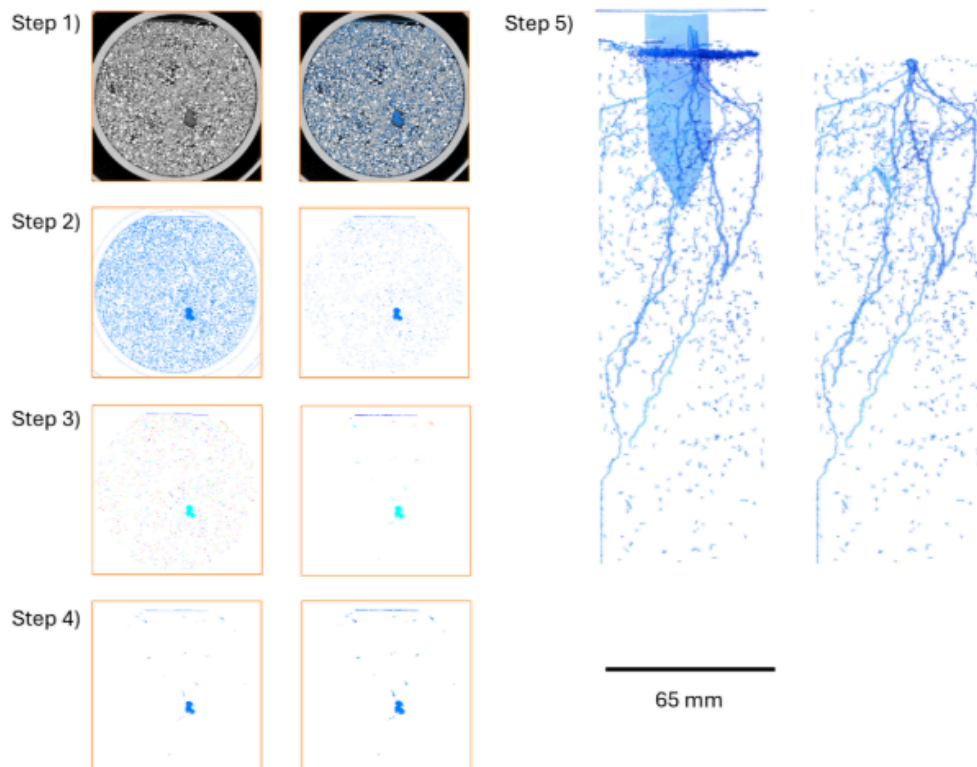


Figure 2

Steps in the workflow for root segmentation from micro-CT scans. 1) 2D horizontal slice just below air-sand interface (left) highlighting low density material segmentation in blue (right); 2) Low density segmentation (left) is eroded (right); 3) connected objects are labelled (left) and small volume objects filtered out (right); 4) remaining structure (left) is dilated (right) back to the same extent it was before erosion in step 2; 5) 3D render of remaining structure (left) is manually edited to remove artefacts (right).

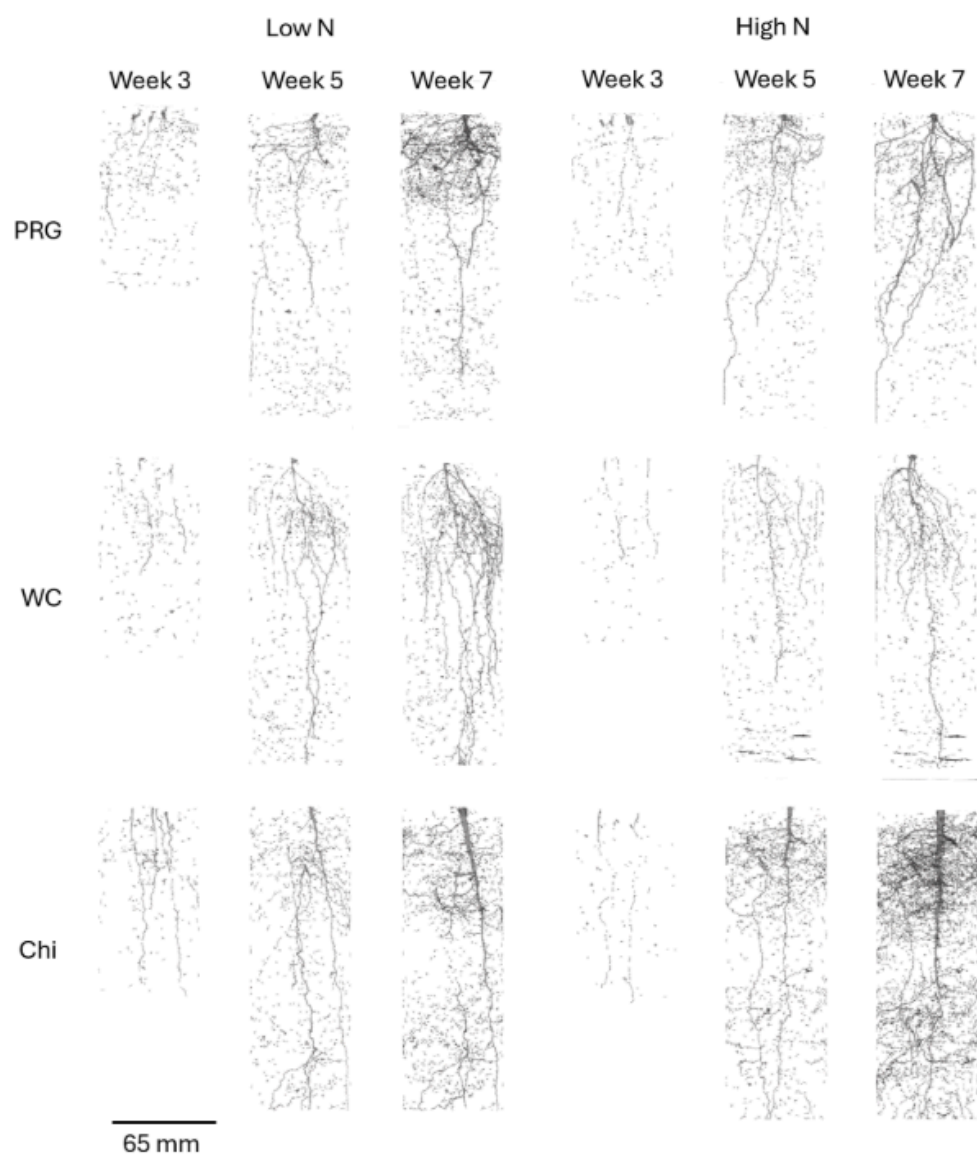


Figure 3

Reconstructed root segmentations from micro-CT scans of perennial ryegrass (PRG), white clover (WC) and chicory (Chi) grown under Low N and High N supply at weeks 3, 5 and 7. For each species $\times$ N-treatment combination, the images show the same replicate monitored across all three scanning time points. Although three replicates per combination were scanned at week 7, only the replicate previously scanned at weeks 3 and 5 is presented.

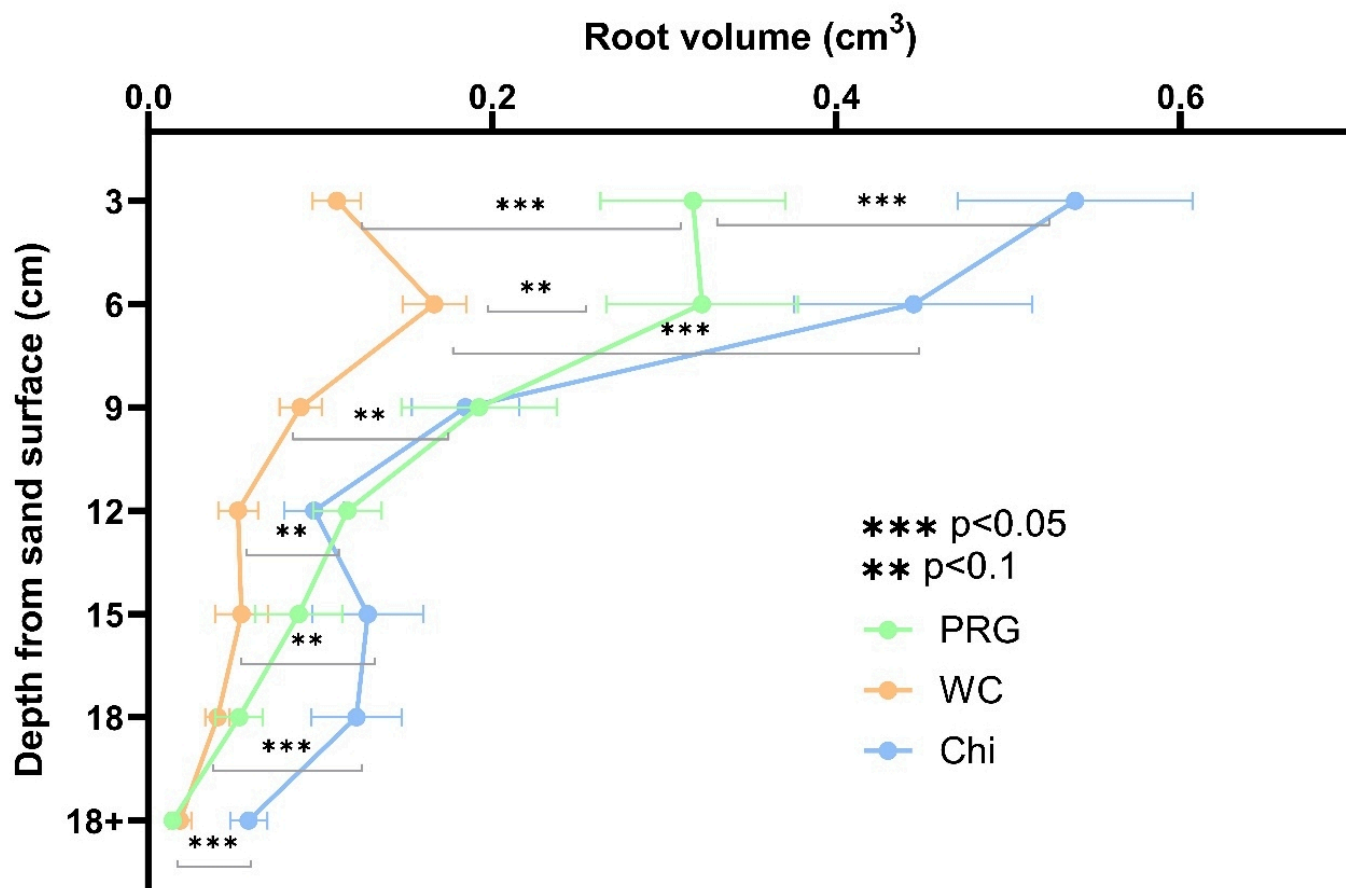


Figure 4

Root volume distribution by depth at week 7, calculated from micro-CT scans of intact sand columns. Values are means $\pm$ SEM. Pairwise differences among species within each depth interval are labelled in the figure (***: $p < 0.05$; **: $p < 0.10$). PRG = perennial ryegrass; WC = white clover; Chi = chicory.