

Belowground links between root properties of grassland species and N₂O concentration across the topsoil profile

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ABSTRACT

Plants can affect N₂O emissions by enhancing nitrogen (N) uptake and other below-ground interactions. However, the specific effect of the root systems of different plant species on the production and accumulation of N₂O within the soil profile remain largely unknown. The aim of this study was to investigate how plant species from different functional groups, their productivity and root traits affect N₂O emissions and N₂O concentrations within the soil profile in a fertilised grassland. We conducted a field experiment with two grasses (*Phleum pratense*, *Lolium perenne*), two legumes (*Trifolium repens*, *Trifolium pratense*), two forbs (*Cichorium intybus*, *Plantago lanceolata*), and the six-species mixture in a fertilised grassland. The effects of these plant communities on N-cycling processes were then assessed through the measurement of above- and below-ground plant traits, plant productivity, soil nutrient availability, N₂O emissions and its distribution in the soil profile. We found that *C. intybus* and *P. pratense* had the lowest N₂O emissions from the soil, which was mainly related to higher root biomass. The six-species mixture also showed lower N₂O emissions compared to *L. perenne* monoculture which was explained by complementary effects between the different plant species. We did not find a relationship between N₂O emission and its concentration in the soil profile. Higher specific root length and root length density coincided with higher N₂O concentrations at 10–20 and 20–30 cm soil depths. Since these two traits have been previously linked to reductions in N₂O emissions emitted from the soil, our results show that the relationships between root traits and N₂O emissions may not be reflected down in the soil profile. Overall, this study underscores the often-neglected importance of root traits for N-cycling and emphasises the need to better understand how root traits modify N₂O consumption within the soil profile to design more sustainable grasslands.

1. Introduction

Nitrous oxide (N₂O) contributes about 6 % to the anthropogenic greenhouse effect and is the most potent ozone-depleting gas (Ravishankara et al., 2009; IPCC et al., 2019). It has approximately 300 times the global warming potential of carbon dioxide on a mass basis (IPCC, 2022). Agriculture is the main source of anthropogenic N₂O emission, accounting for 50–70 % of the total emission in the world (Tian et al., 2020). To improve agricultural sustainability, we must urgently reduce N₂O emissions, and this requires an improved understanding of the drivers and dynamics of its production in the soil, and of the role played by plant-soil interactions as regulators of such emissions.

Manipulating plant community composition is a promising strategy to promote better nitrogen utilisation while reducing N₂O emissions from managed grasslands (Abalos et al., 2021). Plant functional groups (e.g. grasses, legumes, forbs) can be used to select species with different effects on soil properties and to promote productivity (Cummins et al., 2021; Fernandez Pulido et al., 2023). This is because contrasting plant functional groups have different root structures, growth patterns and niche occupation in the rhizosphere, which might affect water and nutrient uptake as well as soil pore distribution and therefore soil gas diffusivity (Holtham et al., 2007; Lucas et al., 2023), which in turn control N₂O production and consumption in the soil (Balaine et al., 2016). Grasses exploit soil nitrogen resources, while legumes are

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particularly important for nitrogen cycling due to their capacity to fix N₂ from the atmosphere, obtaining nitrogen to meet plant requirements and possibly reducing the need for inorganic nitrogen fertilisers application (Fuchs et al., 2020). However, legumes can increase the abundance of N-cycling microbes and N₂O emissions compared to grasses when the application of nitrogen fertiliser is not reduced accounting for the fixed nitrogen (Abalos et al., 2021; Barneze et al., 2023). Forb species are generally deep-rooting and may be able to acquire soil mineral nitrogen from deeper soil layers (Cong et al., 2017), but the importance of reducing N₂O production deep in the soil for N₂O emissions is highly uncertain. Some forb species, in particular *Plantago lanceolata*, may reduce N₂O emissions from grazed grasslands due to the presence of biological nitrification inhibitors (BNI) produced by the plants and released in the rhizosphere (Luo et al., 2018; de Klein et al., 2020).

Combining plants from different functional groups provided an opportunity to maximise complementarity in soil mineral nitrogen uptake, which could reduce N₂O emissions (Abalos et al., 2014). Plant mixtures could also enhance N₂O emissions by increasing root carbon inputs (Chen et al., 2020) stimulating oxygen consumption and denitrification (Philippot et al., 2009), although this will depend on the balance between N₂O production and reduction in a given soil environment (Senbayram et al., 2012; Saggar et al., 2013). It is important to improve our understanding of the specific mechanisms and conditions under which plant species of different functional groups and their combination affect N₂O emissions from the soils.

Trait-based approaches are increasingly used to evaluate the effect of plant species on plant nitrogen uptake and retention (Fowler et al., 2013) and to study soil processes producing N₂O (Abalos et al., 2014, 2018). Most of the studies have focused on shoot traits, despite burgeoning evidence pointing at the key role of root traits for nitrogen cycling and N₂O emissions (Freschet et al., 2021). For instance, plant species with high root length density (and high specific root length, relatively low biomass investment) can take up more nutrients compared to species with a precision strategy which can place their roots in nutrient hotspots (Hodge, 2004; Mommer et al., 2011). This can have consequences for N₂O production, as lowering bulk soil mineral nitrogen can limit microbial uptake (de Vries and Bardgett, 2016), nitrification, and potentially denitrification. Unfolding the relationships between root traits and N₂O emissions is thus instrumental for understanding the impact of plant foraging strategies on nutrient dynamics and associated consequences for the environmental sustainability of the agroecosystem.

Soils act both as a sink and a source of N₂O emissions (Chapuis-lardy et al., 2007; Jones et al., 2014). Numerous scientific studies published over the last decades have increased our understanding about N₂O emissions released from the soil to the air. However, the dynamics of gas production and consumption within the soil profile, which result in these topsoil fluxes, are mostly considered a black box. It is therefore crucial to evaluate and quantify the differences between surface and soil profile concentrations and their drivers. Plant species of different functional groups have contrasting root traits with a diverse spatial root configuration, determining the soil volume that can be explored by the roots. The root configuration can affect both N₂O production (e.g. supplying carbon through root exudates), consumption (e.g. decreasing mineral nitrogen availability, which stimulates denitrifiers to use N₂O as electron acceptor (Chapuis-lardy et al., 2007; Wang et al., 2018), and transport of gases throughout the soil profile. Yet, the relationship between N₂O concentration, plant species, and root traits across the soil profile has never been studied before. This knowledge is needed to unravel the dynamics of N₂O within soil profiles, and to explore the fundamental links with topsoil fluxes.

The aim of this field study was to investigate how plant species from different functional groups influence N₂O emissions in fertilised grasslands, with a focus on understanding the links between root traits and N₂O concentrations in the soil profile. The following hypotheses were tested: H1. Legumes will have higher N₂O emissions from the soil than grasses and forbs due to the increase abundance of N-cycling microbes,

while combining plant species from different functional groups will have lower N₂O emissions from the soil due to complementarity in nitrogen uptake; H2. Plant species with higher specific root length will lower N₂O emissions due to larger N uptake; H3. More root biomass allocation at a certain soil depth will be negatively correlated with N₂O concentration at that depth by increasing plant nitrogen uptake.

2. Material and methods

2.1. Study site

The experimental site was located at Aarhus University, Foulum, Denmark (56° 30' N, 9° 35' E, 54 m a.s.l.). The site is under temperate oceanic (Cfb – Köppen classification) climatic conditions, with mean annual temperature of 8 °C and mean annual precipitation of 641 mm between 1985 and 2008. The soil is a loamy sandy soil, classified as Mollic Luvisol (Krogh and Greve, 1999) and Typic Hapludult (USDA Soil Taxonomy System) containing 7 % clay, 10 % silt, and 81 % sand. Initial analyses of the properties of the upper 20 cm of the soil profile were: total N content 0.17 %, total C content 2 %, C/N ratio of 12, available P of 36 mg kg⁻¹, K of 129 mg kg⁻¹, pH of 5.9, and bulk density of 1.1 g cm⁻³.

2.2. Experimental design

The experiment was conducted on a subset of plots belonging to a larger field experiment, focused on the effects of multispecies mixtures on several agronomic properties and ecosystem functions (LegacyNet Project). We used a section of 3 × 1 m (3 m²) within the larger plots (3 × 10 m). The field experiment was established in 2020 when seeds (DLF Seeds & Science, Denmark) from two grasses (*Lolium perenne* L. cv. Ovambo 1 and *Phleum pratense* L. cv. Summergraze), two legumes (*Trifolium pratense* L. cv. Callisto and *Trifolium repens* L. cv. Brianna) and two forbs (*Plantago lanceolata* L. cv. AgroTonic and *Cichorium intybus* L. cv. Choise) were sown in monocultures, two, three, four and six-species mixtures following a simplex design (total of 57 plots). For the current study, we selected the six monocultures and the six-species mixture with three replicates each (21 plots) (Fig. S1). The seeding density was based on the recommended rates: 30 kg ha⁻¹ (*L. perenne*), 10 kg ha⁻¹ (*P. pratense*), 6 kg ha⁻¹ (*T. repens*), 6 kg ha⁻¹ (*T. pratense*), 8 kg ha⁻¹ (*C. intybus*) and 8 kg ha⁻¹ (*P. lanceolata*). For the six-species mixture, 1/6 of the seeding rate for each of the individual species were mixed.

The plots were established in April 2020, and were harvested three times in 2020 (June, August, and October), followed by fertilisation with 750 kg ha⁻¹ PK (0N 4P 21K), and with 75 kg N ha⁻¹ using calcium ammonium nitrate (CAN; 27 % N) before the first cut (June 2020). In 2021, the plots were harvested three times (May, June, and July), fertilised with 750 kg ha⁻¹ (0N 4P 21K), and with 75 kg N ha⁻¹ in the form of CAN (27 % N) in April 2021. At the start of the experiment reported in this paper (September 2021), plots were harvested followed by N-fertilisation of 50 kg N ha⁻¹ with CAN diluted with 6 L of water (approx. 2 mm of water) and applied homogeneously with a cross bar watering can. The plots were further irrigated with 20 mm water per plot at the end of day 7 of the experiment to simulate a rainfall event.

2.3. Nitrous oxide emissions from the soil

The closed static chamber method was used to measure N₂O emissions. A 20 cm diameter, 14 cm high gas sampling base ring was inserted into the soil to a depth of 5 cm one month prior to the measurements (Abalos et al., 2021; Oram et al., 2021). For each flux measurement, an opaque polypropylene plastic chamber was attached to the base ring and the headspace samples were taken approx. 30 min after chamber closure with tubes connected to a photoacoustic infrared spectroscopy GaseraOne gas analyser (Gasera Ltd, Turku, Finland). Samples of the

ambient-air entering were also measured (one every 10 samples) and used to correct the gas fluxes (Barneze et al., 2023). The linearity of the gas concentration increase in the headspace was tested regularly (Chadwick et al., 2014). Gases were sampled before the start of the experiment, daily after N-fertilisation application, and then three times per week up to day 29, when emissions subsided (Fig. S2). Cumulative N_2O flux was calculated by linear interpolation of the average N_2O emissions between the measurements and integrating the results over the total time period.

2.4. Nitrous oxide concentrations in the soil profile

Soil N_2O concentrations in the profile were determined according to the method used by Petersen et al. (2011). In each plot, next to the N_2O flux measurement chambers, soil N_2O concentrations at 5, 10, 20 and 30 cm soil depth were determined on 7, 8, 11, 15, 22 and 29 days after fertiliser application (Fig. S2). Samples were taken with custom-made stainless-steel needles (dual 0.7 mm diameter sideport openings at 10 mm distance from the tip) (Mikrolab, Højbjerg, Denmark) which were inserted into the soil via a plastic template (5 × 10 × 1.5 cm) with 15 holes that were used as a guide to guarantee vertical insertion. Only four holes were used, and the extra holes ensured that an alternative placement could be chosen if stones were encountered, or needles got clogged and had to be reinstalled.

For each measurement, 5 mL plastic syringes were mounted on each needle. Then 1 mL gas samples were taken and discarded using 3-way valves, and 2.1 mL gas samples taken from each depth in 1 mL steps, alternating between the four syringes. The gas samples (2 mL, as 0.1 mL was discarded) were transferred into pre-evacuated 6 mL exetainer vials (Labco, High Wycombe, UK) re-filled with He. Samples were analysed using a dual-inlet Agilent 7890 interfaced with a CTC CombiPal autosampler (Agilent, Nærum, Denmark) having a 2 m backflushed pre-column with Hayesep P and a 2 m main column with Poropak Q. This was connected to an electron capture detector; the carrier gas was N_2 with a flow rate of 45 mL min⁻¹ and the make-up gas that was used was Ar-CH₄ (95 %/5 %) with a flow rate of 40 mL min⁻¹. Temperatures of injection port, column, and detector were 80, 80, and 325 °C, respectively. The chromatograms were processed using EZ Chrom Elite software.

2.5. Plant productivity and nitrogen uptake

At the end of the experiment, plots were harvested (October 2021) (Fig. S2). Above-ground biomass from all plots was cut 3 cm above-ground level, mixture plots were sorted per plant species, dried at 60 °C for 72 h and weighed. Root biomass was collected by taking one soil core (Ø = 8 cm) at two different depths per plot: 0–10 and 10–30 cm. The soil cores were stored in plastic bags at 4 °C and washed over a 0.5 mm sieve. A representative subsample of roots was stored in 70 % ethanol at 4 °C for root morphology measurements. The rest of the clean roots were dried (60 °C for 72 h) and weighed.

The expected mixture productivity (PE) was calculated based on Loreau and Hector (2001) as shown in Eq. (1):

$$PE = \sum (RPE_i \times M_i) \quad \text{Eq. (1)}$$

where PE is the expected productivity of the mixture, based on the productivity of the monocultures of the component species; RPE_i is the expected relative contribution of species i to productivity in the mixture (the expected contribution of each species was assumed to be proportional to the proportion of seed sown for each species in the species mixture, i.e. 1:6 in the six-species mixture); M_i is the productivity of species i in monoculture.

Leaf and root subsamples were ground, ball-milled into a fine powder, weighed into tin cups (approx. 4 mg cup⁻¹) and analysed for leaf and root C and N content using an Elementar Vario MAX Cube

(Elementar, Germany). Plant and root N uptake (g N m⁻²) was estimated as the product of percentage N content and dry matter production.

2.6. Plant traits

Plant and root traits were measured following the guidelines described in Pérez-Harguindeguy et al. (2013) and can be seen in Table S1. The youngest fully expanded leaves from seven plants per species per plot were sampled to measure leaf traits. Specific leaf area (SLA, cm² g⁻¹) was calculated as the plant leaf area divided by the leaf dry weight using a LI-3100C area meter (Li-Cor Inc., NE, USA). Leaf dry matter content (LDMC, mg g⁻¹) and root dry matter content (RDMC, mg g⁻¹) was estimated as the ratio of dry to saturated weight. The stored root samples were scanned (Epson Perfection V700/750 3.92), root diameter and length (mm) were estimated with WINRHIZO (Regent Instruments Inc., Quebec City, QC, Canada), and scanned roots were dried (60 °C for 48 h). Subsequently, specific root length (SRL, m g⁻¹) and root length density (RLD, cm cm⁻³) were calculated. All root traits were measured for 0–10 and 10–30 cm soil depths at the end of the experiment (Fig. S2).

2.7. Soil sampling and analyses

Three bulk soil cores (Ø = 2 cm) were taken from each plot to determine soil mineral N, soil moisture and calculate water-filled pore space at three soil depths: 0–5 cm, 5–10 cm, and 10–20 cm. The soil samples were taken before the fertiliser application and then every week after nitrogen application (Fig. S2). Soil samples were sieved moist (2 mm), mixed and stored at 2 °C. Soil gravimetric moisture content was determined in a subsample after drying at 105 °C for 24 h. Mineral N (NH_4^+ and NO_3^-) was assessed with 1 M KCl in a 1:4 (soil weight: extractant volume) ratio extraction by analysis with a spectrophotometer (Autoanalyzer AA500, SEAL Analytical, Germany) (Keeney and Nelson, 1992). Water-filled pore space was calculated by the ratio of volumetric soil water content to total soil porosity.

2.8. Statistical analyses

Linear mixed effects (LME) models (*nlme* package, Pinheiro et al. (2017)) were used to test the effect of plant functional group (grass vs forb vs legume), community composition (each species monoculture and the six-species mixture) and root traits (specific root length, root dry matter content, root length density, root biomass and root N uptake) on daily and cumulative N_2O emissions, soil mineral N (soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$), and plant and root N uptake. Plot was included as a random effect. Auto-correlation of daily N_2O emissions due to repeated measurements were accounted for with the *corAR1* function. Some root samples from the 10–30 cm depth (i.e., plots 1, 3, 6, 7, 8, 11, 13, 14, 16 and 19) did not have enough material to be analysed for root N content, thus, they were not included in the analyses of root N uptake from this specific depth.

A principal component analysis (PCA) was carried out (FactoMineR, Lê et al. (2008)) to group the plant species on the basis of their root traits (0–10 and 10–30 cm depth), cumulative N_2O emissions, N_2O concentration in the soil profile (0–5, 5–10, 10–20 and 20–30 cm depth), and soil moisture in the soil profile (0–5, 5–10, and 10–20 cm). Spearman rank correlation analysis was conducted to examine relationships between cumulative N_2O emissions, N_2O concentration in the profile (0–5, 5–10, 10–20 and 20–30 cm depth), root biomass and root traits at 0–10 and 10–30 cm depth. In addition, Spearman rank correlation analysis was conducted to examine relationships between daily N_2O emissions and N_2O concentration in the profile (0–5, 5–10, 10–20 and 20–30 cm depth) for every sampling day.

All data were checked for normality and equal variances using residual plots, and log-transformed where necessary before analysis (i.e. cumulative N_2O emissions, RDMC, RLD, root biomass). Weight functions

(*varIdent*) were used to account for unequal variances following [Zuur et al. \(2011\)](#), i.e. cumulative N_2O emissions, SRL, RDMC, RLD. Tukey post hoc testing was performed using pairwise comparisons at $P \leq 0.05$ with the *emmeans* package ([Lenth, 2020](#)). All statistical analyses were carried out in the R programming language 4.0.2 ([R Core Team, 2020](#)).

3. Results

3.1. N_2O emissions from the soil

Nitrous oxide emissions increased slightly on the first day following nitrogen fertiliser application for all plant treatments ([Fig. 1](#)). However, emission rates then declined rapidly to background values by day 5. There was a much larger increase in N_2O emission rates following addition of water to bring soil WFPS to 80 % on day 9. That day, although there were no statistically significant differences, N_2O emissions from *L. perenne* and *T. repens* were numerically the highest. *T. repens* continued to exhibit the highest numerical emissions until the end of the measurement period. Emissions reached background levels by day 18 and remained there until the end of the experiment 29 days after fertiliser application.

Functional groups had no effect on the cumulative N_2O emissions from the soil ($P > 0.05$, [Fig. 2](#)), but the N_2O emissions differed significantly among the individual species in monocultures ($P < 0.05$, [Fig. 2](#)). *P. pratense* and *C. intybus* reduced N_2O emissions from the soil compared to the other species. The six-species mixture also reduced emissions compared to the legumes and *L. perenne*. The forb *P. lanceolata* did not reduce N_2O emissions compared to *L. perenne* ([Fig. 2](#)). The observed cumulative N_2O emissions in the six-species mixture was approximately 10 % lower than the expected cumulative N_2O emissions based on the monocultures ([Fig. 2](#)).

3.2. Soil mineral N

Soil mineral nitrogen (both soil $\text{NH}_4^+\text{-N}$ and soil $\text{NO}_3^-\text{-N}$) varied strongly over the measurement days and between the different plant communities ([Figs. 3 and 4](#)). Soil $\text{NH}_4^+\text{-N}$ was consistently low during the experiment period (around 5 mg kg^{-1}), and both soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ peaked just after the simulated rainfall event, eight days after fertiliser application (T8) (27 and 100 mg kg^{-1} , respectively). In this day, the highest soil $\text{NO}_3^-\text{-N}$ concentration was measured for the legume *T. pratense*. Soil mineral nitrogen remained at a lower level for the other

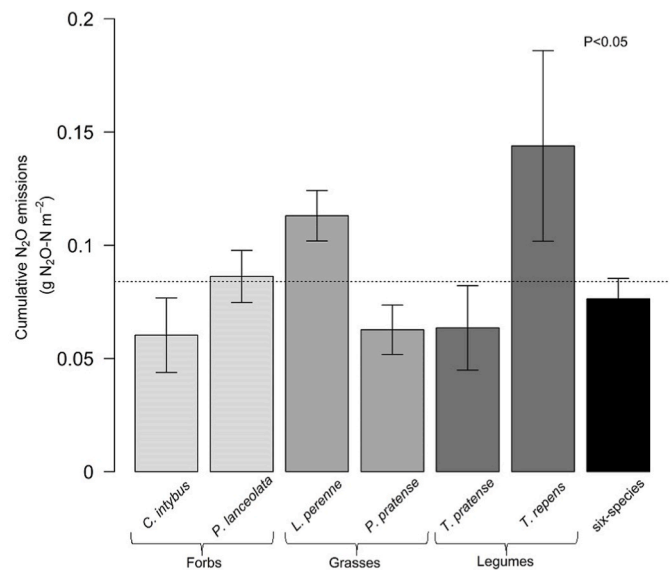


Fig. 2. Cumulative N_2O emissions ($\text{g N}_2\text{O-N m}^{-2}$) from the soil in relation to different grassland plant species. Vertical bars show ± 1 standard error ($n = 3$). The horizontal dashed line represents the expected cumulative N_2O emissions in the six-species mixture based on the monocultures.

legume species, *T. repens*. In general, soil $\text{NH}_4^+\text{-N}$ concentration was not higher for *P. lanceolata* compared to the other treatments.

3.3. Root traits

Root traits showed different patterns at 0–10 and 10–30 cm soil depth between the different plant communities due to the contrasting root systems of the different plant species. At 0–10 cm depth, the grasses (*L. perenne* and *P. pratense*) had the highest root length density, while the forb *C. intybus* had the lowest ($P < 0.05$, [Fig. 5, Table S2](#)). Despite having high root length density at 0–10 cm, *L. perenne* had the lowest root N uptake, whereas the six-species mixture and *T. repens* had the highest root N uptake in this soil layer, which might represent nitrogen from fixation or nitrogen assimilated from soil ($P < 0.05$, [Fig. 5, Table S2](#)). At 10–30 cm depth, *P. pratense* had the highest specific root length ($P < 0.05$, [Fig. 6, Table S2](#)), while root length density was very similar for all

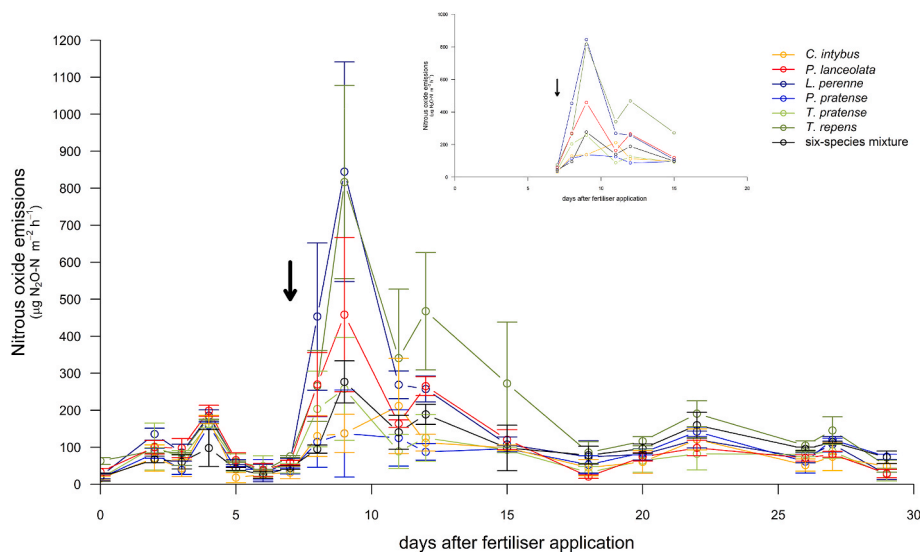


Fig. 1. Soil N_2O emission dynamics following fertiliser application to the field experiment in relation to different grassland plant species. The arrow indicates the rainfall event simulation. Vertical bars show ± 1 standard error ($n = 3$).

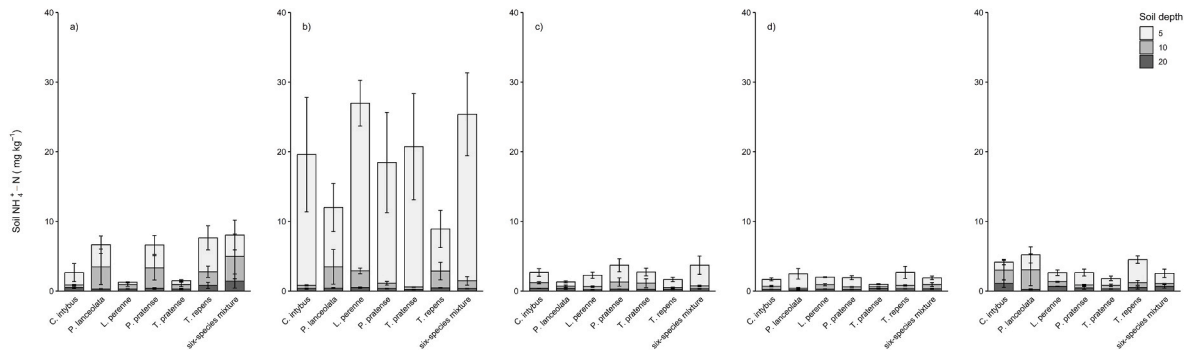


Fig. 3. Soil $\text{NH}_4^+\text{-N}$ concentration for different grassland plant species a) before fertiliser application, b) 8 days, c) 15 days, d) 22 days and e) 29 after fertiliser application in three different soil depths: 0–5, 5–10 and 10–20 cm.

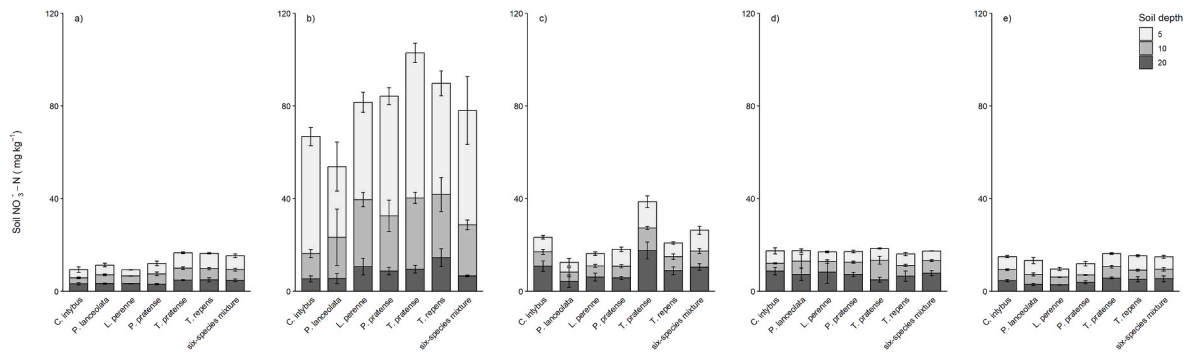


Fig. 4. Soil $\text{NO}_3\text{-N}$ concentration for different grassland plant species a) before fertiliser application, b) 8 days, c) 15 days, d) 22 days and e) 29 after fertiliser application in three different soil depths: 0–5, 5–10 and 10–20 cm.

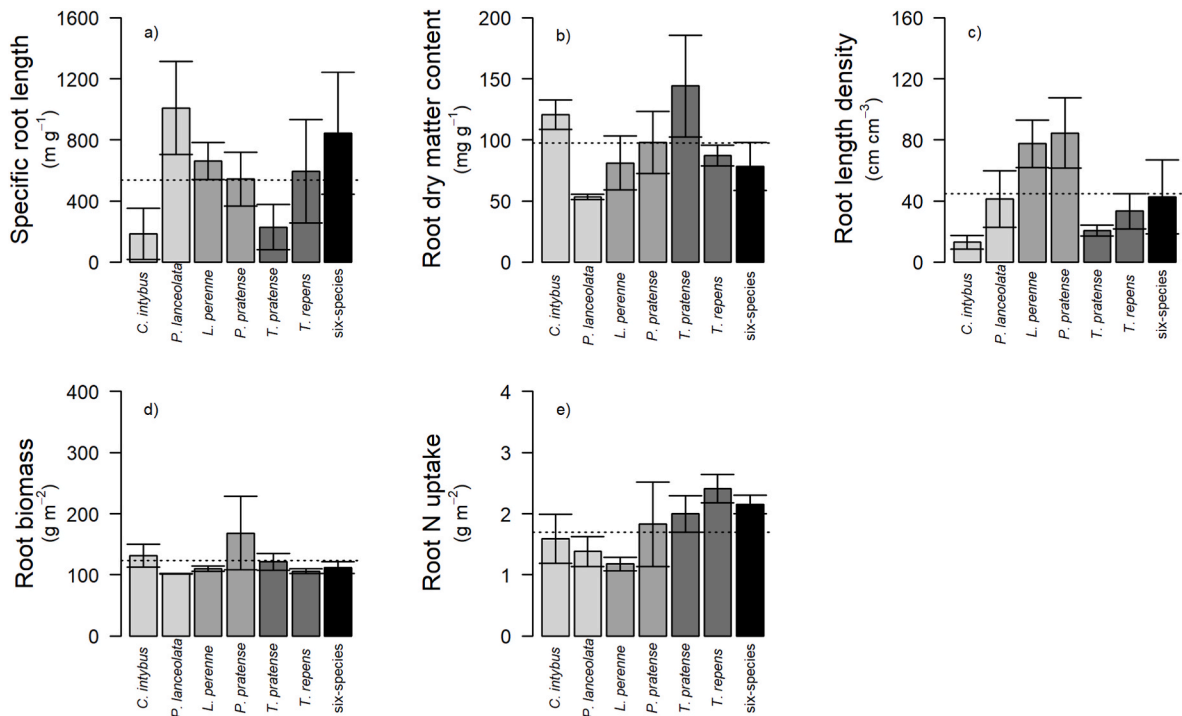


Fig. 5. Root traits at 0–10 cm soil depth from different grassland plant species, a) specific root length, m g^{-1} ; b) root dry matter content, mg g^{-1} ; c) root length density, cm cm^{-3} ; d) root biomass, g m^{-2} and e) root N uptake g N m^{-2} . Vertical bars show ± 1 standard error ($n = 3$). The horizontal dashed line represents the expected root traits in the six-species mixture based on the monocultures.

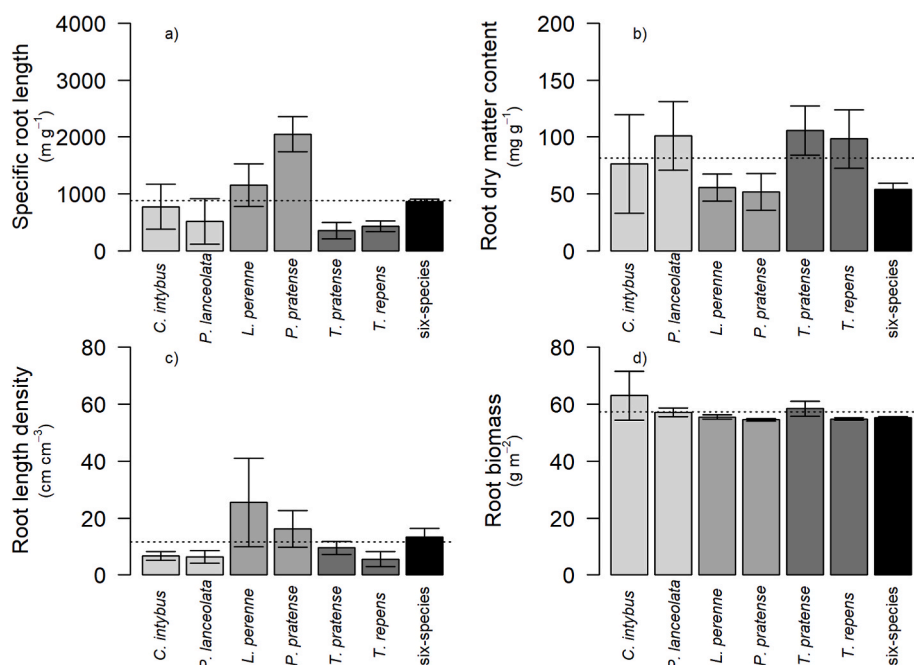


Fig. 6. Root traits at 10–30 cm soil depth from different grassland plant species, a) specific root length, m g^{-1} ; b) root dry matter content, mg g^{-1} ; c) root length density, cm cm^{-3} and d) root biomass, g m^{-3} . Vertical bars show ± 1 standard error ($n = 3$). The horizontal dashed line represents the expected root traits in the six-species mixture based on the monocultures.

plant communities ($P > 0.05$, Fig. 6). Across all plant communities, the monocultures of the two forbs *P. pratense* and *C. intybus* had numerically highest root biomass, although the differences were not statistically significant ($P > 0.05$, Fig. 6, Table S2).

3.4. N_2O concentrations in the soil profile

In general, N_2O concentrations in the soil profile were higher 11 and 25 days after fertiliser application, 4 and 18 days after the rainfall event,

respectively (Fig. 7). The lowest N_2O concentrations were almost always measured at the upper soil layer (5 cm depth). *L. perenne* showed a very high accumulation of N_2O at 10 cm depth while *P. pratense* had the highest N_2O concentrations at 20 and 30 cm (Fig. 7).

3.5. Testing the relationship between root traits and N_2O production and emission

From the PCA and Spearman rank correlation analyses, we found

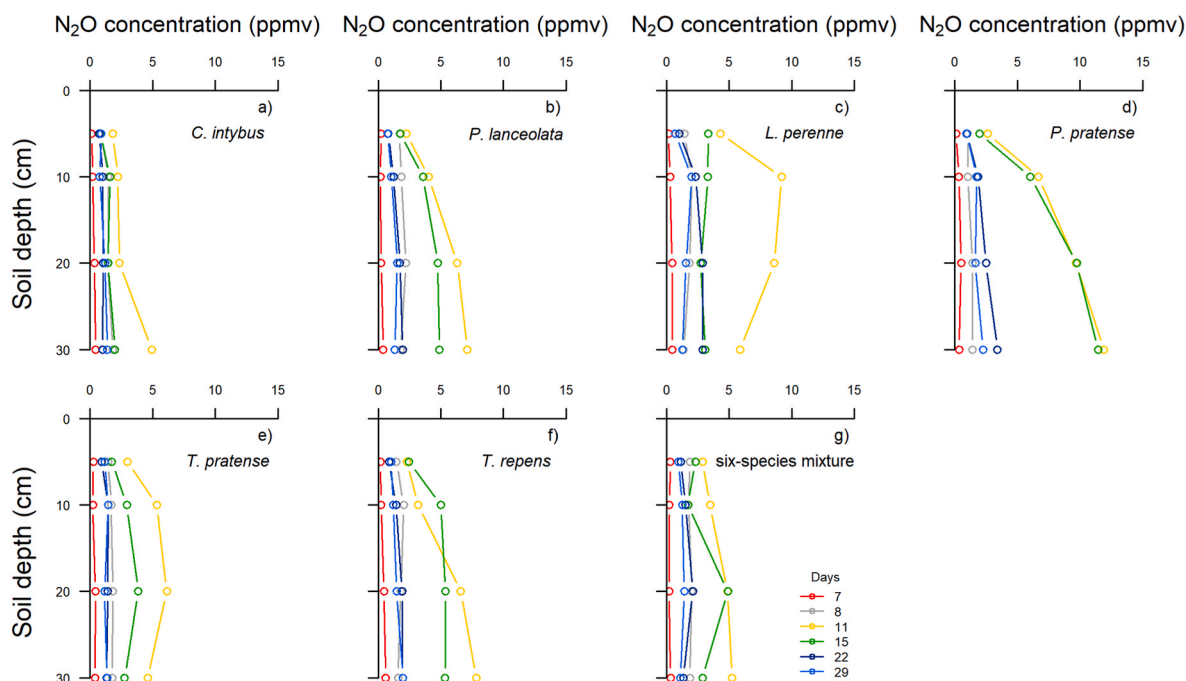


Fig. 7. Distribution of N_2O concentration in the soil profile measured by soil gas probes in relation to different grassland plant communities. The measurements were taken at 5, 10, 20 and 30 cm soil depth on day 7, 8, 11, 15, 22, 29 after nitrogen fertiliser application.

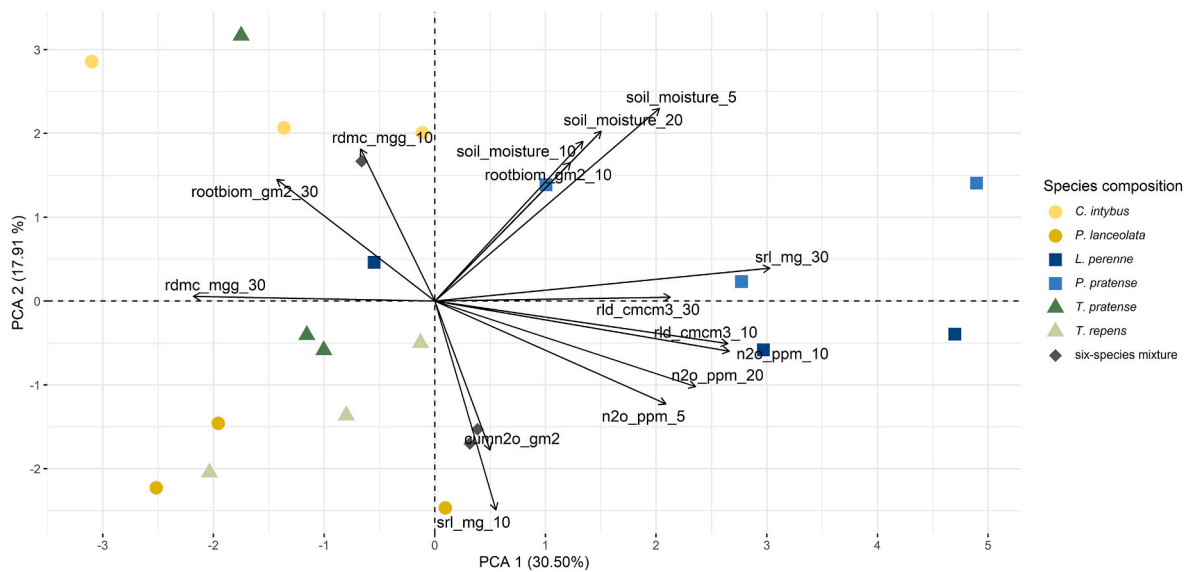


Fig. 8. PCA biplots of the cumulative N₂O emissions over the experimental period (cumn2o_gm2), N₂O concentration in the profile (n2o_ppm_5, n2o_ppm_10, n2o_ppm_20), root traits for 10 cm depth (rootbiom_gm2_10, rdmc_mgg_10, rld_cmcm3_10, srl_mg_10), root traits for 30 cm depth (rootbiom_gm2_30, rdmc_mgg_30, rld_cmcm3_30, srl_mg_30), soil moisture (soil_moisture_5, soil_moisture_10, soil_moisture_20). Rootbiom, root biomass; rdmc, root dry matter content; srl, specific root length; rld, root length density.

that cumulative N₂O emissions or daily emissions from the soil were not correlated with N₂O concentration in the soil profile at any of the soil depths ($P > 0.05$, Table S3, Fig. 8, Table S4). Regressions between N₂O emissions on day 9, the day with the highest N₂O emissions, against soil N₂O concentrations at 5, 10 and 20 cm depth (Fig. S4), further illustrate the weak relationship between N₂O emissions from the soil and N₂O concentrations in the soil. Root biomass allocation to deeper soil layers (10–30 cm) was negatively linked to N₂O concentrations within the soil profile (Fig. 8). Higher trait values for specific root length and root length density were associated to higher N₂O concentrations at 10–20 and 20–30 cm soil depths.

4. Discussion

In contrast to our first hypothesis, there were no differences in N₂O emissions between the plant functional groups, although previous research has shown that legumes may increase emissions compared to grasses and forbs (Abalos et al., 2021; Cummins et al., 2021). Due to its capacity to associate with Rhizobia to acquire nitrogen from the atmosphere, legumes have lower efficiency in acquiring nitrogen from the soil, and this may increase the activity of nitrogen cycling microbial communities from fertilised grasslands potentially enhancing soil N₂O emissions (Abalos et al., 2021; Barneze et al., 2023). One reason for a lack of effect of plant functional group on N₂O emission may be the large trait differences between species within functional groups: the stimulation of N₂O induced by legumes was observed for *T. repens*, but not for *T. pratense*. Another reason might be that under high soil nitrogen availability from fertiliser addition, legumes tend to fix nitrogen less efficiently since nitrogen uptake from the soil is more energy-efficient for the plant. These results are supported by recent studies indicating that plant functional groups are not good indicators to select plants for reducing N₂O emissions from soils due to large within-functional group trait variation (e.g. Fernandez Pulido et al., 2023).

Within the legume functional group, *T. repens* had the highest N₂O emissions, even though soil mineral nitrogen (soil NH₄⁺-N and NO₃⁻-N) was rather low for this species (Figs. 3 and 4), in particular soil NH₄⁺-N when N₂O peaked. As observed in earlier studies with ¹⁵N isotopes, *T. repens* has greater nitrogen deposition to topsoil compared to *T. pratense* (Pirhofer-Walzl et al., 2012; Rasmussen et al., 2012) from exudates as well as from residue decomposition (rhizodeposits), and

since both *Trifolium* species had similar plant nitrogen uptake in our study, this extra soil nitrogen released by *T. repens* may have been used by soil microorganisms thereby stimulating N₂O emissions. Additionally, *T. repens* colonises the topsoil and spreads above-ground mostly through a stolon network (Frame, 2005), and therefore it can release more nitrogen into the soil because it does not need to build up nitrogen reserves in roots for regrowth after defoliation (Pirhofer-Walzl et al., 2012). Furthermore, the finer roots of *T. repens* might have a faster turnover than the ones from *T. pratense*.

The observed cumulative N₂O emission in the six-species mixture was numerically lower than the expected values based on the monocultures. This finding is in line with earlier studies that found that a more diverse sward can promote complementarity effects by enhancing resource utilisation (Nyfeler et al., 2011; Mason et al., 2020) and consequently primary productivity (Finn et al., 2013; Suter et al., 2015; Mason et al., 2020). In our study, the trend for lower N₂O emissions compared to *L. perenne* monoculture could be linked to the higher root nitrogen uptake found in the six-species mixtures, indicating complementarity effects in terms of nutrient uptake (Fig. 3, Table S5). Mixtures which include deep-rooting species such as forbs have been related to stronger complementarity effects (Oram et al., 2017), since different rooting depths, structures and strategies can improve the overall acquisition of nitrogen at the plant community level.

The forb *P. lanceolata* was expected to reduce N₂O emissions based on the reported capacity of this species to release biological nitrification inhibitors (BNI) (Luo et al., 2008; Simon et al., 2019), but we did not find this effect. In our study, *P. lanceolata* did not increase soil NH₄⁺-N concentration compared to other species with a similar nitrogen uptake, suggesting that this species did not inhibit nitrification. A short experimental duration could have compromised the release of a sufficiently high concentration of the biological nitrification inhibitor compounds (Simon et al., 2019), but this is unlikely to be important in our study, since the plants had been growing for 1.5 years already by the time our measuring campaign started. Another possibility could be that the chosen cultivar may not produce enough biological nitrification inhibitors to be able to reduce N₂O emissions from soil under field conditions, but we used the same cultivar that has been used in earlier studies (Simon et al., 2019; Fernandez Pulido et al., 2023; Vi et al., 2023). The deep rooting of *P. lanceolata*'s may have caused a partial mismatch between the location of BNI exudation (deeper soil layers,

below 20 cm) and the presence of soil mineral nitrogen susceptible to be transformed to N_2O and escape from the soil (upper 10 cm). Other recent studies also did not find N_2O emissions reductions with *P. lanceolata*, which questions the potential of this species as an N_2O mitigation strategy under realistic field conditions (Cummins et al., 2021; Fernandez Pulido et al., 2023).

The grass *P. pratense* and the forb *C. intybus* had lower N_2O emissions compared to the other plant species. These two species both had high root biomass, and relatively low soil nitrate (Fig. 4), agreeing with studies that found that plant species that invest more in root biomass can increase mineral nitrogen uptake, thus reducing soil nitrate and consequently N_2O emissions from soils (Abalos et al., 2014; de Vries and Bardgett, 2016). Root biomass allocation to deeper soil layers (10–30 cm) was also negatively related to N_2O concentrations within the soil profile. Root biomass has been used as a proxy for rhizodeposition, and therefore it could be linked to higher N_2O emissions by providing carbon and stimulating denitrification, but we did not observe this effect. On the contrary, our results indicate that the carbon released from the roots could have stimulated microbial respiration triggering localised oxygen depletion, which can further promote N_2O consumption within the soil profile through complete denitrification. Higher carbon availability may have also stimulated ammonium immobilisation into microbial biomass, thereby lowering nitrate and N_2O emissions. Another study also found a negative correlation between root biomass and N_2O emissions either in control or flooded conditions (Oram et al., 2020), which seemed to be related to the quality and quantity of root turnover in the system. This result supports the relevance of root biomass for soil mineral N capture (Abalos et al., 2021).

The grass *P. pratense* had high specific root length, which might have increased the soil exploitation per unit of carbon invested in root length (Ho et al., 2005; Laliberté et al., 2015), reducing nutrient availability. Specific root length usually describes the economic aspects of the root system, thus it is linked to root-nutrient uptake efficiency (Eissenstat, 1992; Eissenstat et al., 2000). This species also exhibited a large root length density, indicating a high root area and therefore a higher capacity to absorb nitrogen (Bardgett et al., 2014).

We observed a higher concentration of N_2O in deeper soil layers compared to the upper layer (0–5 cm), agreeing with findings of Van Groenigen et al. (2005) and Button et al. (2023). This indicates that N_2O is mainly produced at some depth in the soil. The higher production of N_2O in the subsoil might be associated with denitrification, as very little NH_4^+ was found at 10–20 cm depth, and soil anaerobicity increases with depth while gas diffusion rates decrease. We did not find a relationship between N_2O emissions from the soil and concentrations in the soil profile. The reason might be related to the simultaneous diffusion-consumption of N_2O upward in the profile (Van Groenigen et al., 2005). Nitrous oxide diffuses from zones of high concentration in deeper soil layers to zones of low concentration in upper soil layers, and during this process N_2O consumption via complete denitrification may occur in the subsoil. This balance between N_2O production and consumption can be highly heterogeneous spatially and temporally within the soil, particularly under realistic field conditions such as those of our study. Soils are a complex mosaic characterised by a large variety of physical, chemical, and biological properties along the vertical and horizontal axes. Soil structure is characterised by a continuum of diverse aggregate sizes, differing in properties such as pore-size distribution, water retention and hydraulic conductivity, organic matter content, microbial community composition and oxygen demand (Wagner-Riddle et al., 2020; Jayarathne et al., 2021). The interactions between these factors at the microsite scale promote small-scale heterogeneity in microbially mediated processes such as nitrification and denitrification. This heterogeneity is difficult to capture with localised measurements of N_2O concentration at different depths.

In terms of temporal dynamics, all plant species showed the same consistent pattern, with N_2O concentration in the soil profile peaking three days after the rain event, corresponding with the highest N_2O

fluxes from the soil two days after the rain event. This showed a stimulation of N_2O production in the soil profile after the rain event. *L. perenne* and *T. repens* had the highest N_2O emissions, but very different soil N_2O concentration development. *L. perenne* showed a strong accumulation at 10 cm depth and steep gradient four days after the rain event, which suggested intense N_2O production around this depth. The N_2O emission from *T. repens* was even higher, but here the below-ground N_2O accumulation was much smaller. Studies indicate that as much as 70 % of the overall root mass of *T. repens* is concentrated within the upper 10–15 cm soil depth, which may have facilitated N_2O transport to the soil surface (Caradus, 1981; Nichols et al., 2007).

The diffusion-consumption of N_2O in the soil profile can be affected by plant species through their root traits and by interactions with soil microbial communities and nutrients. We found that higher specific root length and root length density were linked to higher N_2O concentrations at 10–20 and 20–30 cm soil depths. These two traits have been previously linked to reductions in N_2O emissions emitted from soils (Abalos et al., 2018; Fernandez Pulido et al., 2023), suggesting that the relationships between root traits and N_2O emissions ultimately emitted to the atmosphere can be highly contrasting down in the soil profile. It is possible that high values of specific root length and root length density may have increased the volume of soil explored by the roots, potentially enhancing gaseous diffusion throughout the soil profile (Button et al., 2023).

Future research should address limitations of our study. For example, plant species with contrasting root architectures may modify soil pore structure and the spatial distribution of organic matter and of rhizosphere microbial communities, ultimately determining the effect of plant species on N_2O concentrations across the soil profile and emitted from the soil (Lucas et al., 2023; Philippot et al., 2023). Further documentation of these variables may help explain the lack of relationship we observed between soil profile N_2O and net N_2O emissions. Furthermore, the nitrogen load used in our experiment was relatively low because it was conducted at the end of the growing season, resulting in low N_2O levels, possibly hindering noticeable differences among different plant species over the experimental period.

5. Conclusions

This is the first study evaluating the relationships between root traits of plants from different functional groups, soil N_2O concentration in the profile, and N_2O emissions. The grass *P. pratense* and the forb *C. intybus* had lower N_2O emissions, and this could be linked to a higher investment in root biomass. Root biomass in deeper soil layers was an important trait related to a decrease in N_2O concentrations within the soil profile. Higher specific root length and root length density were linked to higher N_2O concentrations at 10–20 and 20–30 cm soil depths, contrasting with earlier studies in which these traits reduced N_2O emissions from soils. This indicates that the relationships between root traits and N_2O ultimately emitted to the atmosphere can be highly contrasting down in the soil profile. We also found that N_2O concentration in the soil profile was not correlated with N_2O emissions from the soil. Accordingly, N_2O consumption within the soil profile seems to play a key role for N_2O emissions, and it appears to be modified by the plants growing in the soil. This implies that a better understating of the main root traits driving N_2O consumption and gaseous diffusion throughout the soil profile may provide an opportunity to select plant species and to design plant mixtures that reduce the emissions of N_2O . Overall, this study shows the importance of the often-overlooked role of root traits in nitrogen cycling, emphasising the need to incorporate such considerations into sustainable grassland management.

CRedit authorship contribution statement

Arlete S. Barneze: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal

analysis, Data curation, Conceptualization. **Søren O. Petersen**: Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Jørgen Eriksen**: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Gerlinde B. De Deyn**: Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Jan Willem van Groenigen**: Funding acquisition, Conceptualization. **Diego Abalos**: Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2024.109498>.

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