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Legume-based cover crop mixtures can overcome trade-offs between C inputs, soil mineral N depletion and residual yield effects

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1. Introduction

Management practices in agroecosystems that increase soil carbon (C) sequestration by removing atmospheric carbon dioxide (CO₂) can contribute to climate change mitigation (Lal, 2004). One such practice is to replace fallow between main crops with cover crops that are incorporated or killed off before the next main crop is sown (Poeplau and Don, 2015). Kaye and Quemada (2017) estimate that soil C sequestration is the most important climate change mitigation effect of cover crops, accounting for an estimated soil C sequestration equivalent of 117 g CO₂e m⁻² year⁻¹. In contrast, the estimated changes in N₂O emissions and additional farm operational fuel use account for net negative effects of up to -2 and -4 g CO₂e m⁻² year⁻¹ respectively (Kaye and Quemada, 2017). Yet, the relative importance of N₂O emissions has been proposed to progressively increase over time, offsetting the positive effect of soil C sequestration as the soil C reaches a new equilibrium (Lugato et al., 2018).

While biomass production is a key factor in soil C sequestration (Blanco-Canqui, Shaver, 2015), very few studies have quantified C input including not just aboveground cover crop biomass C, but also root C. Management practices in agroecosystems that incrementally increase soil C sequestration by removing atmospheric carbon dioxide (CO₂) can contribute to climate change mitigation (Lal, 2004). One such practice is to replace fallow between main crops with cover crops that are incorporated or killed off before the next main crop is sown (Poeplau and Don, 2015). Kaye and Quemada (2017) estimate that soil C sequestration is the most important climate change mitigation effect of cover crops, accounting for an estimated soil C sequestration equivalent of 117 g CO₂e m⁻² year⁻¹. In contrast, the estimated changes in N₂O emissions and additional farm operational fuel use account for net negative effects of up to -2 and -4 g CO₂e m⁻² year⁻¹ respectively (Kaye and Quemada, 2017). Yet, the relative importance of N₂O emissions has been proposed to progressively increase over time, offsetting the positive effect of soil C sequestration as the soil C reaches a new equilibrium (Lugato et al., 2018).

While biomass production is a key factor in soil C sequestration (Blanco-Canqui, Shaver, 2015), very few studies have quantified C input including not just aboveground cover crop biomass C, but also root C.
and rhizodeposition C (Austin et al., 2017; De Notaris et al., 2020; Mortensen et al., 2021). Rhizodeposition C can be operationally defined as the C lost by the living plant via roots, and includes both root exudates and fine dead root parts such as root hairs since it is inherently difficult to distinguish between root exudates and the degradation products of fine root parts (Rasmussen, 2011; Wichern et al., 2008). Rhizodeposits are continuously released from the living root and readily decomposed by soil microorganisms (Kuzyakov and Schneckenberger, 2004). The remaining rhizodeposition C after partial turnover (i.e., net rhizodeposition) is therefore mainly of microbial origin and contributes to the mineral-associated organic matter (MAOM), whereas plant shoots and roots contribute relatively more to the particulate organic matter (POM) (Cotrufo et al., 2019, 2013). In a recent study, net rhizodeposition inputs have been estimated to have a MAOM formation efficiency of 46% compared to 9% for roots and 7% for shoots (Villarino et al., 2021). While POM can be protected by inaccessibility for microbial breakdown in larger aggregates for years to decades, MAOM is physiochemically protected from breakdown by sorption to minerals and occlusion in finer aggregates, showing persistence in soil for decades to centuries (Lavellee et al., 2019; Lugato et al., 2021). Therefore, rhizodeposition C inputs are highly relevant from a climate change mitigation perspective, despite only about 7% of C in gross primary production is allocated as rhizodeposition for crops (Pausch and Kuzyakov, 2018).

Carbon allocation patterns to aboveground and belowground pools are highly dependent on species and abiotic factors, while the division between root and rhizodeposition C has been suggested to be more similar across species (Pausch et al., 2013; Pausch and Kuzyakov, 2018). That rhizodeposition C is closely correlated with root C has been known for decades (Shamoot et al., 1968), as it is linked to the specific root surface from where passive diffusion and active exudation of rhizodeposits occurs (Jones et al., 2004). Rhizodeposition remains the most uncertain component of the C cycle (Nguyen, 2003), which can be ascribed to the difficulties of reliable quantification and the low amount of root-derived organic substances against a high background of other organic substances present in the soil (Kuzyakov and Schneckenberger, 2004). Therefore, isotopic tracing techniques, e.g. labeling plants with $^{13}$C or $^{15}$N-enriched CO$_2$, have been developed to quantify rhizodeposition (Nguyen, 2003; Kuzyakov and Schneckenberger, 2004). In field trials, it is usually not possible to differentiate between tracer input to the soil from rhizodeposition and the less studied C deposition from the phyllosphere (leaf-derived inputs from the living plant via (senescent) litter drop and tracer washed out of the leaf with precipitation), hence recent studies have reported on a combined pool designated “phyllo- and rhizodeposition C” (De Notaris et al., 2020; Mortensen et al., 2021; Rasmussen et al., 2019).

When cover crops are established, the preceding main crop and fertilizer management may have left different levels of residual soil mineral N. Higher N availability for the cover crop will increase growth and N uptake, but may also change the relative allocation of C to roots and rhizodeposition (Farrar and Jones, 2000; Kuzyakov and Schneckenberger, 2004). While “a variety of possible mechanisms generate conflicting predictions for effects of N on rhizodeposition” (Bowsher et al., 2018), a recent study has found that the relative net phyllo- and rhizodeposition C decreases significantly under higher N availability (Mortensen et al., 2021).

Until recently, the main foci in cover crop research have been on the ability of cover crops to reduce N leaching and enhance the yield of the subsequent main crop (De Notaris et al., 2018; Thorup-Kristensen et al., 2003). It is widely accepted that appropriate cover crops in general should be effective in depicting residual soil mineral N (Constantin et al., 2010; Olesen et al., 2007; Thorup-Kristensen et al., 2003; Tonitto et al., 2006), and that cruciferous cover crops are more efficient than winter cereals and legumes at doing so (Thorup-Kristensen, 2001). Legumes, which are capable of biologically fixing N from the atmosphere, have been shown to be less effective at reducing N leaching (Valkama et al., 2015; White et al., 2017). While biomass production determines C input, which is desired in a climate change mitigation context, high biomass production corresponds to high soil N uptake by non-legumes and hence to reduced N leaching - but also the risk of pre-emptive competition. If pre-emptive competition is coupled with low litter quality (e.g. high C:N ratio), slow N release or N immobilization during cover crop decomposition can have negative effects on the subsequent crop yield (Thorup-Kristensen et al., 2003). The review by Valkama et al. (2015) shows a significant 3% reduction in grain yield caused by non-legumes, while other studies show neutral or non-significant yield reductions (Miguez and Bollero, 2005; Quemada et al., 2013; Tonitto et al., 2006). Differences in soil type, precipitation, time of sowing, winter hardness and timing of termination all have effects on pre-emptive competition and/or N mineralization/immobilization dynamics (Thorup-Kristensen et al., 2003; Thorup-Kristensen and Dresbøll, 2010; White et al., 2017), and result in a wide variation found between studies.

Legume-based cover crop mixtures have shown over-yielding, i.e. higher biomass production per unit area than the average of pure stands (Couédel et al., 2018; Elhakeem et al., 2021; Smith et al., 2014). Ascribed to the additional biologically fixed N input and generally lower C:N ratio compared with non-legumes, legume-based mixtures have shown enhanced yield of the subsequent main crop compared with a control (Doltra and Olesen, 2013; Miguez and Bollero, 2005; Valkama et al., 2015). In addition, a higher litter quality has recently shown greater accumulation of microbial-derived C in MAOM as an indicator of long-term soil organic carbon (SOC) persistence (Zhang et al., 2022). Cover crop mixtures of species with complementary traits, rather than high species richness, have been suggested to increase cover crop multi-functionality (Blesh, 2018; Finney and Kaye, 2017). Hence, mixtures have been found to lead to as high a soil mineral N depletion as brassicas (Couédel et al., 2018) and downregulated N fixation under high soil N availability (De Notaris et al., 2021).

However, for farmers, selection of cover crop species will mainly depend on agronomic goals and regulatory or economic incentives, why it is imperative to improve our understanding of the agronomic as well as environmental value of different cover crop functional types (Zhang et al., 2022) and whether mixing these types can improve the overall system performance.

The overall aim of this study was to assess aboveground and belowground C input from different cover crops in a conventional cereal crop rotation. The specific objectives were to investigate i) how total C input derived from cover crops and the relative C partitioning to shoot, root and phyllo- and rhizodeposition are affected by cover crops species in pure stands and mixtures under varying soil N availability, and ii) the ability of cover crop mixtures to overcome possible trade-offs between the multiple aims of generating soil C inputs, soil mineral N depletion, and positive residual N effects. Based on the literature review above, it was hypothesized that the C lost via phyllo- and rhizodeposition (qCIVPR) is positively correlated with root C (hypothesis 1), and that the percentage loss of C as phyllo- and rhizodeposition (%CIVPR) decreases with higher soil N availability (hypothesis 2).

2. Materials and methods

2.1. Field site and experimental design

The experiment was carried out from August 2020 to August 2021 on the University of Copenhagen’s research farm in Taastrup, Denmark (55°40′31.3″N 12°17′18.0″E). The soil is a coarse sandy soil with 5.1% clay, 2.6% silt, 39.9% fine sand, 50.9% coarse sand and 1.5% soil organic carbon (SOC) at 0.15 m depth. The climate is temperate oceanic (Cfb, Köppen classification) with an average annual temperature of 9.6°C and cumulative precipitation of 561 mm between August 2020 and July 2021 (Fig. 1). Prior to this experiment, the crop rotation was winter barley (2018), oat (2019) and spring barley (2020), fertilized with mineral NPK fertilizer according to general agronomic
recommendations and without the use of cover crops.

A randomized split plot design (main plot: N level, subplot: cover crops) was established on 10 August after the spring barley main crop (*Hordeum vulgare* L.) was harvested. Forty days prior to the harvest of the main crop, when the spring barley crop was close to physiological maturity (kernel soft dough, Zadoks stage 8.9), on 30 June, four out of the eight field blocks received an application of ammonium nitrate at the rate of 75 kg N ha⁻¹. This was done in order to simulate moderately higher soil residual available N (high $N_{res}$) (e.g. the case of crop failure or high soil N mineralization), while the low $N_{res}$ treatments did not receive any mineral N application. The application was made sufficiently early so that it would take part in the soil mineralization-immobilization cycle before cover crop sowing, but sufficiently late so that the spring barley would not have any significant N uptake. The field was plowed and the seedbed harrowed on 11 August and the cover crops were sown on 12 August in plots of 3 × 12 m. Each plot consisted of two subplots, one for plant $^{14}$C tracing and soil samplings and one kept undisturbed to measure the effect of the cover crops on the subsequent spring barley grain dry matter (DM) and N yield.

The trial involved the following six cover crop treatments: a control without a cover crop, pure stands of winter rye (*Secale cereale* L. var Jobaro), hairy vetch (*Vicia villosa* Roth var. Ostaat Dr. Baumanns C1) and oilseed radish (*Raphanus sativus* subsp. oleiferus var. Baracuda), a mixture of winter rye and hairy vetch, and a mixture of winter rye, hairy vetch and oilseed radish. Control plots were kept reasonably weed free by a single application of herbicide (glyphosate at rate 1.4 kg ha⁻¹) 28 days after sowing (DAS) of cover crops (see Table 1 for nomenclature and seeding rates). The five treatments and the control were established at both low and high residual N, in a four replicate block design, totaling 48 plots.

### 2.2. Isotopic labeling with $^{14}$C-$\text{CO}_2$

Total C inputs were traced for all cover crop treatments except for the two mixtures at high $N_{res}$ (in total 32 plots). Cover crops emerged within a week, and on 19 August 32 PVC cylinders (d=0.3 m, h=0.4 m) were inserted 30 cm into the soil in each plot. Oilseed radish plants were, where necessary, transplanted to the cylinder to represent the plant density in the rest of the plot. One low $N_{res}$ rye replicate was excluded from further analysis due to damage from herbicide spraying in a neighboring control plot.

To enable quantification of C input to the soil from phyllo- and rhizodeposition, multiple-pulse $^{14}$C-$\text{CO}_2$-labeling of the cover crops in the cylinders was initiated 19 days after sowing (19DAS), on 31 August. A transparent labeling tent was made from a plastic bag attached to the cylinders and supported by steel wire bows, creating a temporary closed atmosphere above the cylinders (Fig. 2). A sodium $^{14}$C-carbonate solution (in 1 M NaOH) in a small beaker was placed inside the tent, and $^{14}$CO₂ was released by injecting a surplus of HCl into the beaker. In accordance with increasing cover crop leaf area and hence photosynthesis, increasing $^{14}$C activity was applied per labeling event, ranging from 50 to 750 kBq per cylinder. Uptake of $^{14}$CO₂ in the labeling tent took place for one to three hours, with increasing time for higher cloud coverage and lower temperatures. Labeling was repeated twice a week until 2 November (82DAS), amounting to 19 labeling sessions in total. Each cylinder received a total of 9.1 MBq $^{14}$C activity (Table S1).

### Table 1 Overview of the experimental design including the nomenclature used in this study and the seeding rate in kg ha⁻¹ and, for mixtures, the percentage share of pure stand seeding rate.

<table>
<thead>
<tr>
<th>Species/mixture</th>
<th>Nomenclature</th>
<th>Seeding rate (kg ha⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cover crop (bare soil)</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>Winter rye</td>
<td>Rye</td>
<td>140</td>
</tr>
<tr>
<td>Hairy vetch</td>
<td>Vetch</td>
<td>80</td>
</tr>
<tr>
<td>Oilseed radish</td>
<td>Radish</td>
<td>12</td>
</tr>
<tr>
<td>Winter rye + hairy vetch</td>
<td>Ry-Ve</td>
<td>56 (40%) + 48 (60%)</td>
</tr>
<tr>
<td>Winter rye + hairy vetch + oilseed radish</td>
<td>Ry-Ve Ra</td>
<td>42 (30%) + 32 (40%) + 3.6 (30%)</td>
</tr>
</tbody>
</table>

![Fig. 1. Mean monthly temperature (°C) and total monthly precipitation (mm) from August 2020 to July 2021 from the Taastrup Climate and Water Balance Station.](image1)

![Fig. 2. Illustration of the transparent labeling tent (plastic bag) when attached to the cylinder. HCl is added to the sodium $^{14}$C-carbonate solution with a syringe through the plastic bag to release $^{14}$C-$\text{CO}_2$.](image2)
2.3. Plant and soil sampling

Plants and soil cylinders were destructively sampled on 9 November (89DAS, one week after the last labeling), at a time of the season when not much further growth was expected. The inserted cylinders, each containing approximately 30 kg of fresh soil, were removed by digging around the cylinder, inserting a level spade horizontally at the lower edge of the cylinder, lifting it carefully out and placing it on a board inside a plastic bag, and brought back to the laboratory. As few roots were visible at the bottom of the cylinders, soil samples (0.30–0.50 m) were taken for subsoil $^{14}$C determination. However, the majority of samples were below the limit of detection and were therefore excluded in the present study.

In the laboratory, the plant-soil column was gently pushed out of the cylinder. The subsequent procedure for isolation of specific belowground fractions was inspired by Mortensen et al. (2021) and undertaken as follows. The main roots were carefully taken out of the soil, and shoots and roots were separated by cutting at the point clearly identifying the change from stem to root. Identifiable senescent leaves on the soil surface were added to the shoot fraction. The soil was then first passed through a coarse sieve (grid size 1.8 cm) to collect the larger soil surface were added to the shoot fraction. The soil was then gently shaken on a 1 mm sieve to separate soil from roots without adhering to the roots, total ash was determined on 500 mg of the main root material (with adhering soil) and 3 mg of shoot material were ground in a ball mill. For C and N determination, 30 mg of soil, 15 mg of plant and root samples and 100 mg of shoot material (with adhering soil) was analyzed with a PYRO Cube elemental analyzer interfaced to an IsoPrime 100 mass spectrometer (Elementar, Germany). To correct for soil leaching, Nitrate N made up 72% and 40% on average at 36DAS and 60DAS, respectively. A grain subsample was dried in the oven at 60 °C and ground in a ball mill. For C and N determination, 50 mg of plant and root samples and 100 mg of soil samples were combusted in a sample oxidizer (Packard Sample Oxidizer model 307, Packard Instrument company, Meriden, CT, USA), where the CO$_2$ evolved was trapped in a 20 mL vial containing CarboSorb E® mixed with scintillation fluid (Permaflour E + ®) at a 1:1 ratio. The activity of $^{14}$C was then analyzed on a liquid scintillation counter (Tri-Carb® 2910 TR, PerkinElmer, Waltham, MA, USA), where the disintegrations per minute (DPM) were determined for 10 minutes per sample.

2.4. C input to the soil via phyllo- and rhizodeposition

The tracer mass balance approach described by Rasmussen et al. (2019) and De Notaris et al. (2020) was used to determine the net C lost via phyllo- and rhizodeposition (ClvPR), i.e. what net remained in the pool, and the mass of the pool (g DM). The 14C root fragments is subtracted from the 14C soil pool in order to avoid overestimation of phyllo- and rhizodeposition C as described by Mortensen et al. (2021). The 14C root fragments is part of the total 14C expressed in the denominator, thus it is only subtracted from the numerator. Based on the %ClvPR, the quantitative ClvPR (qClvPR) is calculated from the total C found in shoot and root biomass (kg C ha$^{-1}$): qClvPR = ($C_{\text{shoot}} + C_{\text{root}}$) / (100 - %ClvPR) x %ClvPR

N soil mineral N

Soil samples from 0 to 25 cm, 25–50 cm, 50–100 cm and 100–150 cm were taken in the main plots on 36DAS and 96DAS to quantify the effect of cover crops on soil mineral nitrogen content during early and late cover crop growth, respectively. Six core samples were taken from each plot with a tractor-mounted mechanical auger (0–150 cm), divided into the abovementioned depth segments, and mixed into one composite sample per depth for each plot. Soil samples were stored in a cooling box in the field and then kept at −18 °C until extraction of mineral N. Mineral N was extracted using a representative 10 g subsample of fresh soil (4 mm sieved) mixed with 40 mL 1 M potassium chloride (KCl), shaken for 30 min on an end-over-end shaker and filtered through a 2.5 µm filter (Whatman no. 5). The content of nitrate N and ammonium N in the extracts was measured using a flow injection analyzer (FIAnalyst 5000 Analyzer, FOSS, Denmark), and reported together as total mineral N (N$_{\text{min}}$) as a proxy for the potential leaching. Nitrate N made up 72% and 40% on average at 36DAS and 96DAS respectively. The percentage reduction (N$_{\text{red}}$) by CCs was calculated as follows:

N$_{\text{red}}$ (%) = (N$_{\text{min control}}$ − N$_{\text{min CC}}$) / N$_{\text{min control}}$ x 100

where N$_{\text{min control}}$ and N$_{\text{min CC}}$ is the measured soil mineral N in control and cover crop treatments, respectively.

2.6. Grain yield of the subsequent main crop

Cover crops were terminated naturally by the frost during winter (radish) or by plowing on 8 March 2021 (rye and vetch). On 30 March the field was fertilized with 108 kg N (20% below the recommended N rate for spring barley to allow for any residual N effect of the cover crops), 16 kg S, 19 kg P and 60 kg K ha$^{-1}$ (formulated as NS and PK fertilizers). On 31 March, the subsequent spring barley main crop (Hordeum vulgare L. var. Laureate) was sown and managed according to common spring cereal management. On 6 August, the spring barley was harvested with an experimental combiner in the undisturbed subplots of all treatments. A grain subsample was dried in the oven at 60 °C for 48 h and DM grain yield corrected for moisture content and impurities such as pieces of chaff, straw and weeds. Grain samples were pulverized in a grain-grinding mill, homogenized and analyzed for total N content with an elemental CNS analyzer (CNS Vario Macro cube, Elementar).
variables. A linear mixed model (LMM) was used to include both fixed and random effects (field block effects to account for the split-plot design). Interactions between species and residual N level were tested with a likelihood ratio test and included in the analysis independently of p-value. Pairwise comparisons of the means were conducted using the post-hoc Tukey HSD test, using the functions emmeans and compact letter display (CLD) from the multcomp package. The significance level was set to p = 0.05. The assumptions of normality and homogeneity of variance were confirmed by a quantile-quantile plot and a residual plot, respectively. To comply with these assumptions, root:shoot C ratio, root C, total plant C, qClvPR, total C input, total Nmin at 36DAS and 96DAS were log-transformed and plant C:N was reciprocally transformed.

3. Results

3.1. Cover crop biomass, quality and shoot and root C input

Cover crop shoot biomass ranged from 1.4 to 2.2 Mg DM ha⁻¹ (Table 2). At low Nres, vetch and the mixtures produced the highest DM, while rye, radish and vetch showed similarly high DM at high Nres. Shoot C ranged from 510 to 960 kg C ha⁻¹ (Fig. 3a; Table S2). At low Nres, vetch and the mixtures showed significantly higher shoot C than radish (p<0.011). At high Nres, there was no difference between the species. Both non-legumes showed significantly higher shoot C at high than at low Nres availability. Root C ranged from 360 to 1500 kg C ha⁻¹, with rye and the mixtures showing significantly higher root C than radish and vetch for both Nres levels (Fig. 3b; Table S2). Root C accounted for between 29% (vetch low Nres) and 63% (rye high Nres) of total plant C (shoot and root C). In contrast to shoot C, higher N availability did not increase root C significantly. There was no correlation between shoot C and root C when all the treatments were pooled (R² = 0.01, Fig. 5a) or when split into species treatments.

Shoot N ranged from 34 to 110 kg N ha⁻¹ (Table 2), with vetch clearly highest at both Nres levels. When also accounting for N in roots, the mixtures accumulated as much total N as vetch at low Nres, whereas rye had an N content between vetch and radish at high Nres. There was a significant effect of Nres level on both shoot and total plant N for all treatments pooled (p < 0.01). Vetch showed a significantly lower shoot:root C ratio than the other treatments for both Nres levels. The root:shoot C ratio of the pure stands responded differently to Nres level (interaction effect, p = 0.02): radish allocated significantly less C to roots (p = 0.03) at high Nres compared with low Nres, whereas vetch and rye showed no difference. The C:N ratio based on whole plant C and N ranged from 9.3 to 20.6, and was lowest for vetch and highest for rye among pure stands. The results for the mixtures fell in between, and in accordance with their relative proportion of vetch in the mixture.

3.2. Net C input to soil via phyllo- and rhizodeposition

The relative net C loss via phyllo- and rhizodeposition (%ClvPR) ranged from 7% to 14% (Fig. 3c). There was a trend of declining %ClvPR with increased N fertilization across all species (p = 0.078), with a slightly stronger tendency for non-legumes (p = 0.058). The net quantity of C lost via phyllo- and rhizodeposition (qClvPR) ranged from 110 to 330 kg C ha⁻¹ (Fig. 3d; Table S2). At low Nres, rye and the mixtures had significantly higher qClvPR than vetch, with radish showing intermediate qClvPR among the pure stands. The same ranking appeared for high Nres, but showed no significant differences. The lower proportion of net phyllo- and rhizodeposition Cat high Nres (Fig. 3c) was compensated for by higher plant C assimilation for non-legumes at high Nres, resulting in similar qClvPR across Nres levels (Fig. 3d).

Total C derived from cover crops (sum of shoot C, root C and qClvPR) ranged from 1250 to 2580 kg C ha⁻¹, with an average of 1880 kg C ha⁻¹. Total C was significantly higher for the two mixtures than for radish and vetch at low Nres (p<0.0001) and significantly higher for rye when compared with radish and vetch at high Nres (p<0.001) (Fig. 4, Table S2). Higher Nres resulted in significantly higher total C for non-legumes.

Root C showed the best correlation with qClvPR across Nres level (R² = 0.51, p-value = 6.2×10⁻⁶) (Fig. 5b). The qClvPR-to-root C ratio averaged 0.24 ± 0.02, with no significant effect of species nor Nres level (Table S2). Shoot + root C and shoot C demonstrated slightly lower and no predictive power of qClvPR compared with root C respectively (Fig. 5d,c).

3.3. Soil mineral nitrogen and leaching prevention potential

The data presents total soil mineral N, i.e. the sum of nitrate and ammonium N in order to include the full leaching prevention potential of the treatments. By mid-September (36DAS), the majority of soil mineral N was concentrated in the upper 0.5 m of the soil profile (Fig. 6a,b). At this time, the Ry + V + Ra mixture at low Nres and radish and rye in pure stands at high Nres had already significantly decreased soil mineral N throughout the whole soil profile (0–150 cm) compared with the control (Table 3). By mid-November (96DAS), the soil mineral N had moved down the soil profile in the control plots, while it had substantially decreased in plots with cover crops (Fig. 6c,d). Specifically, at 96DAS, soil mineral N (0–150 cm) was significantly reduced by radish and the mixtures at low Nres and by all cover crop treatments at high Nres compared with the control (Table 3). There was a significant effect of cover crop treatment for both days (p < 0.001), but no significant difference was seen between low Nres and high Nres.

3.4. Effects of cover crops on subsequent spring barley grain dry matter and N yield

The grain yield of subsequent spring barley averaged 4.3 ± 0.4 and 4.1 ± 0.2 Mg ha⁻¹, and the total N content of grain averaged 87 ± 5 and 89 ± 3 kg N ha⁻¹ at low and high Nres, respectively. There were no significant differences between cover crop treatments or N levels for either

<table>
<thead>
<tr>
<th>Species</th>
<th>Shoot biomass (Mg DM ha⁻¹)</th>
<th>Shoot N (kg N ha⁻¹)</th>
<th>Total plant C (kg C ha⁻¹)</th>
<th>Total plant N (kg N ha⁻¹)</th>
<th>Root:shoot C ratio</th>
<th>Plant C:N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Nres</td>
<td>Vetch</td>
<td>2.0 (0.2)ab</td>
<td>105 (9)abc</td>
<td>1240 (70)abc</td>
<td>134 (8)b</td>
<td>0.42 (0.07)</td>
</tr>
<tr>
<td>Rye</td>
<td>1.4 (0.2)ab</td>
<td>34 (5)abc</td>
<td>1110 (140)abc</td>
<td>64 (11)c</td>
<td>1.20 (0.12)c</td>
<td>17.8 (0.6)c</td>
</tr>
<tr>
<td>V + R</td>
<td>1.5 (0.2)ab</td>
<td>38 (7)d</td>
<td>1680 (360)abc</td>
<td>89 (25)bc</td>
<td>1.61 (0.22)b</td>
<td>19.5 (1.3)c</td>
</tr>
<tr>
<td>High Nres</td>
<td>Vetch</td>
<td>2.2 (0.1)ab</td>
<td>85 (6)abc</td>
<td>2110 (140)c</td>
<td>145 (7)c</td>
<td>1.22 (0.19)b</td>
</tr>
<tr>
<td>Rye</td>
<td>2.0 (0.2)ab</td>
<td>63 (4)bc</td>
<td>2120 (200)bc</td>
<td>130 (5)b</td>
<td>1.50 (0.31)bc</td>
<td>16.2 (0.8)bc</td>
</tr>
</tbody>
</table>

Table 2

Shoot biomass, shoot N, total plant C and N (shoot and root), shoot:root C ratio (calculated based on root and shoot C), and plant (shoot and root) C:N ratio. Means and standard error (n = 4, except for rye at low Nres, where n = 3) in parentheses. Lower and upper-case letters denote significant differences within the Nres level respectively. One and two stars denote significant differences between Nres level within species at p < 0.05 and p < 0.01 respectively.
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grain dry matter or N yield in spring barley, but there was a tendency for a lower yield after the rye cover crop at the high N<sub>res</sub> level (Fig. 7).

4. Discussion

4.1. Cover crop shoot and root C input

The cover crops in this trial were sown in the first half of August, following a relatively early main crop harvest. Early sowing is known to be one of the main determining factors for biomass production and the N leaching reduction potential of cover crops (Cottney et al., 2022). The autumn provided sufficient temperatures and precipitation for well-established cover crops, resulting in biomass production and quality parameters (Table 2) within the range of other Danish studies looking at similar species and mixtures with sampling of cover crops in November (De Notaris et al., 2020; Hansen et al., 2021; Mortensen et al., 2021; Thorup-Kristensen, 2001). This trial can therefore be considered valid and representative for Danish cereal-based cropping systems on sandy soils.

The significantly higher shoot C for rye and radish under high N<sub>res</sub> suggests that non-legumes were limited by N availability at low N<sub>res</sub>, which is often the case (Thorup-Kristensen et al., 2003). The individual cover crop species showed different C allocation patterns (root:shoot C ratio, Table 2), which was also reflected by the lack of correlation between shoot C and root C across all treatments. Rye in pure stands and in mixtures showed significantly higher root C than radish and vetch at both N<sub>res</sub> levels. In fact, rye showed root C amounting to 62–63% of total plant C. For rye in pure stands and mixtures, the root:shoot C ratio was

Fig. 3. C derived from cover crops (kg C ha<sup>-1</sup>) via (a) shoots and (b) roots (0–30 cm), (c) relative and (d) quantitative C lost via net phyllo- and rhizodeposition (% ClvPR and qClvPR respectively). Means ± standard error (n = 4, except for rye low N<sub>res</sub>, where n = 3). Lower- and uppercase letters denote significant differences between cover crop treatments within the low and high N<sub>res</sub> levels respectively. One and two stars denote significant differences between N<sub>res</sub> levels within species at <i>p</i> < 0.05 and <i>p</i> < 0.01 respectively.

Fig. 4. Total C derived from cover crops (kg C ha<sup>-1</sup>) via shoots, roots and net phyllo- and rhizodeposition (qClvPR) in soil. Means ± standard error (n = 4, except for rye low N<sub>res</sub>, where n = 3). Hatched bars denote high N<sub>res</sub>. Lower- and uppercase letters denote significant differences between cover crop treatments within the low and high N<sub>res</sub> levels respectively. One star denotes significant differences between N<sub>res</sub> levels within species at <i>p</i> < 0.05.
substantially higher than that seen in Thorup-Kristensen (2001), De Notaris et al. (2020a) and Mortensen et al. (2021), where the ratios were all well below 1. The root sampling procedure might have led to a contribution of non-cover crop-derived C from older undecomposed straw tangled up in the root system, which was more pronounced for the more fibrous rye roots. However, from a visual inspection during sampling and post-inspection of images (Fig. S1), this contribution was expected to constitute a minor share. However, such a potential source of error could be corrected for by using the specific activity of a clean (free of non-cover crop-derived organic material) root sample. In the present study, root C may also have been underestimated by not including C from root fragments. Liang et al. (2022) show that the amount of C in root fragments is comparable to C in the recovered roots, using the same sieve sizes as the current study. To include root fragments, more robust sampling is needed (e.g. using more than 100 g for decantation or replicated decantation) which should also be free of old POM. This is also highly important when making comparisons across species, as plant root morphology and resistance to breakage differ markedly and could give rise to sampling biases. In the current study, the root fragments subtracted in Eq. 1 differed between the species, with vetch showing a significantly higher share of \(^{14}\)C in root fragments relative to its main roots compared with the thick taproot of radish (\(p < 0.03\)) (data not shown).

No correlation was identified between shoot C and root C with all treatments pooled, or when split into species treatments. Mortensen et al. (2021) report significant species-dependent correlations between shoot and root C but, in line with earlier studies (Chirinda et al., 2012; Taghizadeh-Toosi et al., 2016), suggest that shoot C might not be a good predictor of root C across species.

4.2. \(C\) input to soil via phyllo- and rhizodeposition

Relatively few studies have quantified \(C\) inputs from cover crops that also include belowground inputs from roots and rhizodeposition (Austin et al., 2017; De Notaris et al., 2020; Mortensen et al., 2021). The net \(C\) lost via phyllo- and rhizodeposition in the present study was in the range of 110–330 kg C ha\(^{-1}\), which is similar to the study of De Notaris et al. (2020) (70–170 kg C ha\(^{-1}\)), but higher than that of Mortensen et al. (2021) (20–80 kg C ha\(^{-1}\)). The latter study experienced overall low cover crop growth due to the late harvest of the main crop and relatively cool and wet growing conditions (ibid.).

The qClvPR showed a significant positive relationship with root C and no correlation with shoot C, supporting that phyllodeposition through shredded leaves constituted a minor input. Hypothesis 1 that \(C\) lost via phyllo- and rhizodeposition (qClvPR) is positively correlated with root C can therefore be accepted. Given the linear relationship between qClvPR and root C, it is meaningful to report the qClvPR-to-root C ratio (Table S2). This ratio did not differ significantly between species or soil N availability level, and had a mean value overall of 0.24. Pausch and Kuzyakov (2018) suggest that the establishment of qClvPR-to-root C ratios from various agroecosystems with different biogeophysical characteristics and management enables estimation of qClvPR from root data already available. However, other studies disapprove of the applicability of constant ratios as environmental factors and plant phenological stage strongly induce root responses, resulting in altered root growth, morphology, and turnover (Poorter et al., 2012). As rhizodeposition by definition also consists of finer roots, root hairs and decomposed roots (Wichern et al., 2008), factors affecting roots also alter the net qClvPR-to-root C ratio (Hüpe et al., 2019). Yet, in the present study, we accounted for (and subtracted) the contribution from finer root fragments (Eq. 1) by decantation over a 250 µm sieve to limit quantification bias of qClvPR.
The current study found the relative allocation of C to phyllo- and rhizodeposition (%ClvPR) to range between 7% and 14%, which is within, although in the upper end, of the range found for crops (Pausch and Kuzyakov, 2018) and in accordance with other recent studies on cover crops. For instance, De Notaris et al. (2020) found 7% for vetch and 12% for a Ry+Ve mixture, while Mortensen et al. (2021) found %ClvPR ranging between 2% and 11% for mixtures with and without vetch. Austin et al. (2017) found an 18–19% C allocation to rhizodeposition from winter rye, but did not correct for root fragments in the bulk soil. If it had not been corrected for root fragments, the %ClvPR in the current study and in Mortensen et al. (2021) would have been in the same range as that in the study by Austin et al. (2017).

In the present study, the %ClvPR tended to be lower at higher soil N availability than at low soil N availability (Fig. 3c) which is in accordance to Mortensen et al. (2021), though showing more pronounced and significant differences than in the present study. The more pronounced differences in %ClvPR in Mortensen et al. (2021) could be due to the more contrasting growing conditions between the mineral N and manure-based crop rotation in their long-term trial, affecting cover crop growth during the whole growing period, whereas the summer-applied N in the current study was diminished during early cover crop growth (Fig. 6).

This tendency is consistent with the general resource optimization hypothesis that higher nutrient availability reduces the C cost of nutrient acquisition (Farrar and Jones, 2000). Hence, high N availability increases the relative C allocation to aboveground C pools (Kuzyakov and Schneekenberger, 2004; Phillips et al., 2011). In the review by Bowsher et al. (2018), increased rhizodeposition C was found for higher N availability, but there was decreased rhizodeposition C per unit of fixed C (comparable to %ClvPR). This could be ascribed to lower relative C allocation to roots (Phillips et al., 2011; Poorter et al., 2012) and mycorrhizae (Phillips et al., 2012) and the associated lower root surface area for C-diffusion losses (Jones et al., 2004). However, in the present study, only radish showed a significantly lower root shoot C ratio at high \( N_{\text{res}} \), and root C explained only 51% of the variation in the qClvPR (Fig. 5b). Therefore, N-limitation effects on root system architecture and functioning could play a role via increased root hair production and cortical cell death, with the larger surface area caused by higher specific

**Fig. 6.** Soil mineral N (mg N kg\(^{-1}\) soil) in the 0–25, 25–50, 50–100 and 100–150 cm layer at low \( N_{\text{res}} \) (a+c) and high \( N_{\text{res}} \) (b+d) measured at 36 days after sowing (DAS) (a+b) and 96 DAS (c+d). Values presented are means ± standard error (n = 4).
4.3. Leaching prevention potential

To simulate a situation where the previous crop leaves a higher amount of residual N in the soil (e.g. due to poor crop uptake) we applied mineral N during summer in the preceding cereal crop (which was pure stand and a legume-based mixture on soil mineral N, and higher mineral N depletion, which is ascribed to faster root growth and deeper rooting of crucifers than monocot cover crops (Laine et al., 1993; Thorup-Kristensen, 2001). Nitrogen uptake has indeed been shown to be effective at reducing soil mineral N across sampling dates and N mineralization incubation experiments in Denmark by Vogeler et al. (2017) showed increasing nitrate leaching with the increasing root length and/or lowered membrane integrity following sub-optimal nutrition (Bowsher et al., 2018; Jones et al., 2004). Lastly, the contribution of $^{15}$C to soil from phyllodeposition (Rasmussen et al., 2019) could be affected by N availability through effects on cover crop leaf production and senescence. However, from a visual inspection of the cover crops twice a week in the current study, decomposing litter on the soil surface appeared minimal.

While the second hypothesis that %Cleaf decreases significantly with higher soil N availability could not be verified, there was a tendency pointing to this. Further studies are needed to quantify C-allocation patterns and allow a deeper mechanistic understanding of the direction and magnitude of the processes involved in regulating rhizodeposition C dynamics.

### 4.3. Leaching prevention potential

To simulate a situation where the previous crop leaves a higher amount of residual N in the soil (e.g. due to poor crop uptake) we applied mineral N during summer in the preceding cereal crop (which was ripening, presumably with little N uptake at this time) while the cover crops were sown after harvest more than a month later. Notably, at the first sampling date (36DAS) there was no difference in mineral N between the low and high Nres level in the control plots (Table 3), which we consider to be caused by microbial immobilization of the applied N during decomposition of barley roots, stubble and straw over the two months since application. Overall, the results showed that all cover crop treatments were capable of reducing soil mineral N. While the assumed differentiated Nres levels did not appear as different Nmin in the control treatments, higher total plant N (Table 2) indicated an actually higher N available for the cover crops under high Nres. This gave rise to similar or lower Nmins under cover crops at high Nres compared to low Nres and correspondingly similar or higher percentage N reductions relative to the control at 96DAS.

In the early growing period (36DAS), soil mineral N reductions were seen in the upper 0.5 m, whereas differences in the late growing period (96DAS) were apparent at 0.25–1.5 m, where all cover crop treatments had reduced mineral N significantly, except for vetch and rye at low Nres (Table 3, Fig. 6). Radish proved to be the most efficient species for mineral N depletion, which is ascribed to faster root growth and deeper rooting of crucifers than monocot cover crops (Laine et al., 1993; Thorup-Kristensen, 2001). Nitrogen uptake has indeed been shown to be more strongly correlated with rooting depth than root density (Thorup-Kristensen, 2001). Among the pure stands, vetch was the least effective at reducing soil mineral N across sampling dates and Nres level in absolute numbers, but generally showed surprisingly similar results to rye. These results suggest that grouping all non-legumes together, as in the meta-analysis by Tonitto et al. (2006), in a discussion of N-leaching prevention could conceal potential differences between grass-like and cruciferous species.

The present results are similar to the reductions in N leaching by brassica (59%) and legumes (43%) found in a simulation model based on mineralization incubation experiments in Denmark by Vogeler et al. (2022). Furthermore, the percentage reductions were in the same range as Tonitto et al. (2006) who found reductions in nitrate leaching of 70% for non-legumes and 40% for legumes.

The mixtures reduced soil mineral N significantly at 96DAS at both low and high Nres, and did not differ significantly from any of the pure stands. Coudel et al. (2018) found no difference between radish in a pure stand and a legume-based mixture on soil mineral N, and higher depletion by the mixture compared with hairy vetch as sole crop. White et al. (2017) showed increasing nitrate leaching with the increasing

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**Table 3**

Total mineral N ($N_{min}$, kg ha$^{-1}$) and percentage reduction ($N_{red}$, %) at 0–1.5 m depth compared with the control measured 36 days after sowing (DAS) and 96DAS. Means and standard error (n = 4) in brackets. Lower and upper-case letters denote significant differences between cover crop species within the low and high Nres level respectively.

<table>
<thead>
<tr>
<th>Cover Crop Treatments</th>
<th>36DAS</th>
<th></th>
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<th>96DAS</th>
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<tr>
<td></td>
<td>Low Nres</td>
<td>High Nres</td>
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<td>Low Nres</td>
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<td></td>
<td>$N_{min}$ (kg ha$^{-1}$)</td>
<td>$N_{red}$ (%)</td>
<td>$N_{min}$ (kg ha$^{-1}$)</td>
<td>$N_{red}$ (%)</td>
<td>$N_{min}$ (kg ha$^{-1}$)</td>
<td>$N_{red}$ (%)</td>
</tr>
<tr>
<td>Control</td>
<td>90 (7)$^a$</td>
<td>-</td>
<td>94 (16)$^a$</td>
<td>-</td>
<td>84 (17)$^a$</td>
<td>-</td>
</tr>
<tr>
<td>Vetch</td>
<td>78 (11)$^{ab}$</td>
<td>13</td>
<td>67 (3)$^b$</td>
<td>29</td>
<td>59 (16)$^b$</td>
<td>29</td>
</tr>
<tr>
<td>Radish</td>
<td>57 (13)$^{ab}$</td>
<td>36</td>
<td>44 (4)$^b$</td>
<td>54</td>
<td>31 (3)$^b$</td>
<td>63</td>
</tr>
<tr>
<td>Rye</td>
<td>70 (18)$^{ab}$</td>
<td>22</td>
<td>46 (12)$^a$</td>
<td>51</td>
<td>56 (12)$^b$</td>
<td>34</td>
</tr>
<tr>
<td>Ry+Ve</td>
<td>61 (8)$^{ab}$</td>
<td>33</td>
<td>66 (7)$^b$</td>
<td>30</td>
<td>45 (4)$^b$</td>
<td>47</td>
</tr>
<tr>
<td>Ry+Ve+Ra</td>
<td>45 (7)$^b$</td>
<td>50</td>
<td>60 (13)$^b$</td>
<td>36</td>
<td>36 (5)$^b$</td>
<td>57</td>
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</tbody>
</table>

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**Fig. 7.** Grain yield DM (Mg ha$^{-1}$) (left) and total N in grains (kg ha$^{-1}$) (right) in spring barley. Means ± standard error (n = 4). No significant differences were found.
presence of legumes in a mixture, whereas soil mineral N in the present study was only slightly lower for the Ry+Ve+Ra mixture than for the Ry+Ve mixture.

4.4. Effects on subsequent main crop yield

Despite the substantial differences in total plant N and C:N ratio (Table 2), none of the cover crop treatments had any significant effect on the grain dry matter or N yield of the subsequent barley crop (Fig. 7). The 75 kg N ha\(^{-1}\) applied before cover crop sowing in high N\(_{\text{res}}\) treatments showed no effect compared with the low N\(_{\text{res}}\) either. Differences may have been blurred by the mineral fertilizer application of 108 kg N ha\(^{-1}\) to the barley, even if it was kept 20% below the normal recommended N fertilizer rate to allow for detection of residual effects. Supporting this, Suarez-Tapia et al. (2018) demonstrate that residual effects of historical N diminish with increasing fertilization. In addition, differences in subsoil texture caused a high field variation with the very stand counterparts, while Ry+Ve+Ra could not compete with the high efficiency of radish, resulting in 14% lower N depletion than the pure stand average (96DAS). While the mixtures had the highest observed C inputs, the mixtures were outperformed by the best-ranking pure stand, radish, in terms of soil N depletion. Blesh (2018) also found that mixtures simultaneously enhanced more ecosystem services than a cereal rye in a pure stand “although not at the highest observed level of each function”.

The two mixtures, Ry+Ve and Ry+Ve+Ra, did not differ significantly in any of the parameters investigated, however the inclusion of radish (and the corresponding reduction in the relative proportion of vetch) resulted in slightly lower total plant N, a higher root:shoot ratio, higher %ClvPR, and higher soil mineral N depletion for Ry+Ve+Ra than for Ry+Ve.

It is argued that cover crop mixtures can offer a realistic means for overcoming trade-offs among ecosystems functions. However, the present paper does not exhaust the scope of cover crop effects on the agroecosystem, and could potentially include changes in nitrous oxide emissions (N\(_2\)O) and solar radiation reflection (albedo) (Kaye and Quemada, 2017), soil structure and biodiversity (Scavio and Fontanazza, 2022), and phosphorus and sulfur mobilization (Hansen et al., 2021). More research is needed to integrate cover crop management and demonstrate yield effects in subsequent crop (as key to farmers’ decision-making) as well as the expression of functional traits by the individual species in pure stands and in mixtures, especially under varying conditions of soil type, nutrient availability and climate. In future research, it is suggested that cover crop species should be discussed in terms of representing certain functional traits, such as those generally expressed by e.g. grasses, brassicas and legumes (i.e. as in Zhang et al., 2022), rather than simply referring to legumes and non-legumes.

4.6. Implications for future studies quantifying belowground C

There are caveats concerning the estimation of C inputs in this study, which should be considered in future research. Firstly, C input was determined in early November before the risk of cover crops being killed off by frost, which implies that additional growth and N uptake in late autumn, over winter and early spring was not accounted for. Secondly, in order not to lose any cover crop-derived C, root fractions were kept unwashed, with considerable amounts of soil adhering to the roots. A subsequent ashing was carried out to adjust the C and N concentrations, underlining the need to include subsoil root fragments and rhizodeposition in future studies for comprehensive plant C-input estimation. Future studies should aim to investigate the contribution of different cover crop species to long-term soil organic carbon (Villarino et al., 2021) across input quality parameters such as C: N ratio and lignin content and soil parameters such as clay mineralogy, nutrient status and extent of C saturation (Angst et al., 2021; Castellano et al., 2015; Cotrufo et al., 2015).

5. Conclusions

The results of this study revealed that at the low residual soil N level, legume-based cover crop mixtures accumulated significantly higher total C input than vetch and radish in pure stands, with the results for rye in between. The quantity of C lost via phyllo- and rhizodeposition (qClvPR) followed the same pattern, demonstrating a significant positive correlation with root C (hypothesis 1 accepted). The percentage
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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2023.108408.

References


