

## 2 | Materials and methods – 13C labelling for root-C estimation

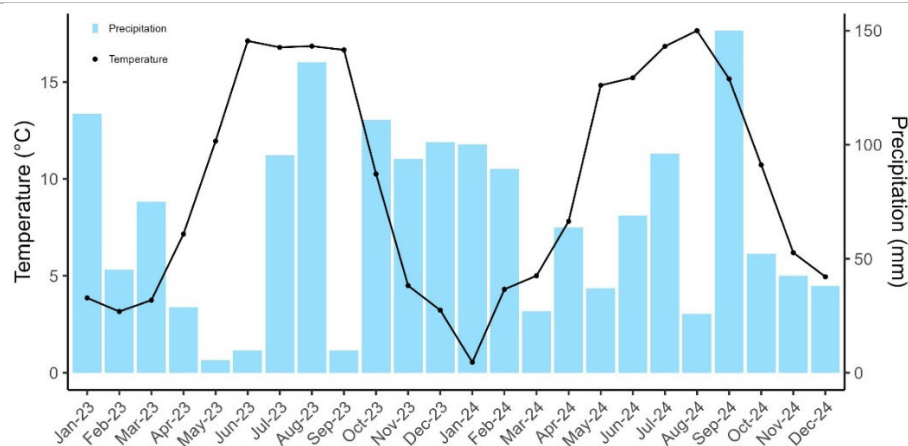
### 2.1 | Experimental site and design

A field experiment was carried out from spring 2023 to summer 2025 in University of Copenhagen's research farms in Taastrup, Denmark (55°40'33.1"N 12°18'05.8"E). The field had been conventionally cultivated with spring barley for the previous three years. The soil is characterized as sandy loam (Table\_) and the climate as temperate oceanic ('Cfb'; Köppen climate classification). Local meteorological data were obtained by a nearby weather station (Svane et al, 2025) and shown in Fig \_.

**Table.** Physicochemical properties of the field soil for the soil depths studied.

Depth (cm)	Clay (%)	Silt (%)	Sand (%)		BD (g cm <sup>-3</sup> )	TOC (%)	pH	P (mg 100g <sup>-1</sup> )	K (mg 100g <sup>-1</sup> )	Mg (mg 100g <sup>-1</sup> )
			Fine	Coarse						
0-25	14.1	12.9	53.1	17.9		1.16	6.3	2.0	9.9	4.8
25-50	17.3	10.0	44.5	26.7		0.85	6.4	1.2	6.6	4.4
50-75	15.3	15.1	42.7	26.0		0.56	6.6	1.1	5.6	4.6
75-100	28.7	16.4	29.9	24.5		0.26	6.9	0.6	7.1	7.5

Three perennial grass species, perennial ryegrass (*Lolium perenne* L. cvs. Bowie, Boyne, Garbor, Nashota, and Sputnik), tall fescue (*Festuca arundinacea* Schreb. cv. Kora), and festulolium (*Festulolium pabulare* cv. Mahulena), and two perennial forbs, chicory (*Cichorium intybus* L. cv. Choice) and plantain (*Plantago lanceolata* L. cv. Tonic), were sown in pure stand plots (11 × 1.5 m) in April 2023. The plots were arranged in a randomized complete block design with three blocks. Seeds were sown in rows at densities commonly used for biomass production, 25–30 kg ha<sup>-1</sup> for grasses and 10 kg ha<sup>-1</sup> for forbs, at a depth of 1.5–2 cm. Prior to sowing, spring barley (*Hordeum vulgare* L. cv. Prospect) was sown as a nurse crop at a rate of 100 kg ha<sup>-1</sup>, and was removed by cutting in mid-July.



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**Figure x.** Daily mean temperature and monthly precipitation sum in the field site.

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## 17 2.2 | Fertilization and biomass cuts

18 Every year, both grasses and forbs were fertilized with an NPK fertilizer (ProMangan 21-3-10;  
19  $\text{NH}_4\text{:NO}_3 = 1.29$ ; with S, Mg and Mn) in spring (before the onset of crop growth) at  $80 \text{ kg N ha}^{-1}$ , after  
20 the first biomass cut at  $60 \text{ kg N ha}^{-1}$  and after the second biomass cut at  $65 \text{ kg N ha}^{-1}$ .

21 Aboveground biomass was cut once in the establishment year on 26 September and three times  
22 in each productive year: year 1 on 12 June, 30 July, and 2 October; year 2 on 13 June, 7 August, and \_.

## 23 2.3 | $^{13}\text{CO}_2$ labelling

24 The aboveground biomass was labeled during the first and second productive years (2024 and  
25 2025) with a single pulse of  $^{13}\text{CO}_2$  at the end of May, typically coinciding with peak root growth (ref.).  
26 In 2024, interspecific differences in belowground  $^{13}\text{C}$  allocation were investigated by labeling one  
27 genotype of perennial ryegrass, tall fescue, chicory, and plantain. In 2025, intraspecific differences  
28 were examined by labeling five perennial ryegrass genotypes, as well as one genotype of tall fescue,  
29 festulolium, and plantain. Labeling was conducted on sunny days, starting at 9:00 AM, to ensure  
30 adequate  $\text{CO}_2$  assimilation.

31 On 22 May 2024, three areas were labeled in each plot (Fig \_). To this end, three transparent  
32 chambers ( $85 \times 85 \text{ cm}$ ) were placed in each plot on top of the vegetation, fitting into pre-made sand-  
33 filled trenches (spaced  $15 \text{ cm}$  apart) to create a temporarily closed atmosphere. A beaker containing  
34  $8 \text{ g}$  of  $^{13}\text{C}$ -labelled sodium bicarbonate (99 atom%) dissolved in  $1 \text{ M NaOH}$  ( $1 \text{ g NaH}^{13}\text{CO}_3$  per  $10 \text{ g NaOH}$   
35 solution) was placed inside each chamber. To release  $^{13}\text{C}$ -labelled  $\text{CO}_2$  within the chambers,  $30 \text{ mL}$  of  
36  $25\% \text{ HCl}$  was injected into each beaker via a syringe, and the injection hole was immediately sealed  
37 with tape. Each chamber was removed  $90 \text{ min}$  after  $\text{HCl}$  injection.

38 On 16–17 May 2025, labeling was conducted as described above, with the following modifications:  
39 only one chamber per plot was used, positioned to avoid previously labeled areas. Each chamber  
40 contained  $12 \text{ g}$  of  $^{13}\text{C}$ -labelled sodium bicarbonate, and to enhance assimilation efficiency the  
41 solution was divided equally between two beakers. Each beaker was injected with  $30 \text{ mL}$  of  $25\% \text{ HCl}$ ,  
42 with the second injection occurring  $30 \text{ min}$  after the first.

## 43 2.4 | Sampling

44 In both years, above and belowground samples were collected from the labeled areas of the plots  
45 in order to determine their C isotopic composition. The samples were taken from a central sampling  
46 area ( $45 \times 45 \text{ cm}$ ) within each labeled area to avoid collecting unlabeled material from neighboring

47 plants. A distant unlabeled area of the same size in the plot was sampled to serve as a baseline for  
48 isotopic calculations (Fig ). Finally, standing root biomass was estimated around the time of labelling.

#### 49 2.4.1 | Year 2024

50 In the first production year, the three labeled areas of each plot were destructively sampled once,  
51 each on a different day: 2, 5, and 8 days after labeling (DAL). Unlabeled control samples were collected  
52 at 12 DAL (Fig ).

53 In each sampling area, aboveground biomass was cut close to the ground. Belowground samples  
54 were collected using percussion gouge augers ( $\varnothing 6$  cm and  $\varnothing 8$  cm), that were mechanically driven into  
55 the soil using an excavator bucket. In each sampling area, augers were inserted at six locations to a  
56 depth of 50 cm: three along the crop row ( $2 \times \varnothing 6$  cm and  $1 \times \varnothing 8$  cm augers) and three between rows  
57 ( $2 \times \varnothing 8$  cm and  $1 \times \varnothing 6$  cm augers). The soil core in the auger was then separated into 25 cm  
58 increments. Samples from the same depth were combined into a single composite sample.

59 To estimate standing root biomass around the time of labelling and  $^{13}\text{C}$  allocation, belowground  
60 samples were collected the same way as described above, from unlabeled areas in the plots, at 7 DAL  
61 (Fig ). The samples were put directly in the freezer ( $-10$  °C) until later processing.

#### 62 2.4.2 | Year 2025

63 In the second production year, the labeled area of each plot was destructively sampled at 5 DAL,  
64 with the unlabeled areas sampled at 12 DAL (Fig ).

65 In each sampling area, aboveground biomass was cut close to the ground. Belowground samples  
66 were collected using the same equipment as in 2024. In each sampling area, augers were inserted at  
67 four locations, two along the crop row and two between rows, to a total depth of 100 cm. At each  
68 location, a soil core was extracted from 0–50 cm using a  $\varnothing 8$  cm auger, followed by a second core from  
69 50–100 cm using a  $\varnothing 6$  cm auger. Each soil core was subsequently separated into 25 cm increments  
70 and samples from the same depth were combined into a single composite sample.

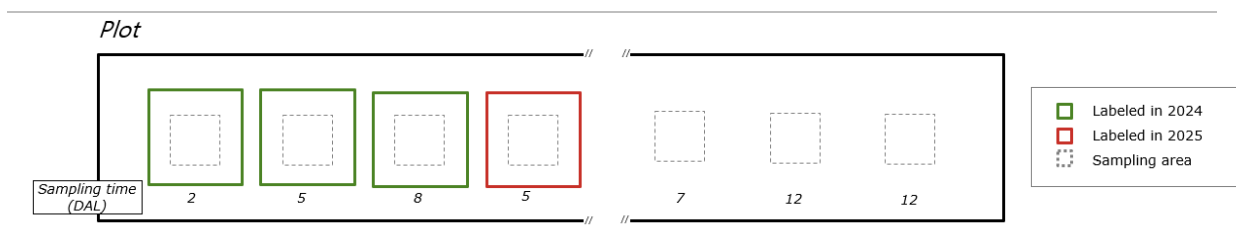


Figure . Schematic representation of an example field plot showing areas labeled in 2024 (solid green squares) and 2025 (solid red squares), and the sampling areas where samples were collected from (dashed squares). Sampling areas on the right side of the figure are located in unlabeled parts of the plot. Within each

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year, all labeled areas received a single  $^{13}\text{CO}_2$  pulse simultaneously. The sampling time of each area is indicated as days after labelling (DAL).

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## 71 2.5 | Sample processing

### 72 2.5.1 | 1<sup>st</sup> year

73 Aboveground biomass samples were oven-dried at 70 °C for 48 h immediately after sampling, and  
74 their dry weight was recorded. Samples were then coarsely ground (Retsch SM 2000), subsampled,  
75 pulverized (MCR KM-1500), and packed into tin capsules for  $^{13}\text{C}$  analysis.

76 Belowground samples were oven-dried for 48 h at 70 °C, and the dry weight recorded. Large visible  
77 stones were manually removed and weighed. Big soil clumps were broken down manually so that the  
78 samples could then be coarsely milled (Humboldt H-4199, internal sieve 2 mm). Stones retained on  
79 the mill's sieve were added to the stone fraction which was weighed and discarded. Any remaining  
80 material on the sieve (mainly roots) was returned to the milled sample. The sample was then  
81 subsampled and pulverized (MCR KM-1500).

82 To determine the  $\delta^{13}\text{C}$  of soil organic carbon (SOC), carbonates—which have a distinct isotopic  
83 signature from SOC (ref)—were removed from belowground samples by direct application of HCl.  
84 Approximately 20 mg of dry soil was added in silver capsules, and 30.5  $\mu\text{L}$  of HCl 4M was added to  
85 each. As no effervescence was observed, no additional HCl was applied. The samples were left to react  
86 for 10 min and then oven-dried at 60 °C to constant weight. To account for chloride formation, samples  
87 were weighed before and after the HCl treatment (Ramnarine et al, 2011), and following  $\delta^{13}\text{C}$  analysis,  
88  $^{13}\text{C}$  measurements were reported on a pre-treated soil mass basis.

### 89 2.5.2 | 2<sup>nd</sup> year

90 Aboveground samples were washed free of dirt immediately after collection with tap water.  
91 Samples were then oven-dried at 70 °C for 48h, and dry weight was recorded. They were subsequently  
92 coarsely milled (Retsch SM 2000), subsampled, pulverized (Retsch ZM 300), and packed into tin  
93 capsules for  $^{13}\text{C}$  analysis.

94 Belowground samples were thawed overnight at 3 °C and homogenized the following day by  
95 breaking up soil clumps, cutting long roots, removing large visible stones, and mixing thoroughly. Each  
96 homogenized sample was divided into two equal aliquots: one for  $^{13}\text{C}$  analysis of the total  
97 belowground pool and the other for  $^{13}\text{C}$  analysis of roots and root mass. For  $^{13}\text{C}$  analysis of the total  
98 belowground pool, the aliquot was oven-dried at 70 °C for 48 h and milled in two steps. First, coarse  
99 milling was done (Humboldt H-4199, internal sieve 2 mm). Stones retained on the sieve were weighed  
100 and discarded, and any remaining material (mainly roots) was returned to the milled sample. Next, a  
101 subsample was pulverized (Retch MM400). For standing root biomass estimation and  $^{13}\text{C}$  analysis of

102 roots, roots were isolated from the other aliquot by root washing: the aliquot was dispersed in water,  
103 swirled and the supernatant was poured over a 1mm sieve. This process was repeated until no  
104 additional roots were recovered on the sieve. For samples from the 0–25 cm soil depth, only one  
105 quarter of the aliquot was used for root washing due to high abundance of roots. Clean roots were  
106 oven-dried at 70 °C for 48 h, their dry mass recorded, pulverized (Retsch ZM300 or mortar and pestle  
107 for samples with low root mass), and packed into tin capsules for <sup>13</sup>C analysis.

108 Carbonates were removed from belowground samples as previously described (1<sup>st</sup> year).

## 109 2.6 | <sup>13</sup>C determination

110 Processed above- and belowground samples were analyzed for <sup>13</sup>C composition and total C, at the  
111 Laboratory for Stable Isotopes in Soil, Plant and Air, UCPH by isotope ratio mass spectrometry (Delta  
112 V PLUS; Thermo Fisher Scientific Inc., MA, USA). The enrichment of the samples is expressed in δ  
113 notations and calculated by eq. 1 as follows:

$$114 \quad \delta^{13}C (\text{‰}) = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000, (1)$$

115 where  $R_{\text{sample}}$  and  $R_{\text{standard}}$  are the <sup>13</sup>C/<sup>12</sup>C ratios of the plant or soil sample and the standard  
116 sample, respectively. The standard sample is Vienna Pee Dee Belemnite (VPDB) with  $R_{\text{standard}} \approx$   
117 0.0112372.

## 118 2.7 | Data analysis

119 The statistical analyses were carried out on RStudio ()