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Specific root respiration of three plant species as influenced by storage time and conditions

Zana A. Lak · Hans Sandén · Mathias Mayer · Boris Rewald

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Abstract

Aims Specific root respiration (RR_S) is a key root trait, determining i.e. nutrient foraging and uptake efficiencies. However, a considerable uncertainty exists regarding the effects of storage time and conditions on RR_S measurements.

Methods Fine root CO_2 efflux rates of three plant types (tree seedling *Carpinus betulus*, legume *Pisum sativum*, grass *Lolium perenne*) were measured as depending on storage time (30–1440 min post-rinsing) and conditions (i.e. attached to plant, warm and cold water storage, and storage under dry conditions).

Results Short-term storage conditions (30 min) had a significant effect on measured RR_S rates, in specific, RR_S rates of all three species were significantly lower under dry storage. Irrespective of plant species or temperature, storage of excised roots in water did not affect RR_S for 300 min., RR_S measurements remained stable for 1 day if roots were stored cold.

Conclusions Our results have important implications on measurement routines of RR_S —a generally understudied root trait. Henceforth it seems reasonable to collect roots in the field and transport them, hydrated but even uncooled, to the laboratory for subsequent measurements for at least 300 min post-rinsing.

Keywords Excised plant roots · Root respiration · Root traits · Sample storage · Specific root respiration

Introduction

Root respiration (RR) is a major component of the terrestrial carbon cycle, contributing 10–90% to total soil respiration (Bond-Lamberty et al. 2004; Hanson et al. 2000). In certain cases, RR can account for up to 75% of the assimilated carbon allocated to roots (Majdi et al. 2007), and extensive carbon costs of RR have been directly related to growth above ground (e.g. Rewald et al. 2016). Specific root respiration (RR_S) is defined as the amount of CO_2 released or O_2 absorbed per unit root during a given time; RR_S originates from three physiological processes: ion mobilization and uptake, growth and defence, and cell maintenance (Van der Werf et al. 1994). Although respiration of root-associated microbes such as mycorrhizal fungi can contribute to measured RR_S rates (Bulgarelli et al. 2012; Nielsen et al. 1998), the specific carbon or O_2 costs per unit root indicate carbon- or O_2 -use efficiencies, respectively. Consequently, RR_S is a key root trait determining i.e. nutrient

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foraging/uptake efficiencies (George et al. 2003; Lynch 2015).

Specific root respiration of various plant species, different root entities and ontogenetic stages have been measured (Freschet et al. 2020; Lambers et al. 2002). Specific root respiration varies largely among plants and are generally considered greater in fast-growing species (Poorter et al. 1990; Rewald et al. 2014). Within root systems, RR_S of young, distal roots are often greater than those of coarser, older roots (which are e.g. lignified, containing less nitrogen, and having a greater stele fraction). Specific root respiration is subjected to considerable environmental influences, including temperature, water availability, ion concentrations in the soil solution, or O_2 availability. For example, with an increase in temperature, RR_S increases and it is generally modelled using a temperature sensitivity coefficient (Q_{10}) near 2; however, large differences between species and root types regarding Q_{10} values and the rate and degree of temperature acclimation have been reported (Atkinson et al. 2007; Freschet et al. 2020). Specific root respiration is often decreasing as soil moisture deficits are increasing; similar, O_2 deficiency or high CO_2 concentrations may inhibit RR_S (Freschet et al. 2020). Regarding the composition of the soil solution, acquisition and assimilation of ammonium is often less carbon costly than that of nitrate (Bloom et al. 1992; Rewald et al. 2016) and concentrations of other nutrients and heavy metals alter RR_S rates depending on concentrations (e.g. Otgonsuren et al. 2016). Potentially related to the amount of carbon assimilates available, RR_S was found to vary or not over the course of a day (e.g. Trolldenier and von Rheinbaben 1981; Widén and Majdi 2001).

Direct measurement of CO_2/O_2 fluxes of roots are frequently performed in closed chamber systems on excised samples—limiting confounding effects by heterotrophic soil respiration, and allowing for measurements on clearly defined root entities (see Freschet et al. (2020) for a recent review on root respiration measurements in the context of standardized root trait measurements). Sampled roots are either rinsed (with tap water) or brushed free of substrate (Makita et al. 2012). Despite the growing popularity of RR_S measurements, possibly fostered by the broad availability of infra-red gas-analyser (IRGA) systems, it is currently uncertain how long and under which conditions (different) roots can be stored without affecting RR_S . This information is key to harmonize RR_S measurement protocols, and thus to

improve the comparability of measurements; this is of particular importance due to the increasing emphasis on trait-function relationships in plant ecology (Westoby and Wright 2006) and the ongoing assembly of large plant trait databases (e.g. FRED, the largest fine-root trait database to date; Iversen et al. 2017).

Thus, the aims of this study were to clarify how pre-measurement conditions, in specific the time span until measurements after root rinsing/excision, and the storage conditions (incl. media and temperature), influence the measurements of specific root respiration rates, and if this differs between three contrasting plant species.

Material and methods

Plant species and experiment set-up

The plants for the experiment were grown in a ventilated polytunnel greenhouse, under slightly increased temperature and near ambient lighting conditions, at BOKU Tulln, Austria (48°19'05.0"N, 16°03'58.2"E). End of March 2017, ~1.5-year-old, bare-rooted *Carpinus betulus* L. seedlings (European hornbeam; Murauer Forstpflanzen GmbH, Ort im Innkreis, Austria), and *Pisum sativum* L. cv. 'Retrija' (Pea; Institute of Agricultural Resources and Economics, Priekulji Research Centre, Latvia) and *Lolium perenne* L. cv. 'Jubilee' (Perennial ryegrass; terrasan GmbH, Rain am Lech, Germany) seeds were obtained. *Carpinus betulus* is a broadleaved, deciduous, small to medium-sized tree with a wide natural distribution range across Central-Southeast Europe and commonly cultivated as an ornamental tree across Europe. *Pisum sativum* is an annual, cool-season legume grown in many parts of the world as crop. *Lolium perenne* is a fast-growing, perennial grass species that requires a large amount of nitrogen (N); it is a most important pasture and forage grass, and frequently used in gardens, parks and sport grounds. The plants were planted / seeded during early April 2017 in 71 pots (ca. 35 cm in height) filled with washed quartz sand (particle size distribution 0.7–1.2 mm); the upper 10 cm of substrate per pot were amended with 10 g of slow-release fertilizer (Osmocote Pro 3–4 M, Everris, The Netherlands). Sand is allowing for non-destructive, relatively fast root system harvest. One tree individual, 3 peas and ca. 30 ryegrass seeds were planted / placed per pot; germination rates were high (>95%). Thirty pots were established per species. An automated irrigation

system was used to supply all pots with ample and uniform amounts of water; irrigation was adjusted to climate conditions and free drainage of pots was provided. Pots were kept free of weeds and no pests were observed. Plants were grown under the described conditions from early April to the end of June 2017.

Root system rinsing, and pre-measurement storage conditions

At the beginning of July 2017, all pots were transported to a growth room at BOKU Wien and acclimated to the ‘indoor’ conditions for at least 7 days (20 °C, 60% relative humidity, 12 h light (fluorescence light bulbs, 400–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$)) before measurements started; plants were irrigated and weeded as needed. About >3 h after light was turned on (to avoid potential diurnal effects), pots were randomly selected and brought to the laboratory. There the root systems were carefully cleaned from quartz sand and rinsed under tap water (2–3 min); the time the root systems were rinsed is considered time ‘0’. Four different storage conditions (treatments) were subsequently established: Attached (A), Warm (W), Cold (C), and Dry (D; Table 1). The establish storage conditions aimed to provide most contrasting storage conditions in terms of excision, storage media and temperature rather than emulating specific storage conditions being used by individual research groups. Roots of treatment A remained connected to the shoot(s) after rinsing and were placed in an aerated water bath at room temperature (22–23 °C); the leaves were illuminated (400–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) to allow photosynthesis to continue. After ~30, 60, 120, 180, and 300 min in the water bath, subsamples of roots close to the shoot (in *P. sativum* and *L. perenne*) or of fine roots (diameter ≤ 2 mm; *C. betulus*) were excised and subjected to immediate respiration measurements. In treatment A, each excised root subsample was measured only once after excision; time point 1440 min was not measured in treatment A. Accordingly, measurement cycles are denoted as A1 to A5. Roots from treatments W and C were excised immediately (i.e. 1–2 min) after rinsing and stored in tap water-filled plastic tubes at 28 °C and 4 °C until respiration measurements, respectively. W and C root samples were subjected to repeated (‘R’) measurements (~30, 60, 180, 300 and 1440 min after excision); after each measurement cycle, roots were placed back into water-filled plastic tubes for in-between measurement storage. In addition, some roots

of treatment C were stored for 60 (C2) and 180 min (C4) without intermediate measurements to determine the effects of repeated warming (CR) on RR_S of cold stored roots. Roots from treatment D were also excised directly after rinsing but were stored on petri dishes under room conditions (~22–23 °C, ~40–60% relative humidity) until and in-between respiration measurements (~30, 60, 180, 300 and 1440 min after excision). Accordingly, the first measurement cycles are denoted as W1, C1, and D1, while the following, repeated cycles are denoted as WR_x, CR_x, and DR_x (R for ‘repeated’ measurements on a sample used at a previous time point; x = 2–6). See Table 1 for details.

Root respiration

Prior to respiration measurements ~2 g (fresh weight) roots of treatments A, C, and W were placed in a beaker filled with 20 °C tap water for ~2–3 min (to facilitate temperature acclimation); fine roots of D were placed in a climate cabinet at 20 °C. Root fresh weights of ~2 g per sample correspond to average dry weights of 450 ± 6 mg (*C. betulus*), 135 ± 2 mg (*P. sativum*), and 246 ± 4 mg (*L. perenne*) as determined at the end of the experiment. Subsequently, A-, W- and C-stored roots were gently blotted surface dry. All roots were then placed in acclimated (20 °C), 55 ml plastic tubes (‘chambers’) itself placed in a climate cabinet (20 \pm 0.1 °C); the CO₂ efflux from roots (i.e. root respiration) was determined in air by means of the closed-chamber technique, connecting the chambers to an infra-red gas analyser (IRGA, EGM-5; PP-Systems International, Inc. Amesbury, MA, USA; 65% pump speed). After 60 s of equilibration (air mixing within chamber), changes in CO₂ concentration (ppm; ΔCO_2) within the chamber was recorded every 30 s for 2–3 min until $\Delta\text{CO}_2 > 50$ ppm. In general, measurements started at the earliest 30 min after IRGA start-up allowing for stable IRGA temperatures; ‘zero setting’ was performed regularly. Air CO₂ concentrations in chambers at the start of recordings were in the range of 450–600 ppm. Root respiration rate was calculated from a cubic regression fitted to the relationship between measurement time and CO₂ concentration (Kutzbach et al. 2007). Specific (fine) root respiration rates per biomass (RR_S ; $\text{nmol CO}_2 \text{g}^{-1} \text{s}^{-1}$) were calculated, taking headspace, air pressure, temperature, and root dry mass into account (Lamouroux 2008). According to the specific storage conditions / time points, root samples were carefully

Table 1 Respiration measurements on *Carpinus betulus*, *Pisum sativum*, and *Lolium perenne* roots per storage conditions (Treatments), defined by storage media (tap water or air) and temperature (4 °C, 22–23 °C*, or 28 °C), repeated or single ('once')

measurements per root sample, and time of excision / measurements relative to the time of root system rinsing (time '0'). Time intervals measured are marked by the specific sample number ($n = 25-30$); n.a. not available

Treatment (Abbreviation)	Storage	Measur. per sample	Rinsing (min)	Time of excision (min)	Realized measurement intervals, after rinsing the root system (± 5 min)
	Media Temp. (°C)				30 60 120 180 300 1440
<i>Attached (A)</i>	Water [§] 22–23*	Once	0	$\sim 5 \pm 2$ (before each measurement)	A1 A2 A3 A4 A5 n.a.
<i>Warm (W)</i>	Water 28	Once	0	<2 after rinsing	W1 n.a. n.a. n.a. n.a. n.a.
<i>Warm Repeated (WR)</i>	Water 28	Repeated	0	<2	WR2 WR3 WR4 WR5 WR6
<i>Cold (C)</i>	Water 4	Once	0	<2	C1 C2 n.a. C4 n.a. n.a.
<i>Cold Repeated (CR)</i>	Water 4	Repeated	0	<2	CR2 CR3 CR4 CR5 CR6
<i>Dry (D)</i>	Air* 22–23*	Once	0	<2	D1 n.a. n.a. n.a. n.a. n.a.
<i>Dry Repeated (DR)</i>	Air* 22–23*	Repeated	0	<2	DR2 DR3 DR4 DR5 DR6

[§] aerated; *i.e. room conditions

retrieved from the 55 ml plastic tubes with tweezers and either returned to water-filled storage beakers for repeated measurements or placed in water for root imaging. Subsequently to respiration measurements, root samples were imaged with a flatbed scanner, and images were analysed for morphological root traits (see Supplementary Material for details). Root samples were dried (65 °C) and dry mass was determined to an accuracy of ± 0.1 mg. Dried fine roots were ground to powder, five times pooling five samples per species, and total carbon and nitrogen concentrations were determined (see Supplementary Material for details). Means of morphological and chemical root traits per species were related by linear regression analysis to mean specific root respiration rates (samples A1); relating morphological and chemical root traits to other mean RR_S (samples WR1, CR1, DR1) did not result in improved regressions (data not shown). Means of CR2,4 and C2,4 samples were compared to determine potential effects of interim warming on RR_S after longer cold storage.

Statistical analysis

Statistical analyses were carried out in IBM SPSS 24.0 and Microsoft Excel 2013. The effect of species, time and treatment were analysed by one-way General Linear Model (GLM) followed by Tukey's post-hoc test. Data was log-transformed to reach the requirements of the

GLM. A one sample T-test was used to determine if respiration rates differed from 0. Statistical relationships were considered significant at $p < 0.05$. All data is displayed as mean \pm standard error (SE).

Results

Species specific root respiration rates after 30 min of storage under different conditions

Among the three species and across the four different storage conditions (A1, W1, C1 and D1; Table 1), *P. sativum* possessed the greatest RR_S rates, ranging from 4.0 nmol CO₂ g⁻¹ s⁻¹ after 30 min under D ('Dry') storage conditions to 31.1 nmol CO₂ g⁻¹ s⁻¹ after 30 min under W ('Warm') storage conditions (Table 2). Similarly, RR_S rates of *C. betulus* and *L. perenne* varied between 2.7 and 2.3 nmol CO₂ g⁻¹ s⁻¹ after D-storage conditions, to 12.7.5 and 20.7 nmol CO₂ g⁻¹ s⁻¹ after W-storage conditions, respectively.

Storage conditions had a significant effect on the measured RR_S rates, i.e. RR_S rates were in all three species significantly lower after D-storage when compared to other storage conditions (Table 2). For *P. sativum* and *L. perenne* roots, storage under W conditions led to significantly greater RR_S rates when

Table 2 Specific root respiration rates (RR_S ; $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$, 20°C) of the species *Carpinus betulus*, *Pisum sativum* and *Lolium perenne* 30 \pm 5 min after root system rinsing. Root storage conditions: Attached (A1), Cold (C1), Warm (W1) and Dry (D1), see Table 1 for details. Measurements were conducted 5 \pm 2 min after

excision (A), or 30 \pm 5 min after root excision (C, W, and D). Significant differences between species are indicated by different capital letters, significant differences between storage conditions by small letters (mean \pm SE; Tukey test, $p < 0.05$; $n = 25\text{--}30$)

Species	Specific root respiration (RR_S ; $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$) per storage condition			
	Attached (A1)	Warm (W1)	Cold (C1)	Dry (D1)
<i>C. betulus</i>	9.8 \pm 1.0 Ba	12.7 \pm 1.0 Ca	11.0 \pm 1.3 Ba	2.7 \pm 0.2 ABb
<i>P. sativum</i>	16.2 \pm 1.0 Ab	31.1 \pm 2.2 Aa	20.7 \pm 3.6 Ab	4.0 \pm 0.6 Ac
<i>L. perenne</i>	14.3 \pm 1.0 Aab	20.7 \pm 3.4 Ba	10.3 \pm 1.0 Bb	2.3 \pm 0.3 Bc

compared to C- (both species) and A-storage (*P. sativum* only) conditions; no effect of the 3 different ‘water’ storage conditions on RR_S was found for *C. betulus*. The ranking of species according to absolute RR_S rates and the significance of differences between species varied according to storage conditions. For example, using roots which were ‘attached’ (A) to the plant until shortly before the measurements resulted in non-significant differences between the RR_S of *P. sativum* (16.2 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$) and *L. perenne* (14.3 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$), with *C. betulus* featuring significant lower RR_S (9.8 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$). In contrast, cold (C) storage conditions resulted in non-significant differences between RR_S rates of *C. betulus* (11.0 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$) and *L. perenne* (10.3 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$) but significant greater RR_S rates of *P. sativum* (20.7 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$) compared to both other species. A significant difference between the RR_S rates of all three species was found under W-storage conditions only (Table 2).

Specific root respiration rates after extended storage under different conditions

The RR_S rates of the three species reacted differently to extended storage times and this was additionally modulated by storage conditions (Fig. 1). Compared to the respective measurements at 30 min (9.8–12.7 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$), the treatments A, WR and CR did not result in a significant decline of *C. betulus*’ RR_S within 300 min (8.2–11.6 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$, respectively; Fig. 1A). Interestingly, the RR_S of *C. betulus* roots were significantly increased after 1440 min of WR-storage conditions (17.2 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$). In contrast, RR_S of *C. betulus* roots stored under DR conditions were significantly reduced from 180 min (DR; 1.7 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$)

onwards. Particularly starting from 300 min, significant differences in RR_S could be noticed for *C. betulus* roots between most storage treatments, WR-stored roots having the significant greatest RR_S values (Supplementary Table S1). Similar to *C. betulus*, the RR_S rates of *P. sativum* measured after 30 min remained significantly unaltered under A, WR and CR treatments until 300 min, and even at 1440 min (CR and WR) (Fig. 1B). Roots stored at DR treatment possessed a significant reduction in RR_S after 300 min. In *L. perenne* both WR and CR conditions, although possessing largely different RR_S towards each other, resulted in significantly unaltered RR_S rates until 1440 min (Fig. 1C). Under ‘attached’ conditions, the RR_S of *L. perenne* was significantly reduced by ~30% after 300 min (10.3 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$) of storage after rinsing/excision compare to RR_S measured at 30 min (14.7 $\text{nmol CO}_2 \text{ g}^{-1} \text{ s}^{-1}$). The very low RR_S rates of *L. perenne* under dry storage conditions remained comparably stable until 180 min; after that, they declined significantly. In both *P. sativum* and *L. perenne*, significant differences between storage treatment WR, and both A and CR storage conditions occurred from the first measurement at 30 min, with warm storage continuously resulting in greatest RR_S rates (Supplementary Table S1).

The RR_S rates of cold-stored roots did not show significant differences when comparing repeated measurements CR2 and CR4 to single measurements C2 and C4, respectively (Supplementary Fig. S1).

Root morphological and chemical traits and their relation to RR_S

The three species differed significantly with regard to all morphological and most chemical root traits assessed (Table 3). The specific root length (SRL) as well as the specific root area (SRA) were significantly smallest in

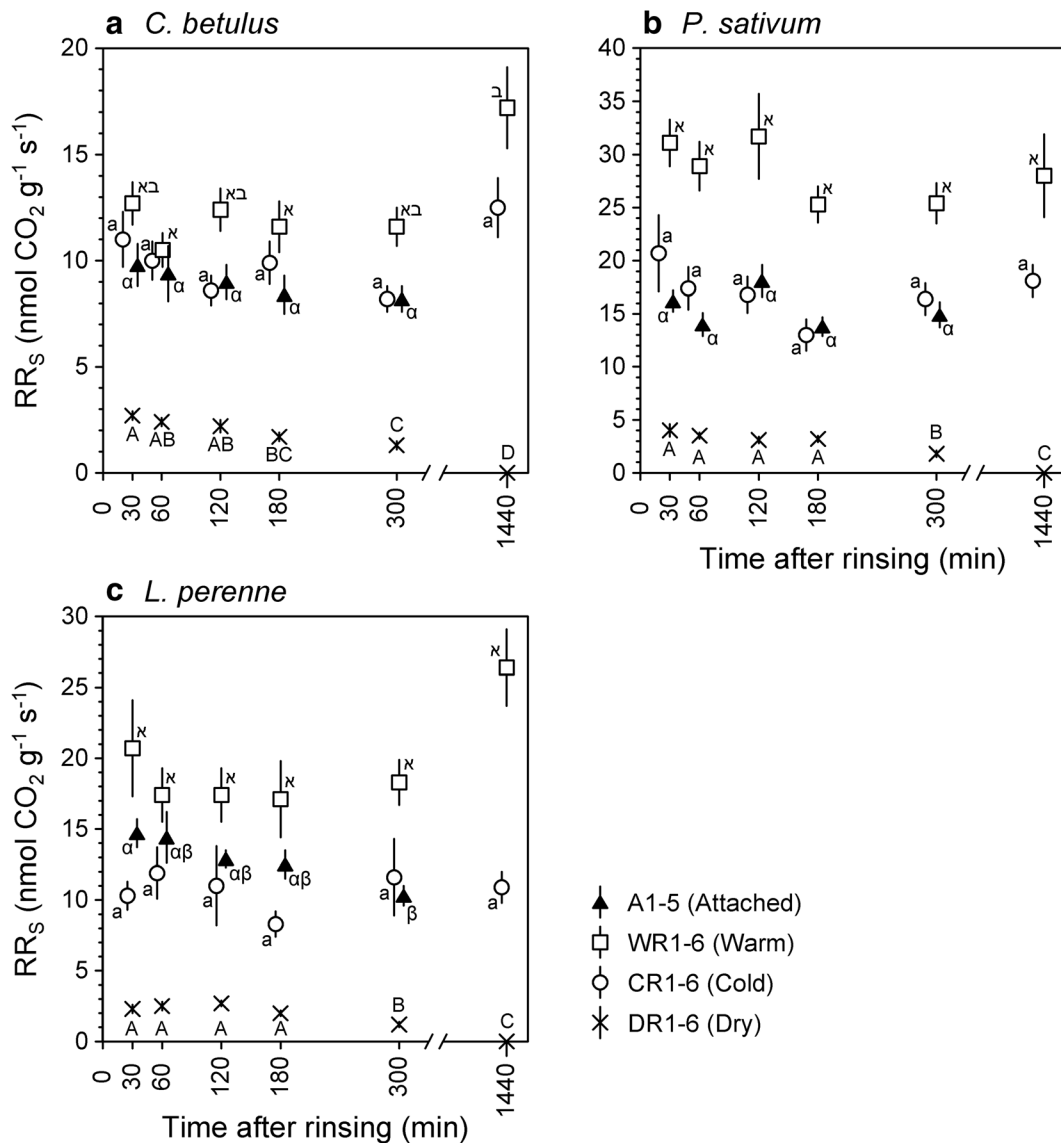


Fig. 1 Specific root respiration (RR_S; 20 °C) of **A** *Carpinus betulus*, **B** *Pisum sativum*, and **C** *Lolium perenne* (fine) roots stored under the conditions (treatments) Attached (A1–5; Triangle), Warm (WR1–6; Square), Cold (CR1–6, Circle), or Dry (DR1–6, Cross) for 30, 60, 120, 180, 300 (and 1440) min after root system rinsing; see Table 1 for details. Please note the

different Y-axis scales. Significant differences between time of measurements within treatments are denoted by different Greek (A), Hebrew (WR), small Latin (CR), and capital Latin (DR) letters (mean ± SE; Tukey test on log-transformed data, $p < 0.05$; $n = 25–30$). See Supplementary Table S1 for statistics comparing RR_S between treatments per time point

C. betulus fine roots ($d < 2$ mm). *L. perenne* was featuring the significant greatest SRL (8024 cm g⁻¹) and SRA (2110 cm² g⁻¹) values of the three species. In accordance, tissue density (TD) was significant greatest in *C. betulus* fine roots (0.099 g cm⁻³) with about 4-fold lower values in *P. sativum* (0.028 g cm⁻³) and *L. perenne* (0.024 g cm⁻³). Nitrogen concentrations were significantly greatest in *P. sativum* roots (2.6%),

followed by *C. betulus* fine roots (1.6%) and the significant least concentrations were found in *L. perenne* roots (1.2%). Carbon concentrations were more similar, but with significant differences between *C. betulus* fine roots (49.4%) and *L. perenne* roots (46.2%). Carbon to nitrogen ratio was clearly lowest in *P. sativum* roots, with nearly twice as high carbon to nitrogen ratios in both other species (Table 3). We found no significant

Table 3 (Fine) root morphological and chemical traits of the species *Carpinus betulus*, *Pisum sativum* and *Lolium perenne*. Morphology traits are specific root length (SRL), specific root area (SRA), and tissue density (TD); root chemical traits are totalnitrogen (N) and carbon (C) concentrations and the C:N ratio. Significant between species are indicated by different capital letters (mean \pm SE; Tukey test, $p < 0.05$; $n(\text{SRL, SRA, TD}) = 25\text{--}30$, $n(\text{N, C, C:N}) = 5$)

Species	SRL (cm g ⁻¹)	SRA (cm ² g ⁻¹)	TD (mg cm ⁻³)	N Conc. (mg g ⁻¹)	C Conc. (mg g ⁻¹)	C:N ratio
<i>C. betulus</i>	2616 \pm 49 A	601 \pm 8 A	99 \pm 0.20 C	16 \pm 0.3 B	494 \pm 4 B	31 \pm 0.4 B
<i>P. sativum</i>	7412 \pm 146 B	1851 \pm 29 B	28 \pm 0.06 B	26 \pm 0.3 C	477 \pm 6 AB	18 \pm 0.3 A
<i>L. perenne</i>	8024 \pm 214 C	2110 \pm 40 C	24 \pm 0.07 A	12 \pm 0.4 A	462 \pm 9 A	38 \pm 1.2 C

linear relation between mean RR_S rates and mean root traits of species, neither for morphological nor for chemical root traits (Supplementary Fig. S2).

Discussion

Specific root respiration of plant species and their dependency on short-term storage conditions

The measured specific root respiration (RR_S) rates are in the range of previously reported values. For example, the studies listed in the FRED fine-root database (<https://roots.ornl.gov>; 09.03.2020) reported RR_S values of 1.7–11.2 nmol CO₂ g⁻¹ s⁻¹ for fine roots of deciduous, broadleaved tree species, 4.9–32.9 nmol CO₂ g⁻¹ s⁻¹ for herbs, and 2.4–22.2 nmol CO₂ g⁻¹ s⁻¹ for C3 graminoids (Iversen et al. 2018). Rewald et al. (2014) reported RR_S rates of ~16 nmol O₂ g⁻¹ s⁻¹ (20 °C) for *C. betulus* fine roots; although the respiratory quotient remains unknown, this is considerable more than the RR_S of *C. betulus* fine roots reported in this study (~10–13 nmol CO₂ g⁻¹ s⁻¹; Table 2, Suppl. Table S1). For *P. sativum*, Ryle et al. (1983) reported RR_S of 6.7 \pm 0.3 mg CO₂ g⁻¹ h⁻¹; ~42 nmol CO₂ g⁻¹ s⁻¹, and de Visser and Lambers (1983) reported RR_S of 5–11 mg O₂ g⁻¹ h⁻¹ (~43–95 nmol O₂ g⁻¹ s⁻¹) largely depending on cultivar, N source and root age; both measurement ranges being much greater compared to the values of this study (~16–31 nmol CO₂ g⁻¹ s⁻¹). This discrepancy could be potentially related to the fact that we did not observe a pronounced Rhizobia establishment, i.e. nodules were infrequent and formed only very small protuberances (by visual observation, not quantified), on the sand-grown, fertilized and non-inoculated *P. sativum* roots; nodules are known to feature great respiration rates (Lambers et al. 2002 and references within). The RR_S of *L. perenne* was

previously determined as ~14 nmol CO₂ g⁻¹ s⁻¹, and thus within the range of values determined in this study (Table 2), and deemed high compared to the RR_S of 12 other perennial grass species (Picon-Cochard et al. 2012). In our study, the greater RR_S of the legume *P. sativum* could be partially related to the greater nitrogen concentration in its roots (Table 3); previous studies have reported strong correlations between root nitrogen concentrations and RR_S (Atkinson et al. 2007; Ceccon et al. 2016; Tang et al. 2019). However, in this study we could not determine a significant linear correlation between RR_S of the three plant species and their respective root nitrogen concentration (Supplementary Fig. S2)—likely because of the great RR_S of the grass *L. perenne* beside its relatively low root N concentration (Table 3). Furthermore, while it can be well assumed that the sum of different traits above- and belowground (e.g. availability of photosynthetic assimilates, or tissue density) is affecting plant species' or even genotypes' RR_S (Lai et al. 2015; Picon-Cochard et al. 2012; Rewald et al. 2014), we did not find any significant correlation between mean RR_S rates (at 30 min, and any storage treatment) and the six morphological or chemical traits tested (displayed for treatment A1 in Supplementary Fig. S2). However, our and previous studies evidenced that the three plant species generally differ in their RR_S (Table 2).

Species-specific RR_S rates were, however, already significantly influenced by short-term differences in storage conditions (i.e. 30 min since root system rinsing). In specific, RR_S rates were significantly lower after dry storage (D), i.e. the placement of surface wet roots on petri dishes exposed to common room conditions for 30 min. This can be explained by reduced physiological activity of tissues from dehydration (Baena-González and Sheen 2008); soil moisture deficits have previously found exerting a substantial negative influence on RR_S (Bryla et al.

2001; Jarvi and Burton 2013). In accordance, Leprince et al. (2000) previously reported gradually decreasing CO₂ efflux rates from *P. sativum* radicles during progressing dehydration.

For *P. sativum*, warm storage (W) led to a significantly higher RR_S rate when compared to A and C treatments (Table 2). This might be related to an incomplete temperature adjustment of W-stored roots (storage at 28 °C) to a flux measurement temperature of 20 °C, as RR_S rates are generally positively related to increasing temperature (Atkin et al. 2000). However, the opposite effect was not noticed for C-stored roots (4 °C), although the temperature difference was much greater. Furthermore, utmost care was taken to prevent root temperatures to deviate from the flux measurement temperature of 20 °C (see Materials and Methods). Thus, we interpret the greater RR_S in W-treated *P. sativum* rather as a ‘lag’ effect of the previous, short-term warm-storage. In this species, the assumed fast acclimation of root respiration to storage at 28 °C, after being exposed to 20 °C for >7 days, might thus indicate rather rapid changes in the demand for ATP for maintenance processes rather than enzyme capacity (Atkin et al. 2000). In contrast, RR_S of cold-stored roots were not significantly different from those subjected to A-storage. Thus, our results might indicate that warming leads to a rapid acclimation of root respiration in some, more active species, with no evidence for a rapid acclimation to cold conditions. Although not the focus of this work, these results are indicative of a strong non-linearity in root Q₁₀'s (see Atkin et al. 2000 and discussion within); this phenomenon requires further studies.

Comparing the short-term storage treatments, our study shows that 1) root storage in water is generally preferable to storage under dry conditions, 2) root storage in warm water may significantly increase RR_S in metabolically very active species such as *P. sativum*, and 3) RR_S rates from roots stored in cold water are comparable to that of recently excised roots (i.e. A treatment). However, our data also shows that the degree of species differentiation is largely influenced by storage conditions. In specific, differences in RR_S among all species were only statistically significant under W-storage; under A- and C-storage conditions RR_S of either *P. sativum* and *L. perenne* (under A) or *C. betulus* and *L. perenne* (under C) were statistically similar (Table 2).

Effects of extended storage and storage condition on RR_S measurements

Information on which carbon pools are respired by roots (i.e. recent assimilates compared to carbon stored in the plant/organ) is not yet very comprehensive, but recent studies indicate that roots may combine recent carbon assimilates with carbon from a stored pool to fuel (fine) root respiration (see Lynch et al. 2013 and references within). The amount of stored carbon used to fuel root respiration is likely differing between species and ontogenetic stages, as well as seasonally and/or even diurnally (Bahn et al. 2006; Hansen 1977; Hopkins et al. 2013; Kuptz et al. 2011). As recent photosynthate relocation ceases after shoot clipping, and stored carbon levels in roots are assumed to decline rapidly, the time span between root sampling and RR_S measurements is usually kept as short as possible (Bloom and Caldwell 1988; Freschet et al. 2020). Labelled photosynthetic carbon can be respired in roots as fast as within 0.5–2 h after photosynthetic assimilation (Yoshida and Eguchi 1992 and references within); Kuzyakov et al. (2001) calculated a rather direct use of recently assimilated carbon for root respiration (95% within 1 day). Accordingly, e.g. Bingham and Rees (2008) reported a ~50% decline of the respiration rates of excised *Trifolium* roots after 24 h. However, Leprince et al. (2000) indicated earlier that incubating isolated *P. sativum* and *Cucumis sativus* radicles on wet filter paper did not induce significant changes in CO₂ emission rates over a period of 4 h. Moreover, Bahn et al. (2006) reported that clipping did not significantly affect root respiration rates of temperate grass species for up to 14 days in situ—indicating that RR_S was largely maintained by carbon reserves in roots or stolons. This coincided with findings of Bazot et al. (2005), which showed on mature *L. perenne* plants that root soluble carbon concentrations even increased for several days after defoliation (but see Smith and Stitt 2007 for *Arabidopsis*). Although we are not aware of long-term measurements of tree RR_S after shoot/stem clipping / root excision, it has been similarly estimated that the amount of carbon stored in tree roots may buffer effects of reduced assimilate supply for several days to weeks (Högberg et al. 2001; Pregitzer et al. 2000). In accordance, e.g. Keith (1998) showed that phosphorous uptake by excised *Eucalyptus nitens* fine roots was significantly reduced 5 days after sampling only. In the light of these findings, our study confirms that appropriate storage conditions

generally enable to measure RR_S after extended storage times, resulting in RR_S rates comparable to those measured 30 min after excision (Fig. 1). The different effects of storage conditions, as outlined above, are largely maintained over time (Supplementary Table S1). Storage in water did generally not result in significant changes in RR_S among plant all three species until 300 min (beside A-storage in in *L. perenne*, see discussion below), and partially until 1440 min (i.e. 1 day) after root rinsing. This was particular surprising for W-storage conditions as we had assumed that the greater metabolic rates maintained in warm water would result in a (faster) depletion of easily available carbon reserves and thus a faster reduction in RR_S rates. Instead, we even noticed a significant increase of RR_S in W-stored *C. betulus* roots and similar tendencies in *L. perenne* roots at 1440 min (Fig. 1). It seem reasonable to speculate that this might (partially) relate to an increased heterotrophic respiration by entophytic microbes. For example, earlier studies have shown that the senescence of plant tissue can rapidly convert microbes from being passive members of the endophytic microbial community to a saprotrophic lifestyle (Porras-Alfaro and Bayman 2011)—with potential effects on their metabolic activity. However, no significant effects of cold storage were found in any of the three species at 1440 min (CR6) compared to C1, indicating a general suitability of extended cold storage to conserve roots for later RR_S measurements. As we did not find significant differences in C vs. CR root samples (Supplementary Fig. S1), we conclude that temporal warming in-between cold-storage periods has only minor effects on RR_S measurements (at least until 180 min with 3 preceding warming periods).

Under extended dry storage, and thus continued dehydration (see discussion above), RR_S continued to decline, particularly starting to be significant from 120 or 180 min compared to D1; however, the rate of decline was at a much slower rate compared to the decline during the first 30 min of dry storage. While Lafta and Fugate (2009) found that cell membrane damage after excessive dehydration accelerated respiration in post-harvest *Beta vulgaris* (storage) roots, this did not apply to the (fine) root respiration measurements conducted here. Leprince et al. (2000) reported that the rates of CO_2 produced by desiccation-sensitive *P. sativum* radicles during progressing dehydration were at least 2-fold higher than those in tolerant radicles. Assuming that fine roots of woody species are more tolerant to desiccation

than roots of herb or grass species, our data does indicate that at 30 min of D-storage the relative decline in RR_S (compared to A) is indeed lower in *C. betulus* than the two other species. However, there is not evidence for a longer ‘maintenance’ of RR_S in D-stored *C. betulus* fine roots compared to the other species as RR_S of all three species was not significantly different from zero at 1440 min (Fig. 1).

The study of Hansen (1977) indicated that the CO_2 release from *Lolium multiflorum* roots in aerated Hoagland solutions was rather dependent on preceding irradiance conditions and diurnal cycles, than to extended storage in aerated nutrient solutions. Thus, we were surprised of the significant decline in RR_S in ‘attached’ *L. perenne* roots 300 min post-rinsing. However, neither for *C. betulus* nor for *P. sativum* the continued cultivation in hydroponics resulted in significant differences. As previous studies have successfully grown *L. perenne* in hydroponic systems, we can only speculate that the cultivar ‘Jubilee’ might hold relative high water-soluble carbohydrate concentrations (Smith et al. 2002)—which may have been leached to a greater extend in the aerated solution and thus led to a reduction in stored carbon available for respiration.

Comparing the long-term storage treatments, our study showed that 1) storage in water for 180 min is generally suitable for delayed determination of RR_S irrespective of plant species or storage temperature. 2) Effects of longer storage periods (>180 min) depend largely on storage conditions, while differences in RR_S caused by the storage conditions remain largely stable over time. Among the tested conditions, 3) cold-storage conditions seem most suitable to achieve stable RR_S measurements on excised roots—as minor differences to A-conditions occurred and values remained stable for 1 day post-excision. In general, our data suggests that the degree of species differentiation in RR_S , i.e. $C. betulus < L. perenne < P. sativum$, is largely maintained by storage periods up to 300 min, although largest differences occurred under warm storage conditions (until 1440 min).

Conclusion and outlook

While wound reactions after root severance have been suggested to result in greater RR_S rates (Makita et al. 2012), we consider this effect minimal in our study as the severed root surface is negligible compared to the large amount of root biomass utilized. Moreover, careful

root rinsing from the sandy substrate did not result in visible damages such as larger amounts of detached root tips. A methodological challenge in determining species' RR_S remain the effects of rooting media and root excision per se on root respiration rates. For example, Cheng et al. (2005) showed that compared to a root excision method, the root respiration rate of longleaf pine measured by a field chamber method was 18% higher when using native soil as rooting medium, was similar in prairie soil, but was 42% lower in sand-vermiculite medium. In addition, the presented data emphasizes that pre-measurement storage conditions of excised roots can have effects on RR_S of a similar magnitude than excision per se—emphasizing the importance of using either highly standardized measurement protocols for determining plant functional traits or providing exact descriptions of pre-measurement conditions. Our findings are also providing evidence that extended storage time (i.e. beyond 180 or 300 min) does not necessarily result in significantly altered RR_S rates. This has important implications on measurement routines of RR_S , a generally understudied root trait; henceforth it seems reasonable to collect roots in the field and transport them, even uncooled, to the laboratory for subsequent measurements (if kept moist). We speculate that this holds especially true for larger plants, which may hold sufficient carbohydrate reserves sustaining root metabolism rates for an extended time (see also discussion in Bahn et al. 2006). Future studies should compare the effects of ontogenetic stages on suitable storage times before RR_S (or any other physiological) measurements, further explore the suitability to supply exogenous sugars to stabilize RR_S rates (Bingham and Rees 2008) or test other relevant storage conditions (e.g. keeping moist roots (potentially wrapped in wetted paper) in closed bags, or 'storage' of roots within soil monoliths). In addition, it seems advisable to further examine the effects of excision and rinsing on RR_S compared to in situ measurements of RR_S per se, particularly considering the effects of epi- and endophytic microbial activities on measured specific respiration rates.

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Author contribution statement BR designed the study. ZL conducted the measurements with help by BR. ZL, HS, MM and BR analysed the data. ZL and BR provided the first draft of the manuscript; all co-authors jointly edited and revised the manuscript and approved its (re-)submission.

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