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$\delta^{13}C$ of organic matter transported from the leaves to the roots in *Eucalyptus delegatensis*: short-term variations and relation to respired CO_2

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Abstract. Post-photosynthetic carbon isotope fractionation might alter the isotopic signal imprinted on organic matter (OM) during primary carbon fixation by Rubisco. To characterise the influence of post-photosynthetic processes, we investigated the effect of starch storage and remobilisation on the stable carbon isotope signature ($\delta^{13}C$) of different carbon pools in the *Eucalyptus delegatensis* R. T. Baker leaf and the potential carbon isotope fractionation associated with phloem transport and respiration. Twig phloem exudate and leaf water-soluble OM showed diel variations in $\delta^{13}C$ of up to 2.5 and 2‰, respectively, with ^{13}C enrichment during the night and depletion during the day. Damped diel variation was also evident in bulk lipids of the leaf and in the leaf wax fraction. $\delta^{13}C$ of nocturnal phloem exudate OM corresponded with the $\delta^{13}C$ of carbon released from starch. There was no change in $\delta^{13}C$ of phloem carbon along the trunk. CO_2 emitted from trunks and roots was ^{13}C enriched compared with the potential organic substrate, and depleted compared with soil-emitted CO_2. The results are consistent with transitory starch accumulation and remobilisation governing the diel rhythm of $\delta^{13}C$ in phloem-transported OM and fragmentation fractionation occurring during respiration. When using $\delta^{13}C$ of OM or CO_2 for assessing ecosystem processes or plant reactions towards environmental constraints, post-photosynthetic discrimination should be considered.

Additional keywords: fragmentation fractionation, phloem transported organic matter, post-photosynthetic carbon isotope discrimination, starch.

Introduction

Carbon isotope discrimination during photosynthetic CO_2 fixation is a comparatively well described and understood phenomenon (Farquhar *et al*. 1982, 1989). Changes in radiation, as well as atmospheric and soil water deficits, affect c_c (CO_2 concentration at the site of carboxylation), and, thus, modify the ratio of ^{13}C to ^{12}C [expressed as deviation from Vienna Pee Dee Belemnite standard ($\delta^{13}C$)] in newly assimilated plant carbon (Ziegler 1979; Farquhar *et al*. 1982; Winter *et al*. 1982). As a consequence, analysis of $\delta^{13}C$ in different chemical compounds in leaf tissues of trees can be used to assess stomatal and photosynthetic responses to environmental constraints, but concerning the other parts of the plant, the functional interpretation of $\delta^{13}C$ values is more delicate. Stomatal closure

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due to water deficits generally reduces c_c, leading to an increase in $\delta^{13}C$ (e.g. Madhavan *et al*. 1991; Korol *et al*. 1999; Keitel *et al*. 2003). In contrast, light limitation of photosynthesis increases c_c, and so $\delta^{13}C$ depends on radiation (Leavitt and Long 1986) and also the combined influences of water and light availability have been observed (Gessler *et al*. 2001). $\delta^{13}C$ of CO_2 respired from forest ecosystems has also been related to the variation of environmental parameters as a close link between assimilation and respiration is postulated (Lancaster 1990; Scartazza *et al*. 2004; Knohl *et al*. 2005).

In contrast, much less is known about isotopic fractionation associated with the metabolic processes following carboxylation (Hobbie and Werner 2004; Badeck *et al*. 2005; Brandes *et al*. 2006). However, carbon isotope discrimination associated with downstream metabolic pathways and with transport processes might alter the original photosynthetic isotopic signal of organic matter (OM). Fractionation due to equilibrium and kinetic isotopic effects causes differences in isotopic signatures (i) among metabolites and (ii) in intramolecular positions (Schmidt 2003) and so bring about differences in the $\delta^{13}C$ signature among chemical fractions if metabolic branching occurs. When a product has a different isotopic composition from the source because of the way the source molecule was fragmented, and not because of any kinetic (or equilibrium) effect, that process has been termed 'fragmentation fractionation' (Tcherkez *et al*. 2004). Inter-organ variation can also occur if the isotopic compositions of exported and non-exported compounds differ (Hobbie and Werner 2004). Temporal variation in $\delta^{13}C$ of OM exported from leaves might be associated with storage and remobilisation of transitory starch (Tcherkez *et al*. 2004) during the day–night-cycle as starch can be up to 4‰ enriched compared with triose-P originating directly from the pentose phosphate cycle (Gleixner *et al*. 1998).

Non-random intramolecular distribution of ^{13}C in hexose phosphates and fragmentation fractionation during respiration is thought to be the main reason for the isotopic enrichment of CO_2 released from respiration (Duranceau *et al*. 1999; Ghashghaie *et al*. 2003; Tcherkez *et al*. 2003, 2004). The isotope enrichment of respired CO_2 can vary in time (Damesin and Lelarge 2003; Hymus *et al*. 2005; Mortazavi *et al*. 2006; Prater *et al*. 2006) and with plant organ (Badeck *et al*. 2005) and is influenced by water availability (Ghashghaie *et al*. 2001) and temperature (Tcherkez *et al*. 2003).

Post-photosynthetic carbon isotope fractionation may be relevant for all approaches that use isotopic signals in OM or CO_2 to elucidate ecosystem processes or plant reactions to environmental constraints (Pataki *et al*. 2003; Helle and Schleser 2004; Badeck *et al*. 2005; Keitel *et al*. 2006).

Whereas previous work on the effects of post-photosynthetic fractionation processes on $\delta^{13}C$ of OM and CO_2 has mainly tackled particular features (e.g. potential carbon isotope fractionation during phloem loading and transport (Gessler *et al*. 2004; Hobbie and Werner 2004); temporal (Hymus *et al*. 2005) or spatial (Badeck *et al*. 2005) variation of carbon isotope discrimination during respiration) more comprehensive studies are scarce. Brandes *et al*. (2006) characterised diel patterns of $\delta^{13}C$ of soluble OM in leaves and phloem exudates at different trunk positions and related carbon isotope composition of phloem exudate OM to $\delta^{13}C$ of CO_2 emitted from the trunk of adult trees in a Scots pine plantation, but did not assess the patterns of diel starch dynamics in detail. Göttlicher *et al*. (2006) determined short-term changes in the carbon isotope composition of soluble carbohydrates and starch over one diel course in leaves and roots of adult beech, but did not assess phloem sap OM or CO_2.

In order to characterise potential temporal (i.e. diel) variation in $\delta^{13}C$ associated with transitory starch metabolism we determined the carbon isotope composition of total and water-soluble OM as well as of lipids, waxes and starch in leaves of *E. delegatensis* during diel courses. In an attempt to trace spatial patterns related to carbon transport we assessed $\delta^{13}C$ in phloem exudate OM at four different positions from the canopy to the trunk base and in the fine root tissue. In addition, we analysed day-to-day variations in $\delta^{13}C$ of CO_2 emitted from the trunk base and the roots of *E. delegatensis* as well as from the soil of the ecosystem.

We developed the following hypotheses.

(1) Starch storage and remobilisation during the diel course strongly influence the carbon isotope composition of OM exported from the leaves into the phloem. Plant metabolites with relatively short turnover times like leaf lipids are similarly affected, albeit with a damped response, whereas longer-term products such as leaf waxes are not significantly affected.
(2) The increasing ^{13}C enrichment from leaves to roots as observed for a multitude of species (see review by Badeck *et al*. 2005) is not only a result of fractionation processes in the source tissues (Hobbie and Werner 2004), but also associated with phloem transport (Gessler *et al*. 2004). Phloem transported sugars are supposed to be subject to leakage out of the transport phloem (Van Bel 2003). As the released sugars are partially metabolised but also partially retrieved into the sieve tubes, additional carbon isotope fractionation steps potentially occur in the trunk.
(3) The temporal variation in carbon isotope signature of trunk phloem exudate and root water-soluble OM determines $\delta^{13}C$ of CO_2 emitted from trunks and fine roots. Based on previous observations of tree stems (e.g. Damesin and Lelarge 2003) and on theoretical considerations on fragmentation fractionation (Tcherkez *et al*. 2003) we assume stem emitted CO_2 to be enriched in ^{13}C as compared with the putative organic source of respiration. There is information that CO_2 emitted from roots of herbaceous plants is ^{13}C depleted as compared with the putative substrates (Badeck *et al*. 2005; Klumpp *et al*. 2005) probably due to CO_2 re-fixation in roots by phosphoenolpyruvate carboxylase (PEPC). We, thus, assume root-respired CO_2 (and potentially soil-respired CO_2 as root respiration should considerably contribute to this CO_2 flux) to be depleted compared with stem emitted CO_2 and to root water-soluble OM. Soil respiration is another important source for total ecosystem CO_2 exchange. We assume that temporal variations in $\delta^{13}C$ of soil respired CO_2 are closely linked to $\delta^{13}C$ of short-term organic matter pools of trees and of tree respired CO_2.

Materials and methods

Field site

Measurements were made on trees located at the Tumbarumba flux station (35°39′20.6″S, 148°09′07.5″E, elevation 1200 m

above sea level) situated in the Bago State Forest in south-eastern New South Wales (Australia), part of a 50 000 ha native forest that has been managed for wood production for more than 100 years. The stand is a moderately open, wet sclerophyll forest. The dominant species are *Eucalyptus delegatensis* R. T. Baker and *Eucalyptus dalrympleana* Maiden with a maximum height of 40 m. The stand is mixed-aged ranging up to 90 years. Leaf area index (LAI) of the forest canopy is $1.4\,m^2\,m^{-2}$, and the understorey has a patchy cover of shrubs and groundcover of grasses and herbs with an LAI of $1.5\,m^2\,m^{-2}$. The soil is classified as an acidic, eutrophic, red dermosol, 2–3 m deep with a weathered C horizon overlying granodiorite, and a moderate nutrient status and amounts of stored carbon (McKenzie and Ryan 1999).

Long-term mean daily maximum and minimum temperatures measured at the Tumbarumba Post Office, which is located 40 km south of the flux station and at the lower elevation of 750 m are 18.7 and 5.0°C, respectively. Annual rainfall (January–December) at the flux station was 1225, 1342 and 1226 mm, for the years 2002–04, respectively, but protracted dry periods from October 2002 to May 2003 and in autumn 2004 caused significant loss of leaf area, tree mortality due to insect damage and consequent reductions in net carbon uptake by the forest between 2002 and 2005 (Leuning *et al*. 2005; Kirschbaum *et al*. 2007).

Meteorological measurements

Fluxes of CO_2 were measured using the open-path, eddy flux technique (Leuning and Judd 1996), making corrections to CO_2 fluxes for the effects of density fluctuations arising from sensible and latent heat fluxes (Webb *et al*. 1980). Components of the turbulent wind vector were measured using a sonic anemometer (Type HS, Gill Instruments Ltd, Lymington, UK), and fluctuations in CO_2 concentrations were measured using an open-path infrared analyser (Type LI-7500, Li-Cor Biosciences Inc., Lincoln, NE). Changes in CO_2 storage in the air column below the eddy covariance instruments were determined using CO_2 concentrations measured at nine heights. The eddy fluxes and change in storage data were used to calculate net ecosystem CO_2 exchange, as described by van Gorsel *et al.* (2007).

Incoming solar radiation was determined with a pyranometer (CM-11, Kipp and Zonen, Delft, The Netherlands). Air temperature and humidity at 70 m were measured using a 50Y RH/T sensor, (Vaisala, Helsinki, Finland) mounted in a fully aspirated radiation shield. Rainfall was measured both above and below the canopy and soil temperature was measured using thermocouples placed at depths of 0.02 and 0.1 m. Further details are given by Leuning *et al*. (2005).

Plant material

We selected three representative dominant individuals of *E. delegatensis* with a maximum height of ~38 m at ~200 m distance from the flux tower. The canopy of the trees and the upper parts of the trunks were accessed using a 40-m hydraulic lift installed on a truck. The measurement campaign began on 7 March 2005 and measurements within the canopy were completed on 13 March 2005. Plant samples were generally collected three times each day during the daylight period: in the morning, at midday/afternoon and in the evening. Between 9 March 2005 and 11 March 2005, samples were taken with higher frequency and night sampling was included. At each sampling time, leaves from the upper third of the canopy (~34 m) were harvested and immediately frozen in liquid nitrogen. In addition, small bark pieces (~150 mg fresh weight) from the same twig where the leaves were collected were sampled using scalpels. Circular trunk bark pieces (diameter 13 mm) were collected at ~26 m (trunk-top), 10 m (trunk-middle) and 0.5 m (trunk-base). Both twig and trunk bark samples were used in phloem exudation studies (see below). The phloem sampling at the lowermost position was continued until 17 March 2005.

Root samples (<10 mm diameter) of *E. delegatensis* were harvested, after they were used for determination of $\delta^{13}C$ in respired CO_2 (see below), from three different positions once per day between 1000 and 1300 h from 7 March 2005 to 17 March 2005.

Collection of emitted CO_2 for the determination of carbon isotope composition

Clear perspex chambers (approximate dimensions $150 \times 150 \times 100$ mm) were sealed with Terostat VI (Henkel Teroson GmbH, Heidelberg, Germany) to the trunks of three trees (at ~1.5 m height) for the collection of CO_2 emitted from trunks according to Damesin *et al*. (2005). The chambers remained fixed to the trees with a strap throughout the campaign and were sealed with airtight rubber septa only during gas sampling periods (Brandes *et al*. 2006). To thoroughly mix the air, a small circulation fan inside the chambers was activated during sampling periods, and paper-backed aluminium foil shielded chambers from direct sunlight. To measure $\delta^{13}C$ in CO_2 emitted from roots, ~10 g of mycorrhizal fine roots were excavated from the soil. After removing adhering soil particles with tweezers the roots were inserted into a Perspex chamber of $120 \times 100 \times 100$ mm. The chamber was sealed with Terostat VI; air was mixed by a fan and the chambers were darkened with aluminium foil.

The chamber design used to determine $\delta^{13}C$ in CO_2 from soil respiration followed the one described by Ekblad and Högberg (2000). Three cylindrical chambers with a removable lid and a gas tight septum were pushed into the upper soil layer of the plot (opaque PVC, ~200 mm diameter; 300 mm height) and a weight of ~2 kg was placed on top of the chamber. We selected positions on the forest floor without understorey vegetation. The lids were closed only during sampling.

CO_2 concentrations in all chamber types (trunk, soil, root chamber) were monitored with an infrared gas analyzer (Li-Cor 6400, Li-Cor Biosciences Inc., Lincoln, NE) connected to the chamber and CO_2 collecting system during the whole measurement period. At the beginning of each measurement, the air space inside the chamber was flushed for 15–20 min with N_2 from a gas cylinder at a flow rate of $150–250\,L\,h^{-1}$ until the CO_2 concentration inside the chamber dropped to $<25\,\mu mol\,mol^{-1}$. Then the system was closed, the emitted CO_2 was allowed to accumulate up to between $600–900\,\mu mol\,mol^{-1}$ (10–30 min) and two 12 mL gas samples were taken with a syringe by piercing the gas tight septa of the chambers with a hypodermic needle (Damesin *et al*. 2005). The 12-mL samples were transferred into sample vials (Exetainer, Labco Ltd, High Wycombe, UK) previously flushed with N_2 to remove CO_2 and stored until measurement. Sampling was performed once a day (between

1000 and 1400 h). At each sampling time the chamber seals were tested by exhaling around the chamber seal.

For method evaluation, $\delta^{13}C$ of respired CO_2 was determined for trunk and soil chambers by additionally applying the Keeling Plot approach on 17 March 2005 in parallel to the N_2 flushing technique described above. For this approach the chambers were not flushed with N_2 before closing. The first of five samples was withdrawn within one minute of closing the chamber. Samples (12 mL) were withdrawn from the closed chambers at $\sim$100 µmol mol^{-1} intervals and stored in Exetainers; sampling was usually completed within ten minutes of chamber closure. The $\delta^{13}C$ of the emitted CO_2 was reported as the intercept of the linear regression between $\delta^{13}C$ of the samples and the inverse of the CO_2 concentration at which the sample was collected (based on the mixing model by Keeling 1958). There was no significant difference between $\delta^{13}C$ of emitted CO_2 as determined by the two different approaches, a result consistent with previous findings by Damesin *et al*. (2005). CO_2 samples in the exetainers were measured within 7 days. CO_2 (400 µmol mol^{-1} in N_2) from a gas cylinder of known carbon isotopic signature (standard gas) was injected into exetainers on all days of the measurement campaign and stored together with the samples. There was no significant change in $\delta^{13}C$ of the standard gas within the maximum storage period.

Plant and soil respiration rates

Trunk respiration rates were measured on nine trees in chambers (volume 0.8 L) permanently attached to the rough bark. A LI-6200 gas analysis system (Li-Cor Inc.) was used to monitor the rate of CO_2 accumulation in the closed chamber. Soil respiration was measured with the static chamber technique using absorption of CO_2 by soda lime as described by Keith and Wong (2006). Chambers had a volume of 6.9 L and respiration rate was measured over 24 h.

Collection of phloem exudates, extraction of water-soluble carbon and starch

The bark pieces obtained from twigs and trunks were washed immediately after sampling with double demineralised water and placed in glass vials containing 2 mL of demineralised water as exudation solution and incubated for 5 h. Gessler *et al*. (2004) concluded that this approach is most suitable for the assessment of stable isotope composition in phloem-transported OM for trees with rough bark from which no bleeding sap can be obtained. Previous studies using the exudation technique with different tree species (Schneider *et al*. 1996) showed that contamination of phloem exudates with cellular constituents was negligible.

Fresh, frozen leaf and root samples were micro-waved for 5 min in order to stop enzymatic activities and oven-dried at 60°C. Plants were homogenised in a ball mill (Mixer Mill M301, Retsch GmbH, Haan, Germany). For the extraction of the water-soluble fraction from the leaves, 50 mg of homogenised leaf samples were incubated for 60 min at 5°C in 1.5 mL demineralised water. The extracts were heated at 100°C for 3 min to precipitate proteins and centrifuged (12 000*g* for 5 min). The supernatants were used for isotope analysis.

Starch concentration in leaves was determined using a commercially available kit for starch analysis ('Starch UV-Method' cat. no. 0 207 748; Boehringer-Mannheim/Roche R-Biopharm AG, Darmstadt, Germany). Starch in the pellets of the water-soluble extracts was solubilised in dimethylsulfoxide and HCl and hydrolysed to *D*-glucose in the presence of the enzyme amyloglucosidase. *D*-Glucose was phosphorylated to *D*-glucose-6-phosphate by ATP in the presence of hexokinase. In the presence of glucose-6-phosphate dehydrogenase, *D*-glucose-6-phosphate was oxidised by NADP to *D*-gluconate-6-phosphate with the formation of reduced NADPH. The amount of NADPH formed in the above reaction is stoichiometric to the amount of *D*-glucose formed by hydrolysis of starch. NADPH was determined by means of its light absorbance at 340 nm.

Determination of $\delta^{13}C$ in starch was performed by modifying the method described by Wanek *et al*. (2001) and Göttlicher *et al*. (2006). 100 mg of oven-dried leaves were incubated at 70°C for 30 min in 1.5 mL of a methanol/chloroform/water solution (12 : 5 : 3, v : v : v) to completely remove soluble carbohydrates. This step was repeated three times, and the samples were kept at 60°C overnight. The pellets were then incubated with 750 µL of demineralised water at 100°C for 15 min to gelatinise starch. Starch hydrolysis was performed by adding 250 µL (equivalent to 1200 U mL^{-1} demineralised water) of a solution of heat stable α-amylase from *Bacillus licheniformis* (Sigma-Aldrich, St Louis, MO). The enzyme solution was cleaned by filtration with a Vivaspin 15 regenerated cellulose membrane with a 5000 Da molecular-weight cut-off (Sartorius, Göttingen, Germany) to remove stabilisers. After cooling, the solutions were centrifuged (12 000*g* for 5 min) and 450 µL of supernatant was filtered with cleaned centrifugal ultrafilters (Vivaspin 500, regenerated cellulose membrane, 10 000 Da molecular-weight cut-off: Sartorius). The filtered samples were used for stable carbon isotope analysis.

Extraction of lipids and separation of the wax fraction

The ground plant material was accurately weighed into a cellulose thimble pre-extracted with dichloromethane (AR grade, Chemsupply) and methanol (ChromAR grade, Mallinckrodt Baker, Inc., Phillipsburg, NJ) (DCM : MeOH, 9 : 1). Dichloromethane was purified before use via distillation using a fractionating column. The top of the thimble was plugged with pre-extracted glass wool. The extraction was performed using a Soxhlet apparatus, with a mixture of dichloromethane and methanol (9 : 1, respectively). Fresh dichloromethane-methanol-solvent was introduced as required, and the extraction was allowed to proceed for at least 72 h or until the solvent was colourless. The solvent was removed using a solvent evaporator apparatus and the dried extract was analysed for $\delta^{13}C$ (bulk lipids).

To separate the waxes from the bulk lipids, 50–100 mg of the dried extract was applied to the top of a large column (20 cm long × 0.9 cm internal diamter) of activated silica gel (0.063–0.200 mm, MERCK, activated at 120°C for at least 8 h, and pre-rinsed with *n*-pentane before use) and 1 cm of activated alumina. The wax fraction was eluted with 35 mL of *n*-pentane (AR grade, APS Chemicals, Seven Hills, NSW) and the solvent reduced on a sand bath (60°C). *n*-Pentane was purified via distillation using a fractionating column

before use. The wax fraction (dissolved in *n*-pentane) was analysed by gas-chromatography-mass spectrometry (GC–MS) before gas-chromatography-isotope ratio-mass spectrometry (GC–IRMS).

Gas chromatography mass spectrometry (GC–MS)

GC–MS was performed for compound identification using an HP 5973 (Hewlett Packard, Agilent, Wilmington, Delaware) mass-selective detector (MSD) interfaced to a HP 6890 gas chromatograph (GC) fitted with a 60 m long × 0.25 mm internal diameter. WCOT fused silica capillary column coated with a 0.25 µm (5%-phenyl)-methylpolysiloxane stationary phase (HP-5 ms, Agilent, Wilmington, DE). The GC oven was programmed from 40 to 310°C at 3°C min^{-1} with initial and final hold times of 1 and 30 min, respectively. Samples (dissolved in *n*-pentane) were injected (split/splitless injector) by a HP 6890 series auto-sampler using pulsed-splitless mode. Ultra-high purity (UHP) helium (further purified using an in-line OMI Indicating Purifier, Supelco, Bellefonte, PA) was used as the carrier gas at a flow rate of 1.1 mL min^{-1} with the injector operating at constant flow. In full-scan mode, the MS was typically operating at an ionisation energy of 70 eV, a source temperature of 180°C, with an electron multiplier voltage of 1800 V and a mass range of 50 to 550 AMU.

Gas chromatography-isotope ratio mass spectrometry (GC–IRMS)

Compound specific isotope analyses (CSIA) were performed on a Micromass IsoPrime mass spectrometer interfaced to a HP 6890 gas chromatograph (GC–IRMS) fitted with the same column used for GC–MS analysis. For samples, the GC oven, carrier gas and injection conditions were identical to those described above for GC–MS analysis. For the external standards, the GC oven was programmed from 50 to 310°C at 10°C min^{-1} with initial and final hold times of 1 and 20 min, respectively.

Individual $\delta^{13}C$ values of waxes with chain lengths of 25, 27 and 29 were calculated by integration of the masses 44, 45 and 46 ion currents of the CO_2 peaks produced by combustion of the chromatographically separated compounds using copper oxide wires (4 × 0.5 mm, Isotope grade, Elemental Microanalysis Ltd, Okehampton, UK) at 850°C. The compositions are reported relative to that of reference gas pulses produced by allowing carbon dioxide (Coleman Instrument grade, BOC Gases Australia Ltd, Canning Vale, WA) of known $^{13}C/^{12}C$ content into the mass spectrometer. The $^{13}C/^{12}C$ content of the CO_2 reference gas was monitored daily using organic standards. Average values of two or three analyses (in limited cases only one) were used per compound. Isotopic compositions are given in the delta notation relative to Vienna Pee Dee Belemnite (VPDB). Since $\delta^{13}C$ of the individual wax components were similar we calculated average $\delta^{13}C$ values of the wax components from individual $\delta^{13}C$ of C_{25}, C_{27} and C_{29} *n*-alkanes.

Isotope analysis (IRMS) of total carbon, water-soluble carbon, starch, bulk lipids, phloem exudate OM and CO_2 and isotope calculations

For $\delta^{13}C$ analysis, ~100 µL of leaf and root extracts, of phloem exudates and of filtered starch samples were transferred into tin capsules (IVA Analysentechnik, Meerbusch, Germany). Water was evaporated at 60°C. A 0.5-mg sample of homogenised dried bulk leaf material and 75–100 µg of dried bulk lipid extract was transferred into tin capsules for $\delta^{13}C$ analysis. All samples contained, on average, ~400 µg organic C and were measured in continuous flow mode. The dried bulk leaf samples, extracts of leaves and roots as well as phloem exudate samples were combusted in an elemental analyser (EuroEA3028-HT, Milan, Italy) coupled to an isotope ratio mass spectrometer (IsoPrime, GV Instruments, Manchester, UK) at Creswick School of Forest and Ecosystem Science, University of Melbourne. $\delta^{13}C$ of CO_2 was determined using a TraceGas Module (GV Instruments, Manchester, UK) for cryogenic focussing and GC separation of CO_2 from N_2O which was coupled to the IsoPrime isotope ratio mass spectrometer. Bulk lipid isotope analyses were performed at Curtin University of Technology in Perth on a Micromass IsoPrime isotope ratio mass spectrometer interfaced to a EuroVector EuroEA3000 elemental analyser. Water-soluble leaf extracts were measured on an Isochrom mass spectrometer (Micromass, Manchester, UK) coupled to a Carlo Erba elemental analyzer (CE Instruments, Milan, Italy) at the Australian National University, Canberra. Starch $\delta^{13}C$ was measured using a Carlo Erba EA (CE Instruments) coupled to a Finnigan Delta plus IRMS (ThermoFischer GmbH; Bremen, Germany) at the University of Freiburg.

Carbon isotopic values are expressed in delta notation (in ‰ units), relative to VPDB. The different isotope working standards used have been inter-calibrated.

$\delta^{13}C$ of carbon released from starch ($\delta^{13}C^{rel}$) within a particular time period was calculated using the following equation (Göttlicher *et al.* 2006)

$$\delta^{13}C^{rel} = \frac{c_{t2} \cdot \delta^{13}C_{t2} - c_{t1} \cdot \delta^{13}C_{t1}}{c_{t2} - c_{t1}}, \quad (1)$$

where c_{t1} and c_{t2} are the carbon contents and $\delta^{13}C_{t1}$ and $\delta^{13}C_{t2}$ the carbon isotope composition of the starch pool at the beginning and the end of the period regarded.

Statistical analyses

All statistical analyses were performed using NCSS 2004 (Number Cruncher Statistical Software, Kaysville, UT). Analysis of variance (ANOVA) was used to determine differences between $\delta^{13}C$ in various chemical fractions or plant positions. Correlations between $\delta^{13}C$ and environmental/physiological parameters were calculated using the bivariate correlation procedure. Significance of correlation has been calculated according to Sachs (1984). Regression lines were determined by linear regression analysis.

A time delay is expected between the formation of substrates in the leaves and their subsequent presence in the phloem exudates of twigs, trunks and roots. Potential time lags between two variables (X, Y) were examined by cross-correlation procedures (Brandes *et al.* 2006). The cross correlation between X_t and Y_{t+k} is called the kth order cross correlation of X and Y. The sample estimate r_k of the cross correlation is calculated using the following equation (Newton 1996; Kendall and Ord 2006):

$$r_k = \frac{\sum_{i=1}^{n-k}(X_i - \overline{X})(Y_{i+k} - \overline{Y})}{\sqrt{\sum_{i=1}^{n}(X_i - \overline{X})^2 \sum_{i=1}^{n}(Y_i - \overline{Y})^2}}, \quad (2)$$

where $\overline{X} = \frac{1}{n}\sum_{i=1}^{n} X_i$ and $\overline{Y} = \frac{1}{n}\sum_{i=1}^{n} Y_i$. Cross correlations were calculated for (i) daily daytime mean values of variables, and hence, the time (lag) index k has the unit 'day', and for (ii) variables from single time points of the diel courses. However, in the latter case the time-differences between data were not exactly equally spaced. Although not strictly correct, we assumed five measurements performed over each 24 h to be representative for (1) morning, (2) midday, (3) afternoon, (4) first part of the night and (5) second part of the night with equal time periods in between. As a consequence, measurements from two time points on 10 March 2005 were omitted for cross correlation analysis. A time lag of e.g. k = 1, means a shift from (i) to (i + 1); i in $\{1, 2, 3, 4, 5\}$. For k = 5 the time lag is then 1 day.

Results

Meteorological variables and net ecosystem CO_2 fluxes

Air temperature dropped by 17.5°C in the 36 h before the start of the measurement campaign on 7 March 2005 and reached a minimum value of ~0.5°C (Fig. 1*A*). The temperature decrease was accompanied by 11 mm of rain. Mean daily temperature increased continuously from 9.4°C on 7 March to 20.0°C on

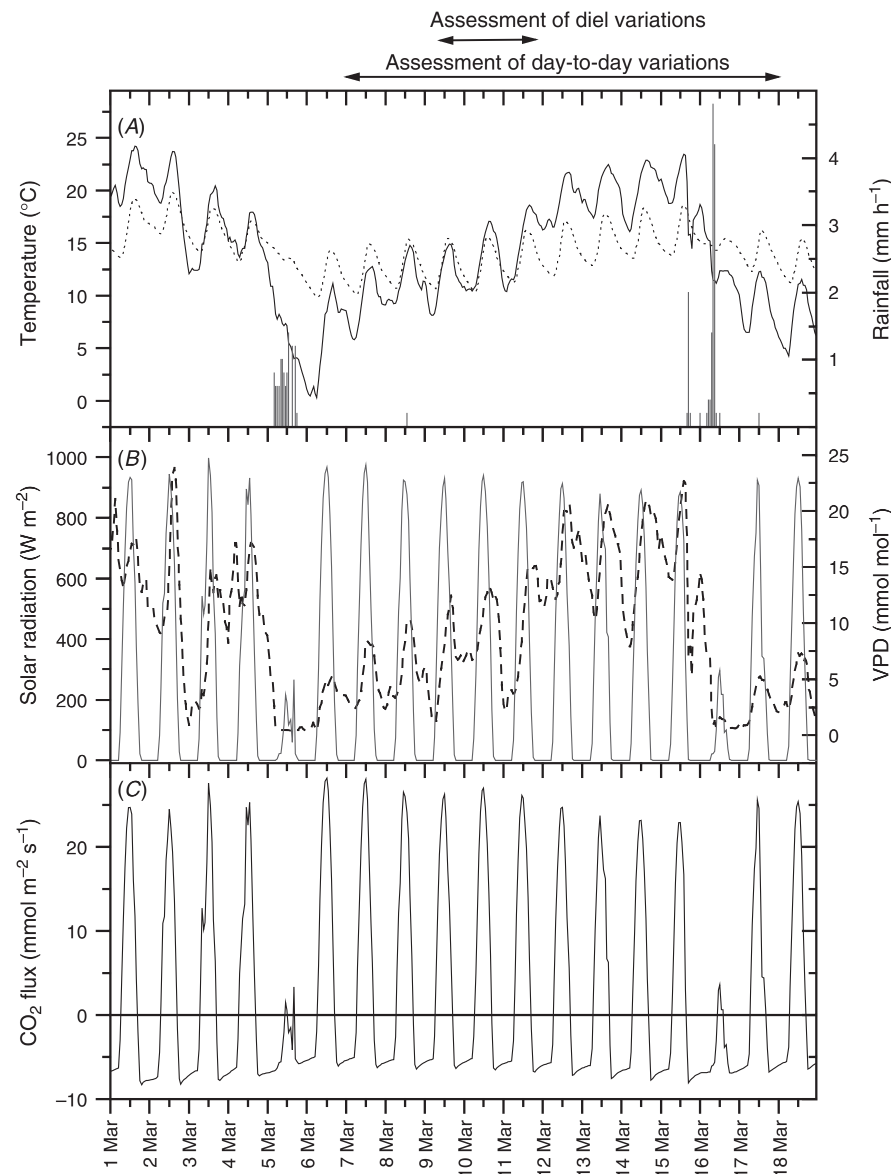

Fig. 1. (*A*) Air temperature (solid line) and soil temperature at a mean depth of 0.05 m (dotted line), hourly rainfall (bars), (*B*) solar radiation (solid line) and water pressure deficit (VPD, dotted line) and (*C*) ecosystem net CO_2 flux at the field site Tumbarumba between 1 March 2005 and 18 March 2005. The arrows at the top graph indicate the duration of the assessment of diel and day-to-day variation of carbon isotope composition. Note that positive CO_2 fluxes denote CO_2 uptake by the ecosystem contrary to conventions in atmospheric studies. The position of the tick label at the x-axis marks midnight.

14 March and decreased again on 15 March, accompanied by 18 mm of rain. The diurnal amplitude of soil temperature was less than that of the air and soil temperature exceeded air temperature during the period of rapid decrease but was lower than air temperature as it increased. Mean daily VPD increased from 5 mmol mol^{-1} on 7 March to 15 mmol mol^{-1} on 14 March and decreased to values as low as 3 mmol mol^{-1} after the second rainfall event (Fig. 1*B*). With the exception of the rainy days, maximum incoming solar radiation was between 880 and 998 W m^{-2}. The ecosystem was a net source of 0.40 mol CO_2 m^{-2} d^{-1} on 5 March and 16 March, and daily net CO_2 uptake ranged between 0.12 and 0.46 mol CO_2 m^{-2} d^{-1} on the other days (Fig. 1*C*).

Diel variations of $\delta^{13}C$

In both total and water-soluble organic carbon, there was a distinct diel variation in the carbon isotopic composition, with ^{13}C enrichment during night and depletion in the light period (Fig. 2) with peak-to-peak differences up to 2‰ in the foliar water-soluble carbon fraction. Water-soluble carbon was significantly ($P < 0.01$) enriched compared with total carbon by 0.8‰ on average. $\delta^{13}C$ of the bulk lipids showed slightly less diel

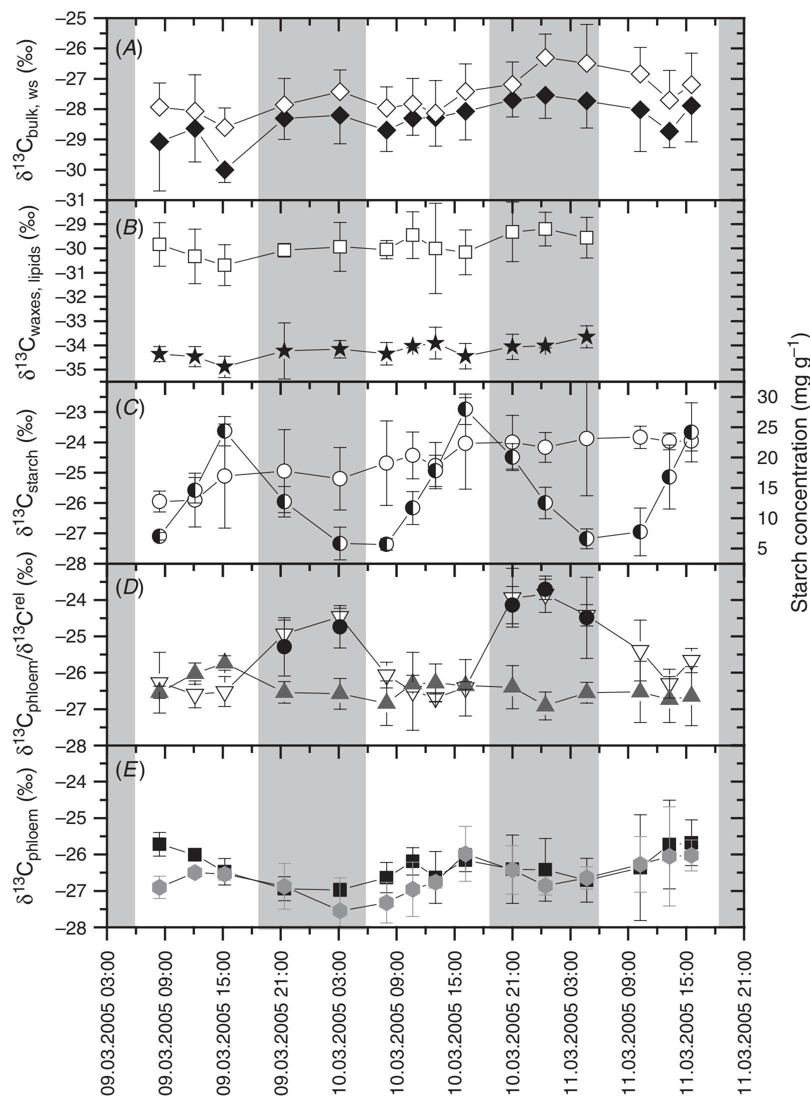

Fig. 2. Diel variations of different carbon fractions in the leaves and of phloem exudate OM. (*A*) $\delta^{13}C$ of foliage total (◆) and water-soluble (◇) OM, (*B*) $\delta^{13}C$ of bulk lipids (□) and average of the individual C_{25}, C_{27} and C_{29} waxes (★), (*C*) diel variation of the carbon isotopic composition (○) and concentration of leaf starch (◐), (*D*) $\delta^{13}C$ of phloem exudate OM from twigs (▽) and trunk top (▲) as well as $\delta^{13}C$ of the carbon fraction mobilised from starch ($\delta^{13}C^{rel}$; ●; calculated according to Eqn 1) and (*E*) $\delta^{13}C$ of phloem exudate OM from the trunk middle (⬢) and base (■). Data shown are mean values ± s.d. from the sun-exposed leaves and phloem of twigs and trunk from three different trees. The shaded fields denote the night period. Note the different scales of the *y*-axes in the different plots (*A–E*).

variation of ~1‰. The wax fraction varied the least diurnally by only ~0.8‰. $\delta^{13}C$ of bulk lipids and waxes increased slightly over 48 h similar to the water-soluble sugars of the leaves. $\delta^{13}C$ values of the water-soluble carbon fraction was significantly correlated with the bulk lipids ($R = 0.85$, $P < 0.0001$) and the wax fraction ($R = 0.75$, $P < 0.0001$), although the $\delta^{13}C$ of the bulk lipids and the wax fraction varied insignificantly over the 48-h intensive measurement period. The wax fraction was most depleted in ^{13}C followed by bulk lipids, bulk leaf, soluble sugars and starch being the most enriched. Bulk lipids were between 3.3 and 5.2‰ (on average 4.3‰) more enriched in ^{13}C than the wax fraction.

The diel variation of $\delta^{13}C$ was most pronounced in the OM from the twig phloem exudates where nocturnal maximum $\delta^{13}C$ values of −23.8‰ and diurnal (daytime) minima of −26.3‰ were observed. In the phloem exudates of the trunk, the diel patterns were less prominent (peak-to-peak amplitude of ~1.3‰) and, in contrast with the twig phloem, trunk phloem exudate OM was slightly enriched (between 0.5 and 1.5‰) during the day compared with night. In the light period, there was no significant difference in $\delta^{13}C$ among the twig phloem and the trunk phloem exudates from different positions. During the night, however, twig phloem exudate was significantly enriched in ^{13}C ($P < 0.01$). Diel variations in $\delta^{13}C$ of leaf and twig phloem exudate OM coincided with the rhythm of decrease and increase in starch concentrations observed in leaves. Starch concentration increased during the day and reached maximum values of up to 28 mg g^{-1} in the evening. During the night, concentrations dropped to ~6 mg g^{-1} before sunrise. Starch was enriched in ^{13}C compared with water-soluble carbon by between 2.0 and 3.7‰. There was a continuous increase in $\delta^{13}C$ of starch during the observation period but no distinct diel patterns were observed.

$\delta^{13}C$ of carbon released from starch during the night was between −25.3 and −23.7‰ and was, thus, comparable to phloem exudate OM during the dark period.

Day-to-day variations

In order to examine day-to-day variations in isotope signatures, diel variations were smoothed by taking daily daytime mean $\delta^{13}C$ values for leaf and phloem exudate OM, and these were related to $\delta^{13}C$ of root OM and CO_2. Fig. 3 shows that there was no clear up- or downward trend in mean daily daytime $\delta^{13}C$ of total or water-soluble OM during the observation period. Mean daytime $\delta^{13}C$ values varied between −28.2 and −29.8‰ for leaf total carbon and −27.2 and −28.2‰ for water-soluble carbon. Even though water-soluble carbon was significantly enriched by ~1‰ above total OM ($P < 0.01$), both fractions showed comparable temporal variations.

OM from the twig phloem exudate was significantly enriched compared with soluble OM from adjacent leaves by 1.6‰ on average. However, there was no significant difference in mean daily daytime $\delta^{13}C$ among different phloem positions. As for leaves, there was no consistent trend in $\delta^{13}C$ values over time. Mean daily daytime values for $\delta^{13}C$ in phloem exudate OM ranged between −25.6 and −26.8‰.

In contrast, $\delta^{13}C$ in water-soluble OM in roots increased by ~1‰ between 7 March 2005 and 17 March 2005. Day-to-day variation was similar for total and soluble OM in roots. The water-soluble fraction was on average enriched by 1.2‰ compared with total root carbon ($P < 0.05$), but was slightly depleted compared with phloem exudate OM. Comparable to root water-soluble carbon, there was a steady increase over the 11-day period in $\delta^{13}C$ of CO_2 emitted from trunks, roots and soil, with a maximum variation of 1.9, 1.7 and 1.8‰, respectively. Although there was no significant difference in carbon isotope composition of CO_2 emitted from trunk and roots, soil respired CO_2 was significantly ($P < 0.01$) enriched by up to 2‰.

Relation between $\delta^{13}C$ of different OM pools

The nocturnal $\delta^{13}C$ values of phloem exudate OM corresponded well with the calculated isotopic composition of carbon released from starch ($\delta^{13}C^{rel}$ calculated according to Eqn 1; Fig. 4). The regression line is characterised by the following linear equation:

$$\delta^{13}C_{phloem}[‰] = 0.71 \cdot \delta^{13}C^{rel}[‰] - 6.7[‰]\,(R^2 = 0.94;\ P < 0.01). \quad (3)$$

The slope of the regression line between $\delta^{13}C^{rel}$ and $\delta^{13}C$ of leaf water-soluble carbon was close to 1 (0.89‰/‰), with this carbon fraction being, however, depleted in ^{13}C by ~2.5‰.

In Fig. 2 it is apparent that $\delta^{13}C$ in leaf OM shows comparable diel patterns with twig phloem exudate OM, whereas the temporal variations in trunk phloem are not only damped but also display a time lag. To estimate the time lag between leaf water-soluble and twig phloem exudate OM on the one hand and twig and upper trunk phloem on the other hand, we applied cross-correlation analyses. The highest correlation coefficient between leaf soluble OM and twig phloem exudate $\delta^{13}C$ was achieved when no time lag was assumed (Fig. 5). When using different time lags in the analysis, the correlation coefficients became smaller and insignificant. The highest correlation coefficient for the relation between twig and upper trunk phloem exudate was obtained for k = 2, which equals ~10 h. When day-to-day variations were assessed (daily average daytime values from Fig. 3), results were comparable to the ones from the diel courses: carbon isotopic composition of both, total ($r = 0.827$; $P = 0.021$) and water-soluble OM ($r = 0.832$; $P = 0.020$) from leaves was significantly correlated with $\delta^{13}C$ of phloem exudate OM from the twig but not from the trunk when no time lag was assumed. In order to estimate potential time lags between mean daily $\delta^{13}C$ determined at different positions along the axis (leaves–phloem twigs–phloem trunk–fine roots), additional cross-correlation procedures were performed. However, the application of variable time lags (k = 1–4 days) did not lead to significant correlations (data not shown).

$\delta^{13}C$ of emitted CO_2 v. $\delta^{13}C$ of OM and environmental conditions

The carbon isotopic composition of CO_2 emitted from the base of the trunk was moderately correlated with $\delta^{13}C$ of phloem exudate OM from the same position and to trunk respiration rate (Table 1). $\delta^{13}C$ of CO_2 emitted from roots was also moderately related to the carbon isotope signature of the water-soluble OM from the root tissue. $\delta^{13}C$ of soil-emitted CO_2 was highly correlated with soil temperature, and $\delta^{13}C$ of root total carbon but there was no significant correlation with soil respiration rate

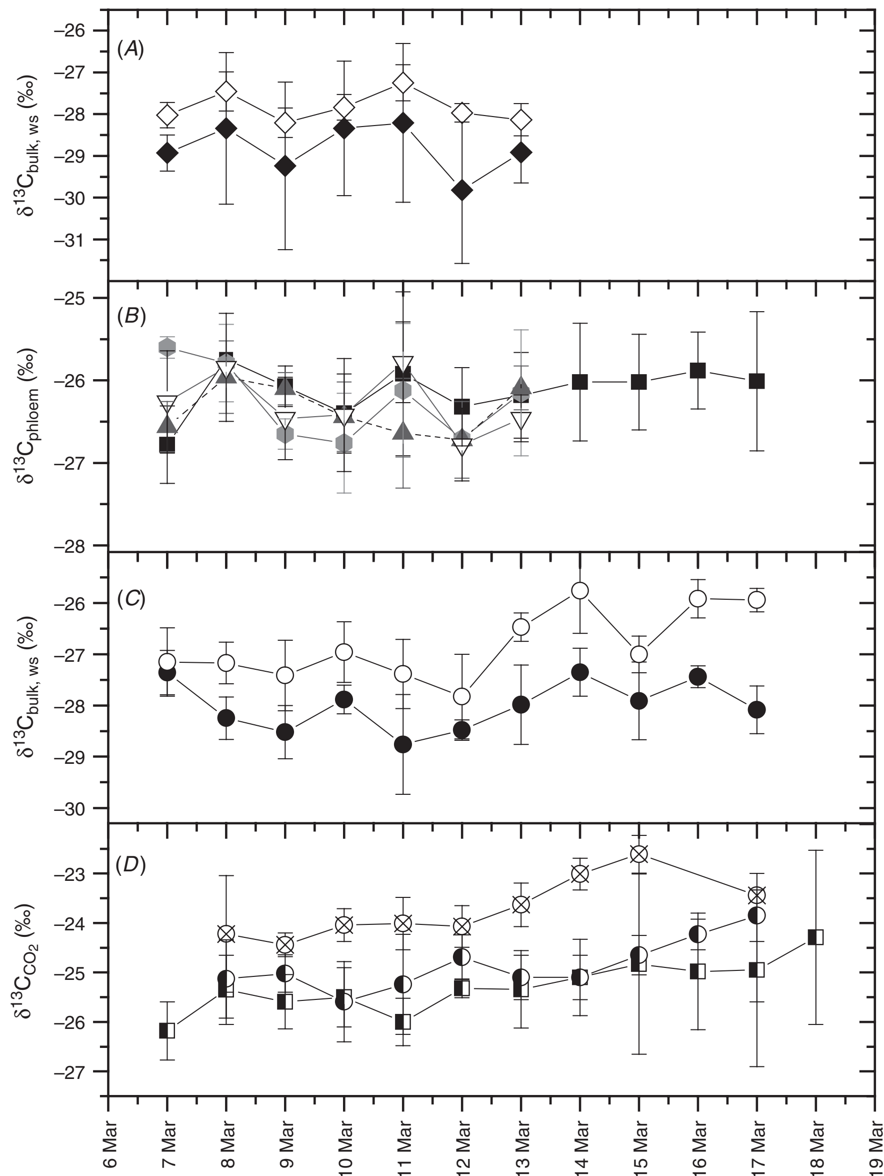

Fig. 3. Daily average daytime $\delta^{13}C$ in (*A*) leaves, (*B*) phloem exudate OM, and $\delta^{13}C$ in (*C*) root OM and (*D*) emitted CO_2. For leaves and roots the water-soluble (white symbols) and total carbon fraction (black symbols) are displayed. Phloem exudates were collected from twigs (▽) and from the three trunk positions [top (▲), middle (⬢), base (■)]. We collected CO_2 emitted at the trunk base (◧), from roots (◐) and from the soil (⊗). Data shown are mean values ± s.d. from three different trees or three different locations (root and soil emitted CO_2).

($P = 0.27$). There was a strong correlation between $\delta^{13}C$ of CO_2 emitted from trunks and from root and soil CO_2 efflux.

$\delta^{13}C$ of CO_2 (emitted from trunks, roots or soil) was not related at $P < 0.1$ to other environmental (air temperature, VPD, radiation, rainfall, ecosystem CO_2 flux) parameters.

To assess potential apparent fractionation during respiration, $\delta^{13}C$ of respired CO_2 was plotted against $\delta^{13}C$ of putative organic substrates (Fig. 6*A*, *B*). CO_2 emitted from trunks had a tendency to be enriched in ^{13}C by between 0.3 and 1.3‰ compared with phloem exudate OM obtained from the same position. This difference between potential respiratory substrate and CO_2 was more pronounced with roots and amounted to between 0.7 and 3.1‰. There was no indication that the isotopic signature of emitted CO_2 differed from trunk to roots (see also Fig. 3). Plotting $\delta^{13}C$ values of CO_2 emitted from the trunk base against values from roots resulted in points scattering around the 1 : 1 line (Fig. 6*C*).

Discussion

We assessed diel and day-to-day variation in $\delta^{13}C$ of water-soluble and total OM in leaves and roots, starch, lipids and waxes as well as of phloem-transported carbon in *E. delegatensis* in an

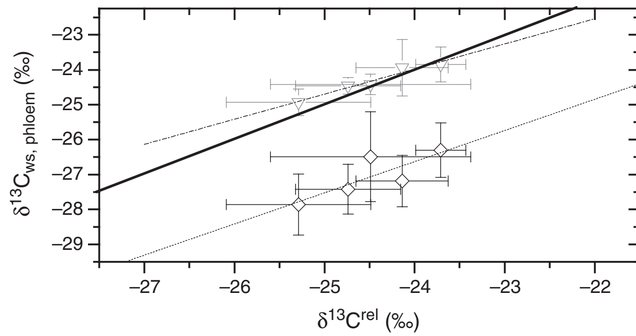

Fig. 4. Stable carbon isotopic composition of leaf water-soluble (◇) and twig phloem exudate OM (▽) as depending on $\delta^{13}C$ of carbon remobilised from starch during the night ($\delta^{13}C^{rel}$). Mean values (± s.d.) of $\delta^{13}C$ in leaf and phloem exudate OM from three trees determined at the different time points during the dark period of the diel courses (as displayed in Fig. 2) are plotted against $\delta^{13}C^{rel}$ which was calculated according to Eqn 1. The bold black line represents the 1 : 1 line.

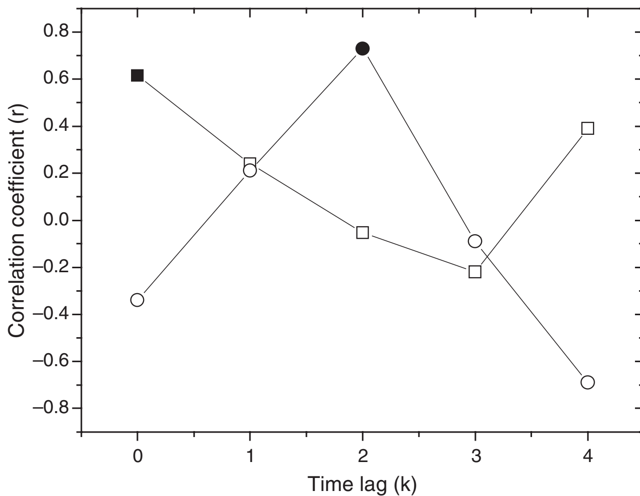

Fig. 5. Correlation coefficients for cross-correlation analysis between carbon isotopic composition of leaf soluble OM and twig phloem exudate (squares) or twig phloem exudate and phloem exudate from the upper part of the trunk (circles). Data from the assessment of diel courses of $\delta^{13}C$ (Fig. 2) were taken for analysis. We assumed five measurements performed over each 24 h to be representative for (1) morning, (2) midday, (3) afternoon, (4) first part of the night and (5) second part of the night. As a consequence measurements from two time points on 10 March 2005 were omitted for cross correlation analysis. A time lag of e.g. k = 1, means a shift from (*i*) to (*i* + 1); *i* in {1, 2, 3, 4, 5}. For k = 5 the time lag is 1 day. Black symbols denote Pearson's correlation coefficients to be significant at $P < 0.05$.

attempt to characterise temporal and spatial patterns of post-photosynthetic carbon isotope discrimination. As the amplitude of mean daily air temperature during the measurement campaign was >10°C, we were able to investigate the carbon isotope composition of fast and slow turn-over carbon pools within a wide range of meteorological conditions.

Diel variations in $\delta^{13}C$ of soluble sugars in leaves and phloem exudates and secondary metabolites

Twig phloem exudate OM, and, to a lesser extent leaf water-soluble OM and lipids, showed a clear diel variation in carbon isotope composition with highest ^{13}C enrichment during the night and strongest depletion during the day (Fig. 2). Only recently, Brandes *et al*. (2006) observed comparable day-night patterns in leaves and in the phloem exudates of twigs from the canopy of *Pinus sylvestris*. The authors attributed the ^{13}C enrichment of phloem sugars during the night to starch remobilisation, and loading of starch derived sugars into the sieve tubes as proposed by Tcherkez *et al*. (2004). Brandes *et al*. (2006) postulated that starch was enriched in ^{13}C compared with primary assimilates in pine, but had no information on the isotopic signature of starch in pine needles.

During the day, starch accumulates in the chloroplasts in leaves but the nature of its control is uncertain. The division of assimilates between starch and sucrose might be under internal control to suit the environmental conditions (especially daylength) (Zeeman *et al*. 2007). Other authors argue that transitory starch accumulates when the utilisation of newly produced triose-phosphates from the chloroplast becomes rate limiting to carbon assimilation, especially when photosynthesis is light saturated and stomatal conductance is high (Beck and Ziegler 1989). Over the measurement period in this study, the leaves of *E. delegatensis* were exposed to high light and transitory starch accumulated during day (Fig. 2*C*). The difference in carbon isotope composition between starch and cytosolic sugars has been attributed to superimposed enzyme equilibrium isotope effects on the aldolase reaction and metabolic branching of triose-phosphate fluxes (Gleixner and Schmidt 1997). Within the chloroplast, triose-phosphates can be isotopically equilibrated by the aldolase reaction. Fructose produced in the chloroplast by aldolase, which is a precursor for intra-chloroplastic starch synthesis, is ^{13}C enriched, and ^{13}C depleted triose-phosphates are exported to the cytosol during the light period (Gleixner *et al*. 1998). During the night, ^{13}C enriched transitory starch is degraded (Fig. 2) to maltose which is exported from the chloroplast (Weise *et al*. 2004). Maltose serves as substrate for the synthesis of sucrose which is, in turn,

Table 1. Correlation between daily (daytime) means of $\delta^{13}C$ of CO_2, root and phloem exudate OM, daily (daytime) average of soil temperature and trunk respiration rate

Pearson's correlation coefficients are displayed (significance levels): *, significant at $P < 0.05$. Only values with $P < 0.10$ are shown

$\delta^{13}C$ CO_2	T_{soil}	$\delta^{13}C$ phloem exudates; trunk base	$\delta^{13}C$ root total carbon	$\delta^{13}C$ root water-soluble carbon	$\delta^{13}C$ CO_2 trunk	Trunk respiration rate
Trunk	–	0.529 (0.094)	–	–	–	0.690* (0.045)
Roots	–	–	–	0.511 (0.096)	0.668* (0.034)	–
Soil	0.893* (0.001)	–	0.756* (0.018)	–	0.745* (0.021)	–

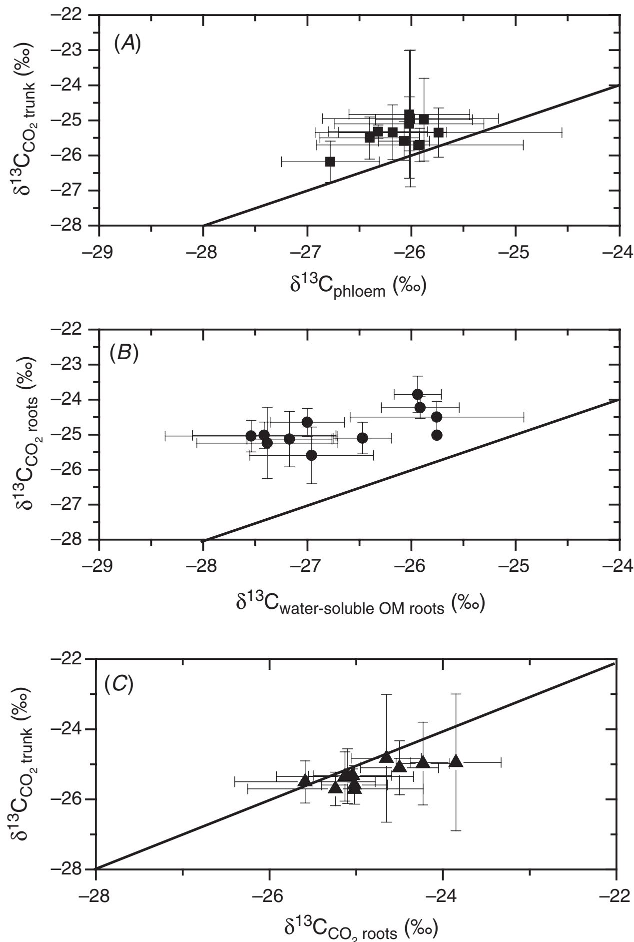

Fig. 6. (*A*) $\delta^{13}C$ of CO_2 emitted from the trunk base plotted against $\delta^{13}C$ of phloem exudate OM (trunk base), (*B*) $\delta^{13}C$ of CO_2 emitted from the roots plotted against $\delta^{13}C$ of root water-soluble OM and (*C*) the relation between $\delta^{13}C$ of CO_2 emitted from both positions. Data shown are mean values ± s.d. ($n = 3$).

loaded into the phloem. As a consequence, sucrose exported from leaves during the night is likely to carry the starch signature and, thus, to be enriched in ^{13}C by up to 4.4‰ compared with day-exported carbohydrates (Tcherkez *et al*. 2004).

In the present study we demonstrated that the isotopic composition of carbon released from starch during the night, as calculated according to Göttlicher *et al*. (2006), matched well that of phloem exudate OM (Fig. 4). Thus, our findings support hypothesis (1) and are in good agreement with the findings by Ghashghaie *et al*. (2001), who observed day-night differences in $\delta^{13}C$ of sucrose in the leaves of *Helianthus annuus* amounting to ~1‰ under controlled conditions. In their study, the carbon isotopic signature of leaf sucrose during the night was comparable to that of transitory starch. In the current study, we observed this 1 : 1 relationship only between starch and phloem exudate OM but not for leaf water-soluble carbon, which was depleted compared with carbon released from starch.

Gessler *et al*. (2004) showed that the phloem exudation technique applied here gains pure sieve tube contents which mainly consist of sugars in various tree species including the genus *Eucalyptus* (Pate *et al*. 1998). The water-soluble carbon fraction in leaves might be influenced more strongly by amino compounds and organic acids, which are supposed to differ in their carbon isotope composition from sugars released from starch (Schmidt and Gleixner 1998; Göttlicher *et al*. 2006).

$\delta^{13}C$ of leaf water soluble or phloem exudate OM during the light period did not match well with values predicted from c_i/c_a which was measured on adjacent leaves simultaneously to leaf sampling with a portable gas exchange measurement device (data not shown). Because of highly variable light conditions (in space and time) within the canopy of *E. delegatensis* we assume that the results of these short-term gas exchange measurements are not representative of c_i/c_a of the harvested leaves or larger parts of the canopy over periods of hours. We assume that the isotope signatures of leaf and phloem sugars integrate physiological traits over longer periods and are not indicative for instantaneous conditions (Barnard *et al*. 2007). We also consider that the variable canopy light conditions can influence $\delta^{13}C$ of leaf OM as even adjacent leaves might differ in longer-term radiation interception. This effect is assumed to be less pronounced in twig phloem as organic matter transported in the sieve tubes integrates over several leaves.

$\delta^{13}C$ of lipids and waxes in this study showed a slight diel variation similar to leaf water-soluble sugars but with a damped response. The bulk lipid extract contains a mixture of lipids including glycerolipids as membrane constituents (e.g. phlospholipids, galactolipids and sphingolipids), storage lipids (triacylglycerols), cuticular lipids, metabolites from the isoprenoid pathway (Ohlrogge and Browse 1995), and waxes which are esters of a long chain fatty acid with long chain acyl alcohols (von Wettstein-Knowles 1993). All lipid components are made from the isotopically light precursor acetyl CoA, and more ^{13}C fractionation occurs at the chain elongation (Collister *et al*. 1994; Lockheart *et al*. 1997); hence, the bulk lipid extract and the wax fraction were the most depleted of all compounds in this study. The average depletion of the wax fraction compared with the bulk tissue falls well within the range reported for C_3 plants (Collister *et al*. 1994). The majority of lipids is found in membranes (Ohlrogge and Browse 1995) and turnover times of membrane lipids are relatively fast (half-life $\tau_{0.5}$ = between 27 and 30 h, Cerbon and Villegas 1983). Thus, a variation in $\delta^{13}C$ of bulk lipids in accordance with new photosynthates is plausible. The diel pattern in the waxes is not significant but a minor change is evident; however, not much is known about the biosynthesis and recycling of leaf waxes throughout the growing season. Waxes are components of membranes which would be released during membrane turnover. Furthermore, waxes on leaf surfaces have shown to be constantly remodelled if weathered by wind or sun exposure and cuticular wax components are able to re-enter epidermal cells to be recycled (Lockheart *et al*. 1997) so there is perhaps a minor imprint of these processes being expressed in $\delta^{13}C$ of leaf waxes.

Leaf-to-root gradients in $\delta^{13}C$

The diel courses of $\delta^{13}C$ were most pronounced in the phloem exudates of twigs, whereas OM transported in the trunk phloem showed less variation and the maxima seemed to be phase-

delayed compared with leaf or twig phloem carbon. Cross-correlation analysis revealed a time lag of ~10 h between the diel variations in the canopy and at the upper trunk. As the distance between the two sampling points is between 8 and 10 m we have to assume phloem-transport velocities for assimilates of ~1 m h^{-1}. This finding is in good agreement with estimates for transport velocities made for adult beech (Gessler *et al.* 2004; Keitel *et al.* 2006) and Scots pine trees (Brandes *et al.* 2006). In addition, the attenuation of the diel oscillation of $\delta^{13}C$ from leaves and twigs to the trunk has also been observed with *P. sylvestris* (Brandes *et al.* 2006). The authors attributed this damping to a strong exchange between transport phloem and surrounding tissues in trunks. As a consequence, trunk phloem exudate OM is thought to consist of a mixture of carbon with different metabolic histories and different residence times within the tree.

Phloem exudate OM was enriched in ^{13}C as compared with total leaf carbon as previously observed by Cernusak *et al.* (2005) and compared with foliar water-soluble OM (Fig. 3), thus, matching the results by Brandes *et al.* (2006).

Hobbie and Werner (2004) and Cernusak *et al.* (2005) proposed that the main carbon fractionation processes responsible for the generally observed ^{13}C enrichment in trunk and root tissues (Badeck *et al.* 2005) are located in leaves and are a result of loading ^{13}C enriched sugars from the soluble carbon pool into the sieve tubes, a hypothesis supported by our data.

Various authors (Damesin and Lelarge 2003; Gessler *et al.* 2004) additionally postulated ^{13}C enrichment in heterotrophic tissues, e.g. associated with phloem transport or tissue synthesis. In contrast with Brandes *et al.* (2006), we did not observe a continuous ^{13}C enrichment in phloem exudate OM from the twigs to the trunk base. During the light period, $\delta^{13}C$ values of phloem exudate OM from all positions were comparable (Figs 2, 3) but during night, phloem-transported OM from the twig was strongly ^{13}C enriched compared with trunk phloem (Fig. 2). $\delta^{13}C$ of OM exhibits strong intra-canopy gradients due to variations in light intensity and hydraulic resistance (Leavitt and Long 1986; Warren and Adams 2000). As carbon transported in sieve tubes from the trunk integrates over the whole canopy it is highly plausible that when $\delta^{13}C$ of trunk phloem exudate is averaged over the whole diel cycle, it is depleted compared with carbon from the twig phloem collected from the upper third of the canopy where leaves were fully sun-exposed.

Along the trunk we observed a more or less constant carbon isotope composition of phloem exudate OM, as previously observed by Pate and Arthur (1998) for *E. globulus*. We, thus, have to reject our second hypothesis and conclude that the proposed mechanism of sugar export and retrieval does not affect carbon isotope composition during phloem transport. It remains to be clarified why such enrichment in ^{13}C of phloem-transported sugars is observed with some species and not with others and which processes (e.g. re-fixation of respired CO_2 by PEPC) are involved.

Root total, and less clearly, water-soluble OM were generally depleted compared with trunk phloem-transported OM, which is supposed to be the main carbon source for root tissue. Cernusak *et al.* (2005) and Pate and Arthur (1998) obtained differing results when comparing phloem-allocated OM with sink tissue total carbon: In *E. globulus*, newly formed wood dry matter did not differ in carbon isotope composition from phloem-transported organic carbon. However, mature xylem tissue was ^{13}C depleted compared with both, phloem and newly formed xylem. The authors attributed that observation to lignification during xylem maturation, as lignin is known to be depleted in ^{13}C compared with cellulose (Wilson and Grinsted 1977). Thus, it is obvious that the chemical composition of a particular sink tissue can be decisive for the carbon isotope composition of the bulk material and might also be responsible for the difference in $\delta^{13}C$ between phloem exudate and root OM observed here.

Stable carbon isotope composition of respired CO_2

Fractionation processes during root respiration may also contribute to differences in $\delta^{13}C$ between source (e.g. phloem) and sink (e.g. root) organs. Duranceau *et al.* (1999), Ghashghaie *et al.* (2001), Tcherkez *et al.* (2003), Xu *et al.* (2004), Ocheltree and Marshall (2004) and Klumpp *et al.* (2005) observed ^{13}C enrichment in respired CO_2 compared with its organic substrates, a finding that could explain the ^{13}C depletion of total root OM compared with phloem exudate as observed in this study. However, the experiments described by the authors cited above were performed with autotrophic (leaf) tissues. In contrast, Badeck *et al.* (2005) observed no change or even ^{13}C depletion of CO_2 released from heterotrophic (stem and root) tissues. Klumpp *et al.* (2005) showed that root respired CO_2 was ^{13}C depleted compared with the putative organic substrate for respiration in three herbaceous species.

There is, however, some contrasting information for heterotrophic tissues of adult trees under field conditions. In both *Fagus sylvatica* (Damesin and Lelarge 2003) and *P. sylvestris* (Brandes *et al.* 2006) trunk emitted CO_2 was ^{13}C enriched compared with the putative organic carbon source for respiration. These results are in agreement with our observations, since trunk and root emitted CO_2 was enriched by up to 1.3 and 3.1‰, respectively, above the water-soluble OM (Fig. 6).

In general, the difference in the isotopic composition between the organic source and the product of respiration is likely to originate from the fragmentation of the substrate molecule with heterogeneous isotope distribution (Tcherkez *et al.* 2003), referred to as fragmentation fractionation (Tcherkez *et al.* 2004). CO_2 emitted from decarboxylation of pyruvate catalysed by the enzyme pyruvate dehydrogenase is ^{13}C enriched, whereas CO_2 emitted from the reactions in the Krebs cycle is ^{13}C depleted compared with glucose or fructose as the putative substrates for respiration (Tcherkez *et al.* 2003). Variations in the contribution of these different processes to total CO_2 emission might help to explain part of the differences in apparent ^{13}C fractionation in heterotrophic tissues between herbaceous and woody species. Both Badeck *et al.* (2005) and Klumpp *et al.* (2005) also assumed re-fixation of respired CO_2 by PEPC to be responsible for the observed apparent ^{13}C discrimination in roots during respiration. It is possible that the mycorrhizal roots of *E. delegatensis* showed less PEPC activity than the ones from herbaceous species, which again could explain the observed differences. In order to understand the differences in respiratory ^{13}C discrimination between trees and herbaceous species, mechanistic approaches, which include the assessment of effects of mycorrhizal associations are necessary.

The difference in the apparent respiratory fractionation between trunk and roots observed here may also be due to the fact that phloem exudate OM mainly consists of sugars, which are the direct substrates for respiration, whereas the water-soluble pool in roots is made up of a more heterogeneous mixture. In the root tissue, $\delta^{13}C$ of the direct respiratory substrates (i.e. sugars) might differ from the carbon isotope composition of the mixture. The close relation between $\delta^{13}C$ of trunk and root-emitted CO_2 also points in that direction.

We observed a positive correlation between $\delta^{13}C$ of CO_2 emitted from soil and trunks with temperature and respiration rate, respectively. This is in contrast to observations made for leaves of *Phaseolus vulgaris* (Tcherkez *et al*. 2003) and trunks of *Quercus petraea* (Maunoury *et al.* 2007). These authors assumed the temperature effect to be caused by a change in the respiratory substrate. In our study, however, $\delta^{13}C$ of CO_2 was correlated with phloem exudate (trunk-emitted CO_2) or root (soil-emitted CO_2) OM so that there is no reason to assume a shift from one organic substrate to another.

We found no indication that CO_2 emitted from different tree tissues (roots, trunks) differed in carbon isotope composition (Fig. 6*C*), whereas soil emitted CO_2 was enriched by up to 2‰. Since there was a strong interrelation between $\delta^{13}C$ of CO_2 emitted from the tree trunks and from the soil as well as between $\delta^{13}C$ of root total carbon and soil emitted CO_2 (Table 1) we assume a significant contribution of autotrophic (root) respiration to soil emitted CO_2 as previously observed by Bhupinderpal *et al*. (2003) and Knohl *et al*. (2005). The difference between the absolute values of $\delta^{13}C$ of CO_2 from soil and root respiration may be attributed to (i) different carbon isotope signature of the substrate for heterotrophic respiration or (ii) different carbon isotope fractionation during heterotrophic respiration.

The differences in $\delta^{13}C$ of CO_2 emitted from different ecosystem compartments (soil *v.* tree tissues) as observed here may potentially violate the assumption of mixing of only two components (ecosystem respired CO_2 and atmospheric background CO_2) which is a prerequisite for the partitioning of respiratory from photosynthetic CO_2 fluxes with the help of stable isotopes (for a review see Pataki *et al*. 2003). In addition, there are indications that $\delta^{13}C$ of CO_2 emitted from plant tissues (Prater *et al*. 2006) and whole ecosystems (Bowling *et al*. 2003) varies during the diel course due to changes in respiratory carbon isotope fractionation (Hymus *et al*. 2005) or to variations in $\delta^{13}C$ of respiratory substrates (Brandes *et al*. 2006), or both, with the time of the day. Thus, we have to consider that the difference in carbon isotope composition between soil and autotrophic respiration might better be considered as a 'snap shot' for the time of the day (1000–1400 h) which was examined here and might vary during the diel course.

Conclusions

Supporting hypothesis (1), our present results strongly indicate that the patterns of transitory starch accumulation and remobilisation govern the diel rhythm of $\delta^{13}C$ in short-term turn-over pools of organic matter in leaves and in phloem-transported OM and that this diel signal is attenuated during basipetal transport. Diel variations in $\delta^{13}C$ are still slightly visible in longer-term products such as leaf waxes. We could not confirm hypothesis (2) as there was no significant ^{13}C enrichment of phloem transported carbon in basipetal direction. We have evidence that respired CO_2 is enriched in ^{13}C compared with the fast turn-over OM pool not only when emitted from autotrophic tissues or stems as observed before and as expressed in hypothesis (3) but also when respired from roots of *E. delegatensis*, possibly due to fragmentation fractionation during respiration.

We conclude that it should be considered that photosynthetic carbon isotope fractionation can be superimposed by post-photosynthetic discrimination when using carbon isotope signature of CO_2 for partitioning of photosynthetic and respiratory fluxes on the ecosystem level or when assessing $\delta^{13}C$ in phloem-transported carbon for integrating the influence of environmental factors on plant performance.

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References

Badeck FW, Tcherkez G, Nogues S, Piel C, Ghashghaie J (2005) Post-photosynthetic fractionation of stable carbon isotopes between plant organs – a widespread phenomenon. *Rapid Communications in Mass Spectrometry* **19**, 1381–1391. doi: 10.1002/rcm.1912

Barnard R, Salmon Y, Kodama N, Weston C, Sörgel K, Rost J, Werner RA, Gessler A, Buchmann N (2007) Diel variation of oxygen isotope enrichment in leaf water and different organic matter pools in *Pinus sylvestris* is controlled by environmental and physiological factors. *Plant, Cell & Environment* **30**, 539–550. doi: 10.1111/j.1365-3040.2007.01654.x

Beck E, Ziegler P (1989) Biosynthesis and degradation of starch in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 95–117. doi: 10.1146/annurev.pp.40.060189.000523

Bhupinderpal S, Nordgren A, Lofvenius MO, Hogberg MN, Mellander PE, Hogberg P (2003) Tree root and soil heterotrophic respiration as revealed by girdling of boreal Scots pine forest: extending observations beyond the first year. *Plant, Cell & Environment* **26**, 1287–1296. doi: 10.1046/j.1365-3040.2003.01053.x

Bowling DR, Sargent SD, Tanner BD, Ehleringer JR (2003) Tunable diode laser absorption spectroscopy for stable isotope studies of ecosystem-atmosphere CO_2 exchange. *Agricultural and Forest Meteorology* **118**, 1–19. doi: 10.1016/S0168-1923(03)00074-1

Brandes E, Kodama N, Whittaker K, Weston C, Rennenberg H, Keitel C, Adams MA, Gessler A (2006) Short-term variation in the isotopic composition of organic matter allocated from the leaves to the stem of *Pinus sylvestris* – effects of photosynthetic and post-photosynthetic carbon isotope fractionation. *Global Change Biology* **12**, 1922–1939. doi: 10.1111/j.1365-2486.2006.01205.x

Cerbon J, Villegas T (1983) Lipid composition and turnover in heterotrophic cell suspension cultures of *Saccharum officinarum*. *Plant Cell, Tissue and Organ Culture* **2**, 317–326. doi: 10.1007/BF00039878

Cernusak LA, Farquhar GD, Pate JS (2005) Environmental and physiological controls over oxygen and carbon isotope composition of Tasmanian blue gum, *Eucalyptus globulus*. *Tree Physiology* **25**, 129–146.

Collister JW, Rieley G, Stern B, Eglinton G, Fry B (1994) Compound-specific $\delta^{13}C$ analyses of leaf lipids from plants with differing carbon dioxide metabolisms. *Organic Geochemistry* **21**, 619–627. doi: 10.1016/0146-6380(94)90008-6

Damesin C, Lelarge C (2003) Carbon isotope composition of current-year shoots from *Fagus sylvatica* in relation to growth, respiration and use of reserves. *Plant, Cell & Environment* **26**, 207–219. doi: 10.1046/j.1365-3040.2003.00951.x

Damesin C, Barbaroux C, Berveiller D, Lelarge C, Chaves M, Maguas C, Maia R, Pontailler JY (2005) The carbon isotope composition of CO_2 respired by trunks: comparison of four sampling methods. *Rapid Communications in Mass Spectrometry* **19**, 369–374. doi: 10.1002/rcm.1799

Duranceau M, Ghashghaie J, Badeck F, Deleens E, Cornic G (1999) $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf carbohydrates in *Phaseolus vulgaris* L. under progressive drought. *Plant, Cell & Environment* **22**, 515–523. doi: 10.1046/j.1365-3040.1999.00420.x

Ekblad A, Högberg P (2000) Analysis of $\delta^{13}C$ of CO_2 distinguishes between microbial respiration of added C_4-sucrose and other soil respiration in a C_3-ecosystem. *Plant and Soil* **219**, 197–209. doi: 10.1023/A:1004732430929

Farquhar GD, O' Leary MH, Berry JA (1982) On the relationship between carbon isotope discrimination and the inter-cellular carbon-dioxide concentration in leaves. *Australian Journal of Plant Physiology* **9**, 121–137.

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 503–537. doi: 10.1146/annurev.pp.40.060189.002443

Gessler A, Schrempp S, Matzarakis A, Mayer H, Rennenberg H, Adams MA (2001) Radiation modifies the effect of water availability on the carbon isotope composition of beech (*Fagus sylvatica*). *New Phytologist* **150**, 653–664. doi: 10.1046/j.1469-8137.2001.00136.x

Gessler A, Rennenberg H, Keitel C (2004) Stable isotope composition of organic compounds transported in the phloem of European beech – evaluation of different methods of phloem sap collection and assessment of gradients in carbon isotope composition during leaf-to-stem transport. *Plant Biology* **6**, 721–729. doi: 10.1055/s-2004-830350

Ghashghaie J, Duranceau M, Badeck FW, Cornic G, Adeline MT, Deleens E (2001) $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf metabolites: comparison between *Nicotiana sylvestris* and *Helianthus annuus* under drought. *Plant, Cell & Environment* **24**, 505–515. doi: 10.1046/j.1365-3040.2001.00699.x

Ghashghaie J, Badeck FW, Lanigan G, Nogues S, Tcherkez G, Deleens E, Cornic G, Griffiths H (2003) Carbon isotope fractionation during dark respiration and photorespiration in C_3 plants. *Phytochemistry Reviews* **2**, 145–161. doi: 10.1023/B:PHYT.0000004326.00711.ca

Gleixner G, Schmidt HL (1997) Carbon isotope effects on the fructose-1,6-bisphosphate aldolase reaction, origin for non-statistical ^{13}C distributions in carbohydrates. *Journal of Biological Chemistry* **272**, 5382–5387. doi: 10.1074/jbc.272.9.5382

Gleixner G, Scrimgeour C, Schmidt HL, Viola R (1998) Stable isotope distribution in the major metabolites of source and sink organs of *Solanum tuberosum* L.: a powerful tool in the study of metabolic partitioning in intact plants. *Planta* **207**, 241–245. doi: 10.1007/s004250050479

Göttlicher S, Knohl A, Wanek W, Buchmann N, Richter A (2006) Short-term changes in carbon isotope composition of soluble carbohydrates and starch: from canopy leaves to the root system. *Rapid Communications in Mass Spectrometry* **20**, 653–660. doi: 10.1002/rcm.2352

Helle G, Schleser GH (2004) Beyond CO_2-fixation by Rubisco – an interpretation of $^{13}C/^{12}C$ variations in tree rings from novel intra-seasonal studies on broad-leaf trees. *Plant, Cell & Environment* **27**, 367–380. doi: 10.1111/j.0016-8025.2003.01159.x

Hobbie EA, Werner RA (2004) Intramolecular, compound-specific, and bulk carbon isotope patterns in C_3 and C_4 plants: a review and synthesis. *New Phytologist* **161**, 371–385. doi: 10.1111/j.1469-8137.2004.00970.x

Hymus GJ, Maseyk K, Valentini R, Yakir D (2005) Large daily variation in ^{13}C-enrichment of leaf-respired CO_2 in two *Quercus* forest canopies. *New Phytologist* **167**, 377–384. doi: 10.1111/j.1469-8137.2005.01475.x

Keeling CD (1958) The concentration and isotopic abundances of atmospheric carbon dioxide in rural areas. *Geochimica et Cosmochimica Acta* **13**, 322–334. doi: 10.1016/0016-7037(58)90033-4

Keitel C, Adams MA, Holst T, Matzarakis A, Mayer H, Rennenberg H, Gessler A (2003) Carbon and oxygen isotope composition of organic compounds in the phloem sap provides a short-term measure for stomatal conductance of European beech (*Fagus sylvatica* L.). *Plant, Cell & Environment* **26**, 1157–1168. doi: 10.1046/j.1365-3040.2003.01040.x

Keitel C, Matzarakis A, Rennenberg H, Gessler A (2006) Carbon isotope composition and oxygen isotope enrichment in phloem and total leaf organic matter of European beech (*Fagus sylvatica* L.) along a climate gradient. *Plant, Cell & Environment* **29**, 1492–1507. doi: 10.1111/j.1365-3040.2006.01520.x

Keith H, Wong SC (2006) Measurement of soil CO_2 efflux using soda lime absorption: both quantitative and reliable. *Soil Biology & Biochemistry* **38**, 1121–1131. doi: 10.1016/j.soilbio.2005.09.012

Kendall SM, Ord JK (2006) 'Time series.' (Oxford University Press: New York)

Kirschbaum MUF, Keith H, Leuning R, Cleugh HA, Jacobsen KL, van Gorsel E, Raison RJ (2007) Modelling net ecosystem carbon exchange of a temperate *Eucalyptus delegatensis* forest using multiple constraints. *Agricultural and Forest Meteorology*, in press.

Klumpp K, Schäufele R, Lötscher M, Lattanzi FA, Feneis W, Schnyder H (2005) C-isotope composition of CO_2 respired by shoots and roots: fractionation during dark respiration? *Plant, Cell & Environment* **28**, 241–250. doi: 10.1111/j.1365-3040.2004.01268.x

Knohl A, Werner RA, Brand WA, Buchmann N (2005) Short-term variations in $\delta^{13}C$ of ecosystem respiration reveals link between assimilation and respiration in a deciduous forest. *Oecologia* **142**, 70–82. doi: 10.1007/s00442-004-1702-4

Korol RL, Kirschbaum MUF, Farquhar GD, Jeffreys M (1999) Effects of water status and soil fertility on the C-isotope signature in *Pinus radiata*. *Tree Physiology* **19**, 551–562.

Lancaster J (1990) ^{13}C fractionation in carbon dioxide emitting diurnally from soils and vegetation at ten sites on the North American continent. PhD thesis, University of California, San Diego, USA.

Leavitt SW, Long A (1986) Stable-carbon isotope variability in tree foliage and wood. *Ecology* **67**, 1002–1010. doi: 10.2307/1939823

Leuning R, Judd MJ (1996) The relative merits of open- and closed-path analysers for measurement of eddy fluxes. *Global Change Biology* **2**, 241–253. doi: 10.1111/j.1365-2486.1996.tb00076.x

Leuning R, Cleugh HA, Zegelin SJ, Hughes D (2005) Carbon and water fluxes over a temperate *Eucalyptus* forest and a tropical wet/dry savanna in Australia: measurements and comparison with MODros. Inf. Serv. remote sensing estimates. *Agricultural and Forest Meteorology* **129**, 151–173. doi: 10.1016/j.agrformet.2004.12.004

Lockheart MJ, Van Bergen PF, Evershed RP (1997) Variations in the stable carbon isotope compositions of individual lipids from the leaves of modern angiosperms: implications for the study of higher land plant-derived sedimentary organic matter. *Organic Geochemistry* **26**, 137–153. doi: 10.1016/S0146-6380(96)00135-0

Madhavan S, Treichel I, O'Leary MH (1991) Effects of relative humidity on carbon isotope fractionation in plants. *Botanica Acta* **104**, 292–294.

Maunoury F, Berveiller D, Lelarge C, Pontailler J-Y, Vanbostal L, Damesin C (2007) Seasonal, daily and diurnal variations in the stable carbon isotope composition of carbon dioxide respired by tree trunks in a deciduous oak forest. *Oecologia* **151**, 268–279. doi: 10.1007/s00442-006-0592-z

McKenzie NJ, Ryan PJ (1999) Spatial prediction of soil properties using environmental correlation. *Geoderma* **89**, 67–94. doi: 10.1016/S0016-7061(98)00137-2

Mortazavi B, Chanton JP, Smith MC (2006) Influence of ^{13}C-enriched foliage respired CO_2 on δ^{13}C of ecosystem-respired CO_2. *Global Biogeochemical Cycles* **20**, GB3029. doi: 10.1029/2005GB002650

Newton HJ (1996) 'Timeslab: a time series analysis laboratory.' (Wadsworth Brooks/Cole: Pacific Grove, CA)

Ocheltree TW, Marshall JD (2004) Apparent respiratory discrimination is correlated with growth rate in the shoot apex of sunflower (*Helianthus annuus*). *Journal of Experimental Botany* **55**, 2599–2605. doi: 10.1093/jxb/erh263

Ohlrogge J, Browse J (1995) Lipid biosynthesis. *The Plant Cell* **7**, 957–970. doi: 10.1105/tpc.7.7.957

Pataki DE, Ehleringer JR, Flanagan LB, Yakir D, Bowling DR, Still CJ, Buchmann N, Kaplan JO, Berry JA (2003) The application and interpretation of Keeling plots in terrestrial carbon cycle research. *Global Biogeochemical Cycles* **17**, 1022. doi: 10.1029/2001GB001850

Pate J, Arthur D (1998) δ^{13}C analysis of phloem sap carbon: novel means of evaluating seasonal water stress and interpreting carbon isotope signatures of foliage and trunk wood of *Eucalyptus globulus*. *Oecologia* **117**, 301–311. doi: 10.1007/s004420050663

Pate J, Shedley E, Arthur D, Adams M (1998) Spatial and temporal variations in phloem sap composition of plantation-grown *Eucalyptus globulus*. *Oecologia* **117**, 312–322. doi: 10.1007/s004420050664

Prater JL, Mortazavi B, Chanton JP (2006) Diurnal variation of the δ^{13}C of pine needle respired CO_2 evolved in darkness. *Plant, Cell & Environment* **29**, 202–211. doi: 10.1111/j.1365-3040.2005.01413.x

Sachs L (1984) 'Angewandte Statistik.' (Springer-Verlag: Berlin)

Scartazza A, Mata C, Matteucci G, Yakir D, Moscatello S, Brugnoli E (2004) Comparisons of δ^{13}C of photosynthetic products and ecosystem respiratory CO_2 and their response to seasonal climate variability. *Oecologia* **140**, 340–351. doi: 10.1007/s00442-004-1588-1

Schmidt HL (2003) Fundamentals and systematics of the non-statistical distributions of isotopes in natural compounds. *Die Naturwissenschaften* **90**, 537–552. doi: 10.1007/s00114-003-0485-5

Schmidt HL, Gleixner G (1998). Carbon isotope effects on key reactions in plant metabolism and ^{13}C-patterns in natural compounds. In 'Stable isotopes – integration of biological, ecological and geochemical processes'. (Ed. H Griffiths) pp. 13–25. (Bios Scientific Publishers Ltd: Oxford)

Schneider S, Gessler A, Weber P, von Sengbusch D, Hanemann U, Rennenberg H (1996) Soluble N compounds in trees exposed to high loads of N: a comparison of spruce (*Picea abies*) and beech (*Fagus sylvatica*) grown under field conditions. *New Phytologist* **134**, 103–114. doi: 10.1111/j.1469-8137.1996.tb01150.x

Tcherkez G, Nogues S, Bleton J, Cornic G, Badeck F, Ghashghaie J (2003) Metabolic origin of carbon isotope composition of leaf dark-respired CO_2 in French bean. *Plant Physiology* **131**, 237–244. doi: 10.1104/pp.013078

Tcherkez G, Farquhar G, Badeck F, Ghashghaie J (2004) Theoretical considerations about carbon isotope distribution in glucose of C_3 plants. *Functional Plant Biology* **31**, 857–877. doi: 10.1071/FP04053

Van Bel AJE (2003) The phloem, a miracle of ingenuity. *Plant, Cell & Environment* **26**, 125–149. doi: 10.1046/j.1365-3040.2003.00963.x

van Gorsel E, Leuning R, Keith H, Cleugh HA, Hughes D, Kitchen M, Suni T, Zegelin SJ (2007) Nocturnal carbon efflux: Reconciliation of eddy covariance and chamber measurements using an alternative to the u_*-threshold filtering technique. *Tellus B*. doi: 10.1111/j.1600-0889.2007.00252.x

von Wettstein-Knowles P (1993). Waxes, cutin and suberin. In 'Lipid metabolism in plants'. (Ed. TS Moore) pp. 127–166. (CRC Press: Boca Raton, FL)

Wanek W, Heintel S, Richter A (2001) Preparation of starch and other carbon fractions from higher plant leaves for stable carbon isotope analysis. *Rapid Communications in Mass Spectrometry* **15**, 1136–1140. doi: 10.1002/rcm.353

Warren CR, Adams MA (2000) Water availability and branch length determine δ^{13}C in foliage of *Pinus pinaster*. *Tree Physiology* **20**, 637–643.

Webb EK, Pearman GI, Leuning R (1980) Correction of flux measurements for density effects due to heat and water-vapor transfer. *Quarterly Journal of the Royal Meteorological Society* **106**, 85–100. doi: 10.1002/qj.49710644707

Weise SE, Weber APM, Sharkey TD (2004) Maltose is the major form of carbon exported from the chloroplast at night. *Planta* **218**, 474–482. doi: 10.1007/s00425-003-1128-y

Wilson AT, Grinsted MJ (1977) ^{12}C-^{13}C in cellulose and lignin as palaeothermometers. *Nature* **265**, 133–135. doi: 10.1038/265133a0

Winter K, Holtum JAM, Edwards GD, O'Leary M (1982) Effect of low relative humidity on ^{13}C value in two C_3 grasses and in *Panicum milioides*, a C_3–C_4 intermediate species. *Journal of Experimental Botany* **33**, 88–91. doi: 10.1093/jxb/33.1.88

Xu CY, Lin GH, Griffin KL, Sambrotto RN (2004) Leaf respiratory CO_2 is ^{13}C-enriched relative to leaf organic components in five species of C_3 plants. *New Phytologist* **163**, 499–505. doi: 10.1111/j.1469-8137.2004.01153.x

Zeeman SC, Smith SM, Smith AM (2007) The diurnal metabolism of leaf starch. *The Biochemical Journal* **401**, 13–28. doi: 10.1042/BJ20061393

Ziegler H (1979) Carbon and hydrogen isotope discrimination – correlations with photosynthetic pathway and environment. *Berichte der Deutschen Botanischen Gesellschaft* **92**, 169–184.

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