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Short-term natural $\delta^{13}C$ variations in pools and fluxes in a beech forest: the transfer of isotopic signal from recent photosynthates to soil respired CO_2

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Abstract

The fate of photosynthetic products within the plant-soil continuum determines how long the reduced carbon resides within the ecosystem and when it returns back to the atmosphere in the form of respiratory CO_2. We have tested the possibility of measuring natural variation in $\delta^{13}C$ to disentangle potential times needed to transfer carbohydrates produced by photosynthesis down to roots and, in general, to belowground up to its further release in the form of soil respiration into the atmosphere in a beech (*Fagus sylvatica*) forest. For these purposes we have measured the variation in stable carbon and oxygen isotope compositions in plant material and in soil respired CO_2 every three hours for three consequent days. Possible steps and different signs of post-photosynthetic fractionation during carbon translocation were also identified. A 12 h-periodicity was observed for variation in $\delta^{13}C$ in soluble sugars in the top crown leaves and it can be explained by starch day/night dynamics in synthesis and breakdown and by stomatal limitations under elevated vapour pressure deficits. Photosynthetic products were transported down the trunk and mixed with older carbon pools, therefore causing the dampening of the $\delta^{13}C$ signal variation. The strongest periodicity of 24 h was found in $\delta^{13}C$ in soil respiration indicating changes in root contribution to the total CO_2 efflux. Nevertheless, it was possible to identify the speed of carbon translocation through the plant-soil continuum. A period of 24 h was needed to transfer the C assimilated by photosynthesis from the top crown leaves to the tree trunk at breast height and additional 3 h for further respiration of that C by roots and soil microorganisms and its to subsequent diffusion back to the atmosphere.

1 Introduction

Tracing the fate of carbon (C) within a plant-soil continuum is relevant for understanding the C source-sink relationships and the ecosystem C budget. External and internal factors affect C allocation patterns. C assimilated by photosynthesis is transported toward

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and reside in different sink organs depending on vegetative stage, environmental conditions and other factors (Gavrichkova and Kuzyakov, 2010; Brueggermann et al., 2011). Thus the time needed for the assimilated C to return back to the atmosphere as respiratory CO_2 may vary widely. Among various methods to study the time-lag between C assimilation and respiration, one of the most commonly used is the pulse labeling of plants in artificial atmosphere ($^{14}CO_2$ or $^{13}CO_2$). However, up to now this technique has been mainly applied to stands not higher than 8 m due to complicated and expensive experimental set-up (Kuzyakov and Gavrichkova, 2010). Recently, the advantages of plant ^{13}C natural abundance and its variation in quantifying the velocity of C translocation were recognized for mature stands, as no limit of plant age and height exists for the application of this method (e.g., Bowling et al., 2002; Wingate et al., 2010). The level of naturally occurring discrimination against ^{13}C in leaves during photosynthesis depends on a number of internal and environmental factors a plant is exposed to, consequently leading to a continuous variation in $\delta^{13}C$ signature in the formed organic matter (Farquhar et al., 1982). Such variations could be detectable on the subsequent stages of C translocation, therefore acting as a natural labeling. To trace the fate of C, the analyses of the time series of $\delta^{13}C$ signature in the interested pools and fluxes is performed, together with the environmental variables potentially responsible for the observed $\delta^{13}C$ changes (e.g., Kodama et al., 2008; Wingate et al., 2010). However, the way the method has been often implemented in previous studies, does not permit to completely solve the issue of time-lags in C translocation. Firstly, many studies were focused on the seasonal variation of $\delta^{13}C$ in ecosystem respiration (Bowling et al., 2002; McDowell et al., 2004; Knohl et al., 2005). Ecosystem respiration (R_E) integrates two major sources of CO_2 with different $\delta^{13}C$ signatures: aboveground and belowground respiration, the relative importance of which varies between different ecosystems and changes during the growing season within the same stand (Law et al., 1999; Mortazavi et al., 2005; Unger et al., 2010). These two main contributors of C are separated by the phloem path through which the C have to be transported. They therefore differ in the timing of evolution of CO_2 with a certain isotopic signal determined by particular leaf

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level photosynthetic discrimination. Consequently, the time lags between CO_2 assimilation and R_E calculated in such experiments are hardly comparable because often the C pool mostly contributing to ecosystem respiratory fluxes, and therefore responsible for the measured $\delta^{13}C$ values, is not known. Secondly, some recent studies suggest that the residence time of C fixed within a specific pool vary during the growing season (Plain et al., 2009; Wingate et al., 2010) and, therefore, the lag time elapsed between its assimilation and its return to the atmosphere by respiratory process is also variable. Low frequency measurements of $\delta^{13}C$ signature in respiration fluxes (both, ecosystem and soil respiration) are not suitable to cover this variability in C residence time and therefore this often results in the absence of correlation between assimilation and respiration or multiple peaks in correlation strength, due to changes in the timing of C translocation over the growing season (e.g., Bowling et al., 2002).

Frequent sampling of plant material and respiration fluxes were performed in several studies on mature tree stands in the field (Brandes et al., 2006; Gessler et al., 2007; Barnard et al., 2007; Kodama et al., 2008; Rascher et al., 2010). Diurnal variation of $\delta^{13}C$ signature in recently fixed organic matter associated with leaf level gas exchange and oscillation in starch content during the day/night cycle has been reported. These approaches gave the opportunity to trace short-term diurnal changes in sink organs and, applying time series analyses, to calculate the velocity of C translocation via the phloem. The results reported by several authors for the C translocation velocity is in the range of 0.3–1 $m\,h^{-1}$ for mature tree stands (Gessler et al., 2004; Keitel et al., 2003; Barbour et al., 2005; Barnard et al., 2007; Rascher et al., 2010). However, in some cases it was not possible to clearly identify the time of translocation or zero lags were reported (Gessler et al., 2007; Kodama et al., 2008). Evidences for post-photosynthetic isotope fractionation on different steps of C metabolism and transport have been observed. Contrasting results have been reported on isotopic fractionation during respiration in heterotrophic tissue. Fractionation favouring ^{13}C enriched substrates, leaving behind depleted components have been observed in some experiments (Cernusak et al., 2001; Brandes et al., 2006; Gessler et al., 2007; Kodama

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et al., 2008), whereas, other authors have reported evidences for an enrichment of the organic matter along the C translocation path (Damesin and Lelarge, 2003; Gessler et al., 2004, 2009; Wingate et al., 2010). Such changes in $\delta^{13}C$ during translocation of organic material are not considered for example when the isotope approach is applied for partitioning net CO_2 ecosystem exchange (e.g., Bowling et al., 2001; Knohl and Buchmann, 2005) into ecosystem respiration and gross primary production, leading to potentially biased results. Furthermore, the extent to which the initial photosynthetic isotope discrimination signal is altered by post-photosynthetic fractionation, mixing and exchange with other storage C pools along the C translocation path strongly affects the reliability of phloem sap and soil respiration $\delta^{13}C$ as tracers of short- and long-term changes in leaf level discrimination (Gessler et al., 2007; Kodama et al., 2008; Unger et al., 2010; Wingate et al., 2010).

Short-term diurnal variation of $\delta^{13}C$ in source C pools (leaves), transport pools (phloem), final sinks (shoot, bark, root) and respiration up to date were studied on conifers (*Pinus sylvestrs*: Brandes et al., 2006; Kodama et al., 2008; *Pinus Pinaster*: Rascher et al., 2010; Wingate et al., 2010) and in evergreen broadleaved trees (*Eucaliptus delegatensis:* Gessler et al., 2007; *Acacia longifolia*: Rascher et al., 2010). To our knowledge, deciduous broadleaved species remain a big plant group not covered by experimental investigations.

The present study aims (i) to assess short-term variation in $\delta^{13}C$ signature of various pools and fluxes along the C transfer route within a plant-soil system in a *Fagus sylvatica* forest; (ii) to check if the $\delta^{13}C$ changes during primary C fixation are subsequently imprinted in the successive stages of C translocation; (iii) to identify steps causing possible ^{13}C post-photosynthetic fractionation process; iv) based on the obtained results to quantify the speed of C translocation from source organs (essentially leaves) to roots and, in general to the soil and therefore, back to the atmosphere as respiratory CO_2.

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2 Materials and methods

2.1 Experimental site

The experiment was conducted at the end of august 2009 in the European beech (*Fagus sylvatica*) forest at the Long Term Ecological Research (LTER) site of Collelongo – Selva Piana (Abruzzo, Italy, 41° 50′ 58″ N, 13° 35′ 17″ E, 1550 m a.s.l.). The site is equipped with an eddy-covariance flux tower since 1993–1994. Stand density is 885 trees ha^{-1} and the average height of trees at the site is 21.5 m. Trees near to the flux tower were chosen for leaf material collection and were slightly higher (~ 24 m) than average; mean tree age at the time of sampling was around 118 years. LAI was regularly measured with LAI-2000 (Li-Cor Biosciences, USA) and in 2009, at season's peak, was $6.03 \pm 0.16\,m^2\,m^{-2}$. The soil is developed on calcareous bedrocks and is classified as humic alisol (FAO classification) with the depth varying between 40 and 100 cm. The climate at the site is Mountain-Mediterranean with an average annual temperature of 6.9 °C and average annual precipitation of 1230 mm. Meteorological measurements are taken from the scaffold tower of 26 m height and cover the whole canopy profile. Eddy-covariance water vapour and CO_2 fluxes are measured 8–10 m above the canopy with the EUROFLUX set-up (Aubinet et al., 2000).

2.2 Sampling

Plant material and soil air were collected every 3 h in the course of three days: from 20 to 22 August. Four target trees accessible from the scaffold tower were chosen for the leaf sampling. To avoid excessive disturbance of the trees near the tower, sampling of phloem exudates and soil air was performed on other representative individuals with similar morphological characteristics within the main footprint of the eddy covariance measurements.

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2.3 Extraction of leaf soluble sugars

At each sampling occasion leaves were collected at three canopy heights: from the top (∼ 24 m), middle (∼ 19 m) and base crown (∼ 15 m) layers. Leaves were immediately frozen into liquid N and conserved at −80 °C until further analyses. Each sample was divided into two subsamples. One subsample was used to extract soluble sugars for further isotopic analyses as in Brugnoli et al. (1998). Shortly, this subsample was reduced to a fine powder, suspended in demineralised water and shacked for 1 h. After 5 min of centrifugation at 12 000 g the supernatant was filtered through ion exchange columns filled with ion exchange resins (Dowex-50 and Dowex-1) to remove all the charged compounds. Extracts were freeze-dried and the remaining leaf soluble sugar fraction (LSS) was analysed for its ^{13}C signature on IRMS (Isoprime, Cheadle, UK) coupled to an elemental analyser (NA1500, Carlo Erba, Milan, Italy). The remaining leaf bulk material after LSS extraction was also freeze-dried and analysed for its isotope composition. Additionally, oxygen isotopic composition ($\delta^{18}O$) was analysed in LSS extracted from the top-canopy leaves. For these purposes LSS subsamples were placed into silver capsules and subjected to pyrolysis thermochemical decomposition on a pyrolitic unit (Euro Pyr-OH, Euro Vector Instruments and Software, Milan, Italy) coupled to the IRMS.

The second subsample of fresh leaf material was treated separately and analysed for starch and soluble sugars (glucose, fructose and sucrose) contents as in Antognozzi et al. (1996). Shortly, carbohydrate concentrations were determined enzymatically, by conversion of sugars, including also glucose originated from the starch hydrolysis, to the units of Glucose-6-Phosphate and by further spectrophotometric determination of NADH quantity in the reaction products of Glucose-6-Phosphate reduction with NAD^+ in the presence of Glucose-6P-dehydrogenase. The readings were performed in dual-wavelength mode (340–405 nm) in an Anthos 2001 plate reader (Anthos Labtec Instruments, Salzburg, Austria).

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Leaves collected from each single tree were analysed separately; the results for each individual tree were averaged for each single height layer.

2.4 Extraction of phloem soluble sugars

Sugars transported with the phloem flow were extracted by phloem exudation technique as described by Schneider et al. (1996) and Gessler et al. (2004).

In brief, pieces of bark, 1 cm in diameter, were collected from the stem breast height of four selected trees. After washing with double demineralised water the bark pieces were placed in 12 ml vials containing 2 ml of pure demineralized water and left for 5 h exudation. After centrifugation at 12 000 g for 5 min the supernatant was freeze-dried and the remaining organic material (PSS) was analysed for its $\delta^{13}C$ signature on EA-IRMS (elemental analyser coupled to an isotope ratio mass spectrometer as described above).

2.5 $\delta^{13}C$ in soil respiration

Four static opaque soil chambers were constructed (ground surface 434 cm^2, volume 7 l) for air sample collection. At the time of measurements chambers were gently placed onto the soil in the previously prepared deepening of approx. 0.5 cm deep and maintained stable for the time of measurement. Replicate gas samples of 10 ml were drawn with a syringe and stored in pre-evacuated glass flasks of the same volume for subsequent isotopic analyses. Samples were taken with a 3 min frequency from time 0 to 12 min after chamber closure. Between two subsequent samplings the chambers were left open. To prevent any possible exchange of air between the flaks and the atmosphere, the lid of each flask was sealed with hot wax. Gas samples were analysed for CO_2 concentration and isotopic composition within a week after the collection on an IRMS (Isoprime, Cheadle, UK) coupled to a Multiflow (Isoprime, Cheadle, UK). The $\delta^{13}C$ isotopic signature of the source of respiratory CO_2 (soil respiration), was calculated using the Keeling plot approach (Keeling 1958, 1961), which allows to separate

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the $\delta^{13}C$ of background atmospheric CO_2 from the $\delta^{13}C$ of CO_2 respired from the soil (root-derived and microbial-derived). Calibration curves between known CO_2 concentrations and the peak area on the mass 44 estimated by IRMS were constructed. The CO_2 concentration in the collected flasks was determined from the obtained calibration curves.

2.6 Calculations and statistical analyses

Canopy weighted $\delta^{13}CO_2$ of LSS was calculated according to Scartazza et al. (2004):

$$\delta^{13}C_{canopy} = \frac{(\delta^{13}C_t \times LAI_t \times S_t + \delta^{13}C_m \times LAI_m \times S_m + \delta^{13}C_b \times LAI_b \times S_b)}{\sum_t^b LAI_i \times S_i} \quad (1)$$

where LAI_i is the leaf area index at the respective height (t = top; m = middle, b = base), S_i is the concentration of the soluble sugars on the same height and $\delta^{13}C$ is the isotope composition of the extracted LSS.

Significance of differences between $\delta^{13}C$ values measured in various pools and fluxes was estimated applying analyses of variances (ANOVA).

Time series analysis was performed to determine the time lags between environmental and physiological variables of interest. For these purposes Pearson correlation coefficient was calculated between two respective time series and tested for the significance. The considered lags were from 0 h to 30 h with the step of 3 h.

Spectral (Fourier) analysis of STATISTICA 7 was used to determine if there is any periodical component in the diurnal course of $\delta^{13}C$ and $\delta^{18}O$ in fluxes and pools.

All statistic analyses were performed using the software STATISTICA 7 (StatSoft).

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3 Results

3.1 Meteo

At the time of sampling vapour pressure deficit (VPD) at 25 m height peaked around 14:00 LT and minimum values were registered during the night (Fig. 1a). VPD generally exceed 2 kPa in the time period from noon to 17:00 LT. The absolute maximum was 2.5 kPa and was reached between 14:00 and 15:00 LT on the second sampling day.

Maximum soil temperatures at 5 cm depth were reached in the evening around 20:00 LT with values close to 13.7 °C (Fig. 1a). Minimum temperatures were observed in the morning. Day/night soil temperature variation did not exceed 1 °C.

Diurnal variations of soil water content (SWC) at 5 cm depth were barely detectable and always not statistically significant. During the period of measurements, a slight gradual decrease in SWC was registered: from 0.24 $m^3\,m^{-3}$ at the beginning to 0.22 $m^3\,m^{-3}$ at the end of the sampling period (Fig. 1b).

Maximum values of photosynthetic active radiation (PAR) exceeded 1800 $\mu mol\,m^{-2}\,s^{-1}$; with a slight dimming due to convective clouds during the central part of the day that was observed only on the last day of sampling (Fig. 1b).

3.2 Leaves

A gradual ^{13}C enrichment of leaf soluble sugars (LSS) was observed going from the base crown to the top leaf layers (Fig. 2a). The average $\delta^{13}C$ values were −29.2‰, −27.0‰ and −25.0‰ for the base, middle and top crown leaves ($p < 0.001$), respectively.

A diurnal dynamic in $\delta^{13}C$ in LSS synthesized at the top of the crown could be observed, with different maximum levels during the night periods and midday hours and minimum (more negative) values in the morning and afternoon. Day/night variation in LSS $\delta^{13}C$ considering all samples measured in the top crown leaf layers was as high as 4.2‰ (1.4‰ variation among the means, Fig. 2).

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Day-night variations in isotopic composition were less pronounced in LSS synthesized at the middle and base crown. Especially, no night enrichment was observed while elevated $\delta^{13}C$ (more positive) could be still detected during the day hours (Fig. 2a).

Current assimilates at the top crown were on average ^{13}C enriched by 3‰ compared to total organic carbon analysed in the same leaf layer ($p < 0.001$) (Fig. 2a). As expected, diurnal variations in $\delta^{13}C$ of the bulk dry material were dampened in comparison to LSS at the respective height.

Oxygen isotopic composition in LSS sampled in the top crown over the three sampling days ranged between 34.2‰ and 37.4‰ among means (Fig. 2b). The most enriched values in ^{18}O were observed during the afternoon and night hours while the least enriched were reached in the early morning. Maximum day-time values of $\delta^{18}O$ were shifted 3 to 6 h respect to $\delta^{13}C$ peaks.

Diurnal dynamics in leaf starch content was analysed for the three crown heights (Fig. 3). The highest starch content was observed at the top crown layer and it was gradually decreasing going down through the crown.

A clear diurnal periodicity in starch content could only be seen in the top crown leaves, with the maximum starch accumulation between 14:00 and 17:00 LT and minimum values during the night and early morning hours. Such a trend was less pronounced for the middle crown leaves and completely absent in the lower crown. A fraction as high as 60% of the average daily starch content measured in the trees was formed in the top crown leaves, while approximately 30% and 10% was originating from the middle and the base crown leaves, respectively. Leaf sugars content (glucose, fructose, sucrose) followed a diurnal dynamic similar to that observed for starch (not shown), with minimum concentrations measured in the early morning and maxima in the afternoon. The contribution of each individual leaf canopy layer to the overall daily sugar productionwere calculated to be 42% in the top leaves, 31% in the middle layer, and 27% in the base crown leaves. Sucrose was the most abundant soluble sugar and accounted for 95% of the total leaf sugars extracted.

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3.3 Phloem

Phloem soluble sugars (PSS) were generally depleted in ^{13}C by 1.5‰ compared to LSS of the top sunlit leaves of the crown ($p < 0.001$) (Fig. 2b).

Similar to the top leaves, a diurnal variation in ^{13}C of PSS was noticed, but the day-night oscillation between the night maximum and day minimum were much smaller and approximately 1‰.

The characteristic $\delta^{13}C$ peaks observed during the central part of the day (11:00–14:00 LT) for top leaf sugars were also reflected in the phloem extracts, confirming its close relation to the supply of photoassimilates from the crown.

3.4 Soil

The mean R^2 for soil Keeling plots was 0.95 and the intercepts of regressions with R^2 lower than 0.9 were excluded from the analyses.

Soil CO_2 was significantly enriched in ^{13}C compared to both phloem and leaf soluble sugars during the night; comparable but still more positive $\delta^{13}C$ values of soil respired CO_2 were observed during the day (Fig. 4).

Diurnal variation in $\delta^{13}C$ of soil respired CO_2 was more evident than in soluble sugar extracted from plant material, with day – night variation of about 5.8‰ on average.

3.5 Correlations between $\delta^{13}C$ of fluxes and pools

The time required for translocation of recently fixed carbohydrates from leaves along the trunk reaching the level of breast height were estimated from the highest correlation coefficient between phloem $\delta^{13}C$ and the time-lagged values of leaf soluble sugars $\delta^{13}C$. The highest and only significant correlation was observed for a 24 h time-lag (Fig. 5a).

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The $\delta^{13}C$ of the soil respired CO_2 showed the strongest correlation with 3 h and 24 h lagged value of $\delta^{13}C$ in PSS. The time needed for C to be translocated from trunk breast height to roots and rhizosphere soil and its further back diffusion to the atmosphere as respired CO_2 was assumed to be 3 h, being the highest correlation coefficient (Fig. 5b).

This latter data was also confirmed by a high correlation coefficient between soil CO_2 $\delta^{13}C$ and the 27 h lagged $\delta^{13}C$ value of leaf soluble material (Fig. 5c), suggesting that the time needed for sugars translocation from phloem breast height to belowground and subsequent respiration is more likely 3 h, rather than 24 h.

A strong diurnal periodicity was observed in $\delta^{13}C$ of soil respired CO_2 (Fig. 6). The highest periodogram units correspond to a cycle step of 24 h. An additional periodicity was registered with a 10 h cycle, but the latter was much less pronounced. Periodical signals in $\delta^{13}C$ were completely dampened in PSS (Fig. 6) and in LSS in middle and base crow layers (not shown). LSS $\delta^{13}C$ from the top leaves had a small increase in periodogram units with 10–12 h cycle step, similar in shape to soil respired CO_2 dynamic. A 20 h periodicity was found for $\delta^{18}O$ in LSS, with a small increase also observed for a 10 h step.

4 Discussion

4.1 $\delta^{13}C$ in LSS

The daily variation in $\delta^{13}C$ observed in the LSS of top crown leaves confirms that a clear diurnal periodicity of ^{13}C signature in recently fixed organic matter exists, according to results obtained by other authors (Brandes et al., 2006; Gessler et al., 2007; Kodama et al., 2008). However, our data show a shorter cycle frequency with respect to the 24 h reported in the literature. Additionally to the common ^{13}C enrichment during the night, already observed by these authors for LSS in different species such as *Pinus sylvestris* and *Eucaliptus delegatensis* we have also observed a midday ^{13}C

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enrichment which was not in phase with the diurnal variation in starch synthesis/breakdown (Figs. 2 and 6). Remobilization and break-down of the ^{13}C-enriched starch and the consequent formation of soluble sugars (sucrose, glucose and fructose) started around midnight (Fig. 3) as confirmed by a decrease in the starch content and by a parallel increase in ^{13}C enrichment of LSS (Fig. 2). Starch enrichment is the result of non-statistical isotopic distribution within the hexose molecule (Rossmann et al., 1991; Gleixner and Schmidt, 1997). The correlation between starch content and δ^{13}C in LSS was significant ($p < 0.001$) although the R^2 was not very high.

The observed midday isotopic enrichment in LSS was probably explained by the especially dry conditions at Collelongo site with VPD values exceeding 2.0–2.5 kPa during the day. Furthermore, at this site, the wilting point derived from the water retention curve was 0.19 $m^3\,m^{-3}$ along the soil profile, a value very close to that measured during the present campaign. The measured VPD is certainly higher than in other experimental sites reported in the literature (e.g., Barbour et al., 2005; Barnard et al., 2007; Kodama et al., 2008). Although neither the photosynthetic rate nor the stomatal conductance were measured in the present experiment, it is quite likely that an increase in VPD led to partial stomatal closure and, consequently, to a midday depression of photosynthetic rates and subsequent ^{13}C enrichment of the newly synthesized carbohydrates. Nevertheless, flux data from the same dates indicate that gross photosynthesis and evapotranspiration maxima were reached in the first part of the morning and during the subsequent hours the values were generally lower. The analysis of δ^{18}O, which does not depend on Rubisco and thus on C fixation activity, but is closely linked to stomatal conductance, may clarify the observed δ^{13}C patterns (Scheidegger et al., 2000; Barbour et al., 2000; Keitel et al., 2003; Barnard et al., 2007). δ^{18}O in LSS of the top crown showed significant day-night variations (Fig. 2b). The most enriched values were registered in the afternoon, in particular on the second sampling day which in turn was associated with highest VPD. δ^{18}O peaks were shifted by about 3 to 6 h compared to similar day-time enrichment found for δ^{13}C in the same plant material. Such diurnal patterns in δ^{18}O of LSS indicates changes in stomatal conductance,

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driven by an increase in VPD, as discussed above. The ^{18}O enrichment of leaf water under such conditions is the result of the reduction in stomatal conductance and consequent decline in evaporation which is coupled to an increase in the intercellular water vapour and leaf temperature, lower Peclèt number and, in parallel, with the decrease in the atmospheric water vapour under low VPD (Farquhar and Lloyd, 1993; Barbour et al., 2000). Dynamics of $\delta^{18}O$ in the leaf water was shown to be imprinted in the $\delta^{18}O$ of the organic matter synthesized in a leaf with a certain fractionation factor (Barbour et al., 2000; Barnard et al., 2007). These authors have reported the time needed for the newly formed organic matter to reflect new isotopic conditions, induced by environmental and consequently intercellular and leaf water chemical changes, to be in the range of 3–12 h. The results of the present study also confirmed a lag in the response of $\delta^{18}O$ as compared with changes in $\delta^{13}C$ and the increase in VPD, with a time to reach the isotopic equilibrium of about 3–6 h.

The pronounced diurnal $\delta^{13}C$ cycle found in the top crown leaves was instead not observed in leaves from the base or middle crown layers (Fig. 2a). The lack of any significant day-time starch accumulation in the base crown leaves is in agreement with the absence of a night-time ^{13}C enrichment of LSS. Although middle crown leaves were accumulating starch in much lower quantities than leaves from the upper layer, diurnal differences in their starch contents were substantial (Fig. 3). Night time ^{13}C enrichment seems to be shifted to the late evening in middle crown leaves, and this shift could be a consequence of different phloem export strategies between different canopy layers. Gottlicher et al. (2006) reported similar results with maxima in sugars and starch concentrations for three crown leaf layers in a beech forest occurring in different times of the day.

The observed gradual ^{13}C enrichment of LSS from the base crown to the top is in agreement with our previous study in the same site (Scartazza et al., 2004) and with other studies both on the same species and in other tree species (Buchmann et al., 1997; Göttlicher et al., 2006). On the other hand, contrasting results were reported for *Pinus sylvestris*, where no intracanopy variations in $\delta^{13}C$ of LSS were found with height

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(Brandes et al., 2006). Differences in LAI distribution in the canopy and canopy density among different species must be taken into account. The dense canopy in beech forest influences significantly the irradiance profile within the canopy and at different leaf layers, resulting in variation of air temperature, humidity and consequently Ci/Ca ratio. Differences in canopy density can also affect the extent of CO_2 recycling within the forest with potential effects on $\delta^{13}C$, although air $\delta^{13}C$ within the canopy was found to have a minor effect on the $\delta^{13}C$ signature of LSS and leaf bulk material (Brooks et al., 1997; Buchmann et al., 2002; Scartazza et al., 2004; Göttlicher et al., 2006).

Bulk leaf material was significantly depleted in ^{13}C compared to leaf soluble sugars sampled in leaves at the same height (Fig. 2a). As previously reported, bulk leaf material include a wide number of compounds differing in turnover time, chemical composition and, thus, in $\delta^{13}C$ signature. ^{13}C depleted molecules, like lipids and lignin, can accumulate in leaves, depleting their total $\delta^{13}C$ (Brugnoli and Farquhar, 2000). Furthermore, most structural C contributing to bulk leaf material is normally assimilated during spring, under environmental conditions (high SWC, low VPD) favouring high stomatal conductance with consequent fixation of ^{13}C depleted C compounds.

A significant variation in $\delta^{13}C$ was found not only in LSS among leaves from different heights, but also among LSS from different trees sampled at the same height as shown by the relatively large error bars (Fig. 2). Diurnal $\delta^{13}C$ variation, as discussed above, with day and night peaks was observed in leaves of all trees at the respective height. However, there were significant differences among individuals in the time the peaks were reached and in the range of $\delta^{13}C$ variation, with some trees being constantly more positive in ^{13}C signature of LSS compared to others. Such differences may be related to slight changes in the radiation regime and exposition of the sampled leaves. In fact, at the top of the crown fully exposed leaves were sampled but there were slight variation in their position with respect to adjacent branches which may result in differences in the exposition time and shifts in the light curve, with consequent variations in the content of sugars and starch (Figs. 2 and 3). Similar results were reported in *Eucaliptus delegatensis*, where $\delta^{13}C$ measured in LSS sampled from certain

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leaves didn't match with values predicted from Ci/Ca measured by gas exchange on the adjacent leaves (Gessler et al., 2007).

4.2 δ^{13}C in PSS

Phloem soluble sugars (PSS) sampled at breast height were depleted in ^{13}C compared to LSS extracted from the top crown, and their δ^{13}C intermediate between that of LSS of top and middle crown leaves. These findings are partially in contrast with previous results in several species, showing that PSS are enriched compared to the leaf organic matter at either layers (Hobbie and Werner, 2004; Cernusak et al., 2005; Scartazza et al., 2004; Brandes et al., 2006; Kodama et al., 2008; Wingate et al., 2010). On the other hand, a significant progressive ^{13}C depletion in PSS downward from the top to the lower part of the trunk in comparison to LSS was reported for *Acacia longifolia* (Rascher et al., 2010). To identify the crown layer exerting the highest influence on the δ^{13}C signature of PSS we have calculated the canopy weighted δ^{13}C according to Scartazza et al. (2004). The top crown leaves, even having a lower LAI than the middle layer, contributed significantly to the whole crown δ^{13}C (not shown) because of their 2–3 fold higher sugars and starch concentrations (Fig. 3). In fact, diurnal variability in PSS δ^{13}C was similar to that of LSS δ^{13}C measured at the top crown, with night and midday enrichment. These two pools of C were significantly correlated with a 24 h time lag (Fig. 5), meaning that 24 h were needed for sugars to be transported from the crown leaves where they are assimilated to the trunk at breast height. The night enrichment, therefore, may be the result of leaf starch remobilisation and sugar transport along the trunk belowground through the phloem flow (Tcherkez et al., 2004; Gessler et al., 2007). Generally, more negative δ^{13}C of PSS could simply derive from the mixing of phloem sugars with isotopically depleted compounds formed in the lower crown layers. Some authors have measured the ^{13}C enrichment in the CO_2 respired from heterotrophic tree tissues (roots and phloem), as compared to its putative substrates, leaving behind isotopically depleted organic material (Cernusak et al., 2001; Brandes et al., 2006; Gessler et al., 2007; Kodama et al., 2008; Rascher et al., 2010).

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In the present study, $\delta^{13}C$ of the trunk respiration was not measured, but the observed $\delta^{13}C$ signature of PSS strongly suggests a fractionation favouring ^{13}C during phloem respiration. The contribution of other processes like fractionation during carbohydrate loading (Hobbie and Werner, 2004; Cernusak et al., 2005; Gessler et al., 2007), enrichment during carbohydrate transport and exchange within the heterotrophic tissues (Damesin and Lelarge, 2003; Gessler et al., 2004), refixation of the respired CO_2 by PEPC (Gessler et al., 2009; Wingate et al., 2010) was probably less relevant in determining the PSS $\delta^{13}C$. Interestingly, $\delta^{13}C$ values of the phloem extracts measured at the same site in 2001 were enriched compared to LSS (Scartazza et al., 2004). These apparently conflicting results may be explained by differences in sampling technique: Scartazza et al. (2004) was collecting PSS in the way that each sample was integrating carbohydrates transported over the whole day. This approach doesn't allow accounting for the diurnal variability in PSS $\delta^{13}C$; in addition they sampled leaves only once a day – before dusk, when LSS are actually depleted.

All mentioned processes could be also responsible for the observed dampening of the day-night variation in PSS $\delta^{13}C$ with respect to that in LSS of the top crown leaves (Fig. 6). The present results confirm previous reports showing that during carbohydrate transport from leaves to the stem their diurnal variation in $\delta^{13}C$ was actually dampened, with smaller range in stem compared to leaves (Brandes et al., 2006; Gessler et al., 2007; Kodama et al., 2008). All these authors have concluded that phloem sap is continuously exchanging C with the surrounding tissues and, therefore, it integrates organic matter synthesized in different layers of the crown with considerable contribution also from storage compounds.

4.3 $\delta^{13}C$ in soil respiration

Among all pools and fluxes analysed for $\delta^{13}C$ dynamics, soil respiration was found to exhibit the most pronounced diurnal variations and it was the most enriched in ^{13}C (Figs. 4 and 6). Such enrichment of soil respired CO_2 was also highlighted in other recent studies (Kodama et al., 2008; Marron et al., 2009; Rascher et al., 2010) and

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might find several different potential causes. The main complication in interpreting soil respiration data arises from the fact that soil cannot be treated as a single source but rather integrates different sources of CO_2 (Kuzyakov, 2006). The following soil CO_2 fluxes directly linked to recent photosynthetic activity can be identified: (i) root respiration, whose substrate is mainly represented by carbohydrates transported from aboveground via the phloem flow and (ii) microbial respiration in the rhizosphere, which is also fuelled by recent assimilates in the form of root rhizodeposits. In contrast, microbial respiration in the root-free zone does not depend on recent photosynthetic supply of C, but rather on soil organic C.

Concerning root respiration, contrasting results about the fractionation associated with respiration in plant heterotrophic tissue were reported for different species. In some non-woody plants, measurements of $\delta^{13}C$ of root respired CO_2 and of the putative respiratory substrate have indicated fractionation favouring ^{12}C, hence resulting in the ^{13}C depletion of root respired CO_2 with respect to the remaining organic matter (Klumpp et al., 2005; Boström et al., 2007; Gessler et al., 2009). Re-assimilation of the respired CO_2 by PEP carboxylase, favouring ^{13}C was proposed to decrease the $\delta^{13}C$ of the remaining CO_2 pool. However, the prevailing dry summer conditions in Mediterranean ecosystems like in this study may inhibit the activity of root and microbial PEP carboxylase, which uses the HCO_3^- dissolved as substrate (Unger et al., 2010). Additionally, most field experiments on tree species like *Fagus sylvatica* (Damesin and Lelarge, 2003), *Eucaliptus delegatensis* (Gessler et al., 2007), *Pinus sylvestris* (Brandes et al., 2006), *Pinus Pinaster and Acacia longifolia* (Rascher et al., 2010), have demonstrated that CO_2 emitted from heterotrophic tissues is ^{13}C enriched compared to its putative C substrate. In contrast, in a recent experiment in a *Pinus pinaster* forest, Wingate et al. (2010) reported opposite results.

Starch and soluble sugars extracted from roots are known to be enriched in ^{13}C compared to the same carbohydrates sampled from leaves and stem of *Fagus sylvatica* (Scartazza et al., 2004; Göttlicher et al., 2006). This observation, coupled with the likely possible fractionation during root respiration favouring ^{13}C, may at least partially

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explain the unusually positive isotopic signatures of the observed soil respired CO_2. However, $\delta^{13}C$ in soil respired CO_2 (Fig. 4) in our site reach much more positive $\delta^{13}C$ values than the range between −20‰ and −28‰ reported for soil and root respiration in the literature (Gessler et al., 2007; Kodama et al., 2008; Kuptz et al., 2010; Unger et al., 2010; Wingate et al., 2010). As also stated in a recent review on ^{13}C fractionation in the root-soil system (Werth and Kuzyakov, 2010) it should be considered that there are not enough experimental data to make any final conclusion about the extent of ^{13}C fractionation during root respiration, and other focused integrated experiments are needed.

The other two components of soil respiration are associated with microbial activity. Soil in *Eucaliptus delegatensis* forest was shown to evolve CO_2 enriched by ∼2‰ compared to trunk and root-respired CO_2 (Gessler et al., 2007). In contrast, CO_2 originating from microbial and soil respiration was found to be significantly more depleted in ^{13}C compared to root respiration in the oak-grass savannah (Unger et al., 2010). Other studies directly or indirectly confirmed this finding: the CO_2 produced during microorganisms activity differs in ^{13}C signature from the CO_2 derived from root respiration (Bhupinderpal et al., 2003; Kodama et al., 2008; Wingate et al., 2010; Werth and Kuzyakov, 2010).

The significant day-night variations in $\delta^{13}C$ of soil respiration (Fig. 4) may be explained by (i) changes in the relative contribution of microbial and root respiration to the total CO_2 efflux over the day/night cycle; (ii) changes in the substrate used for respiration by roots and microorganisms; (iii) changes in isotope fractionation during respiration in roots. The point (iii) would be associated with different relative contribution to the CO_2 formation by glycolysis, with decarboxylation of ^{13}C enriched pyruvate and by citrate cycle, with decarboxylation of ^{13}C depleted acetyl-CoA. These processes were reported for leaf and trunk respiration (Tcherkez et al., 2003; Hymus et al., 2005; Kodama et al., 2008; Kuptz et al., 2010; Unger et al., 2010). Citrate cycle contribution has been shown to be temperature-dependent, whereas pyruvate decarboxylation, being less sensitive to temperature changes, is more likely to prevail at lower temperatures

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(Atkin et al., 2000; Hymus et al., 2005). Since diurnal variations in the soil temperature were very minor in our site (Fig. 1) changes in respiratory C isotope fractionation, similar to the one observed by Kodama et al. (2008) for trunk respiration can be considered unlikely. Additionally, no temperature dependence of respiratory $\delta^{13}C$ in incubated roots of *Fagus sylvatica* was observed by Marron et al. (2009). On the contrary, gradual increase in the involvement of acetyl CoA into the secondary metabolism during the day, which in turn reduce citrate cycle contribution to respiration, may partially explain day-night $\delta^{13}C$ oscillations (Ghashgaie et al., 2003; Tcherkez et al., 2003, 2004).

Similarly, the absence of any significant day-night variations in soil temperature and SWC (Fig. 1) during the sampling period suggest that no marked changes in the availability of substrate pools with different $\delta^{13}C$ signature for microorganisms might be foreseen (Kodama et al., 2008; Marron et al., 2009).

Soil CO_2 efflux was negatively related to $\delta^{13}C$ of the evolved CO_2 (Fig. 4, $R^2 = 0.22$, $p < 0.05$). The most ^{13}C enriched air was evolved from soil during the night and was associated with lower soil respiration rates. During the day, when higher respiration was generally observed, the $\delta^{13}C$ of the respired CO_2 was more depleted. Such variations in CO_2 efflux would imply changes in the relative contribution of root and microbial respiration to the total CO_2 efflux over the diurnal course, and this may also explain the observed night-time significant isotope enrichment. Root activity has been proposed to be sensitive to recent photosynthetic status on a short-term scale (Mencuccini and Hölttä, 2010), resulting in low respiration rates when photosynthesis is declining or absent. Other authors have also proposed that changes in contribution of different sources to soil respiration over a day/night cycle may explain the diurnal ^{13}C patterns of soil respiration (Gessler et al., 2007; Kodama et al., 2008; Marron et al., 2009; Wingate et al., 2010).

Unusually positive $\delta^{13}C$ values of soil CO_2 efflux could be explained not only by variation of its biological source but also by contribution of geological processes, like dissolution of carbonates and exchange of this C with soil CO_2 (Cerling et al., 1984; Quade et al., 1989; Asano, Ph.D. thesis 2007).

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4.4 Time lags

Although, spectral analysis didn't detect any periodic signal for $\delta^{13}C$ variation in PSS (Fig. 6), this component was still significantly correlated with the 24 h lagged $\delta^{13}C$ in LSS (Fig. 5a, $R^2 = 0.52$, $p < 0.01$). It corresponds to the C translocation velocity of approximately 1 m h^{-1} from the sites of C assimilation to the tree trunk at breast height. Significant correlation between $\delta^{13}C$ in soil respiration and lagged $\delta^{13}C$ in PSS (Fig. 5b, $R^2 = 0.40$, $R < 0.01$) indicate that additional 3 h were needed to transport the photosynthates from the trunk at breast height to roots and soil, and further back into the atmosphere in the form of CO_2. The lower translocation speed (0.5 m h^{-1}) for this part of the C transfer path may be explained by the diffusion process of CO_2 from the place of its production through the soil surface to the atmosphere. In fact, it has been demonstrated that diffusion component may substantially prolong C translocation times (Stoy et al., 2007). Furthermore, the length of roots from the tree stumps is variable and hence it is difficult to assess the exact speed of C transfer from breast height to the point of CO_2 emission from roots. It should be taken into account that the experiment duration doesn't allow a reliable detection of lags longer than 1.5 days. In fact 1 m h^{-1} could be considered as relatively fast translocation in comparison to literature reported data for direct C transfer. We are aware that stable climatic conditions in the adjacent days during the sampling period resulted in similar photosynthetic and post-photosynthetic fractionation patterns and may further bring to an increase in correlation strength with leaf soluble sugars synthesized in different days. We do not exclude that velocities of C translocation observed for the lower part of the C transfer path (phloem breast height-soil respired CO_2) might be also true for the upper path section (leaves – phloem breast height).

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5 Conclusions

A strong correlation between $\delta^{13}C$ in leaf recent assimilates and other pools and fluxes along the C transport path confirm the tight correlation between the fluxes of respiration and recent photosynthetic activity. The main determinants of $\delta^{13}C$ signature in the recently fixed organic material in leaves were isotope effects during photosynthesis associated with leaf level gas exchange and starch day/night dynamic. Along C transfer, $\delta^{13}C$ imprinted in this recently fixed carbohydrates, undergo substantial variations due to post-photosynthetic fractionation on various steps of translocation and due to further mixing with other pools of C carrying different isotopic signals. All these processes complicate the application of the natural abundance of isotope for studying the fate of C within the plant-soil continuum. A significant improvement of the method will consist in the identification of the degree to which microbial respiration component overlap with root respiration $\delta^{13}C$ and in the ability to isolate these isofluxes.

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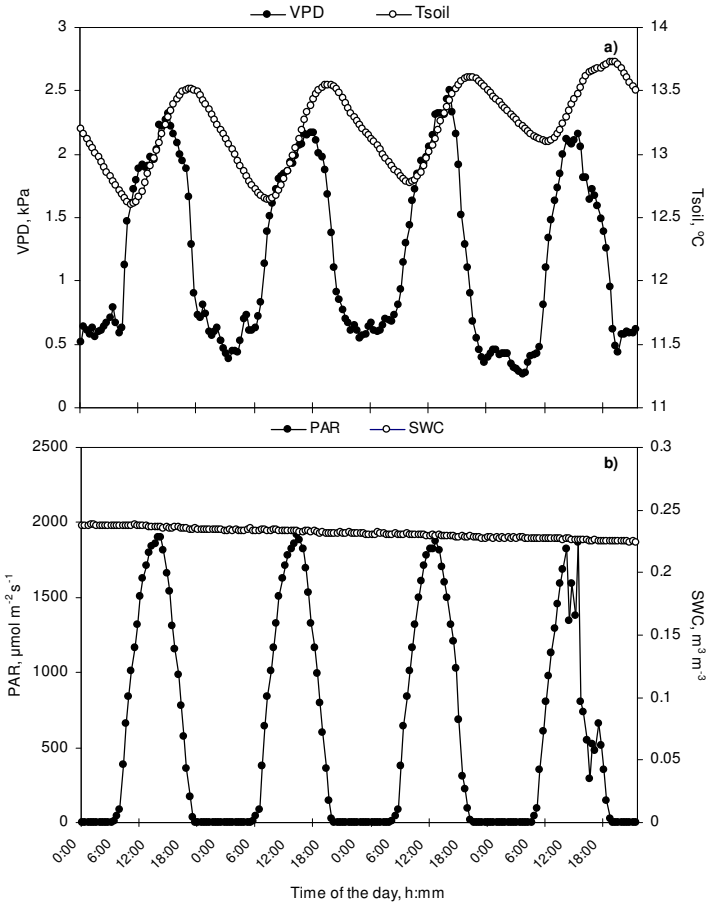

Fig. 1. Variation in **(a)** vapour pressure deficit (VPD, kPa) and soil temperature (T_{soil}, °C); **(b)** soil water content (SWC, $m^3\ m^{-3}$) and photosynthetic active radiation (PAR, $\mu mol\ m^{-2}\ s^{-1}$) measured on the day before and during three days of sampling. VPD was measured at 25 m and PAR at 27 m height, Tsoil and SWC correspond to a depth of 5 cm.

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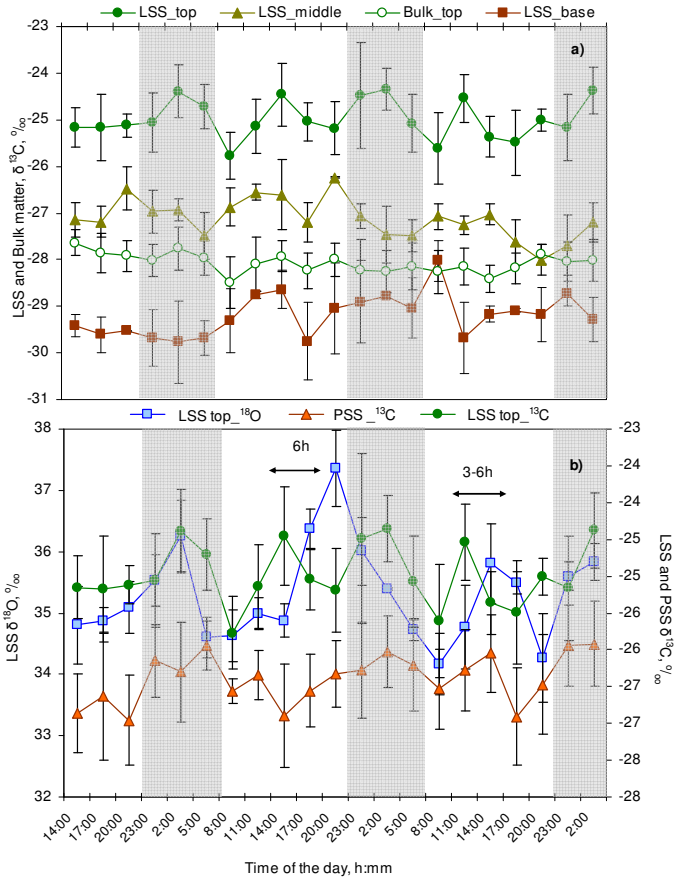

Fig. 2. (a) Dynamic of ^{13}C in leaf soluble sugars (LSS) of beech leaves sampled from the top, middle and base crown. Total ^{13}C in the bulk dry matter for the top crown leaves is also shown; **(b)** diurnal variation in $\delta^{13}C$ in soluble sugars in phloem exudates (PSS), sampled at breast height (1.3 m), $\delta^{13}C$ and $\delta^{18}O$ in LSS of the top crown leaves. White and grey hatching of the graph indicate day and night periods. Bars represent the standard error of mean ($n = 4$).

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Variations in pools and fluxes in a beech forest

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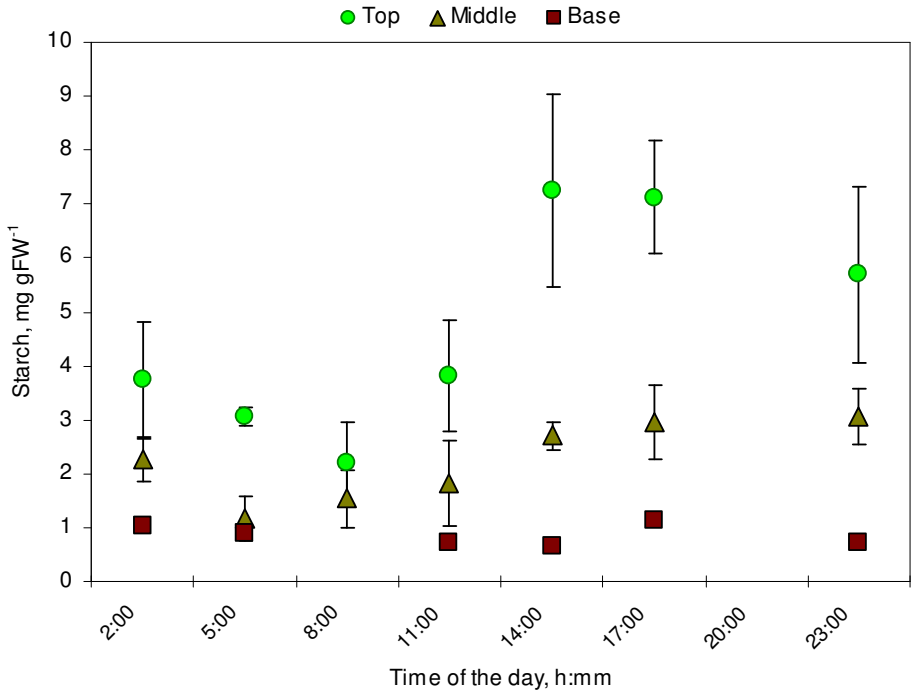

Fig. 3. 24 h time-course of starch content in the top, middle and base crown leaves of beech trees. Bars represent the standard error of mean ($n = 4$).

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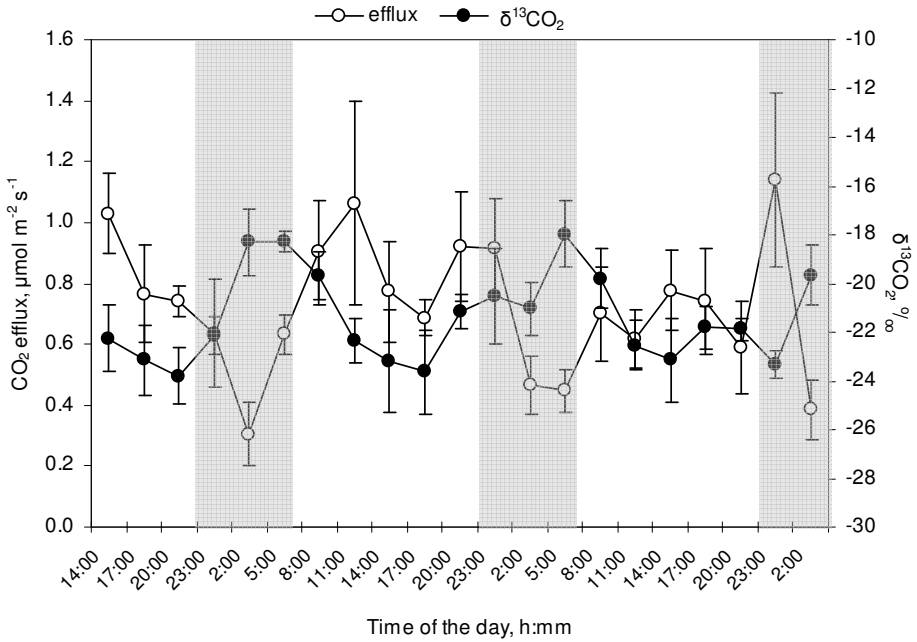

Fig. 4. ^{13}C signature of the soil CO_2 and soil respiration rates, obtained with Keeling plot technique. White and grey hatching of the graph indicate day and night periods. Bars represent the standard error of mean ($n = 4$).

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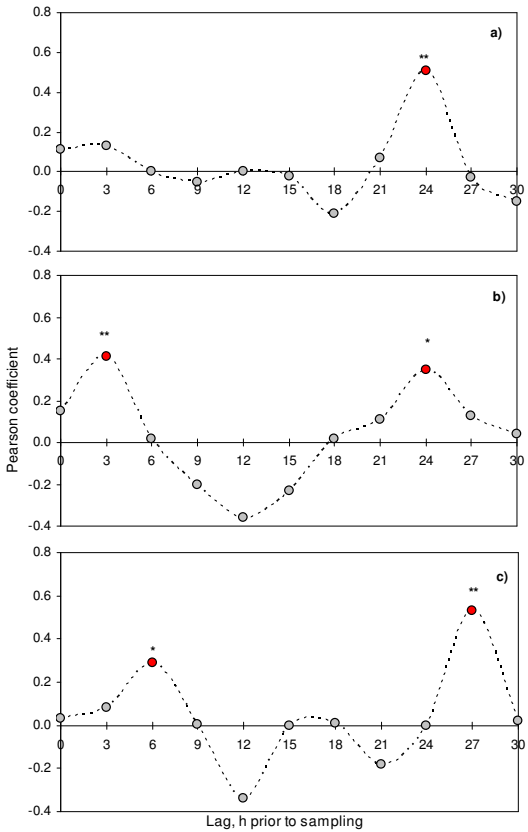

Fig. 5. Pearson correlation analyses among different plant sugar pools and respired CO_2: **(a)** lagged to different hours $\delta^{13}C$ in leaf soluble sugars (top crown) vs. $\delta^{13}C$ in phloem soluble sugars, **(b)** lagged to different hours $\delta^{13}C$ in phloem soluble sugars vs. soil respired $\delta^{13}C$, **(c)** lagged to different hours $\delta^{13}C$ in leaf soluble sugars (top crown) vs. soil respired $\delta^{13}C$. Negative values indicate negative slope of the regression line. The asterisks indicate significance level of P, * corresponding to $P < 0.05$; ** to $P < 0.01$.

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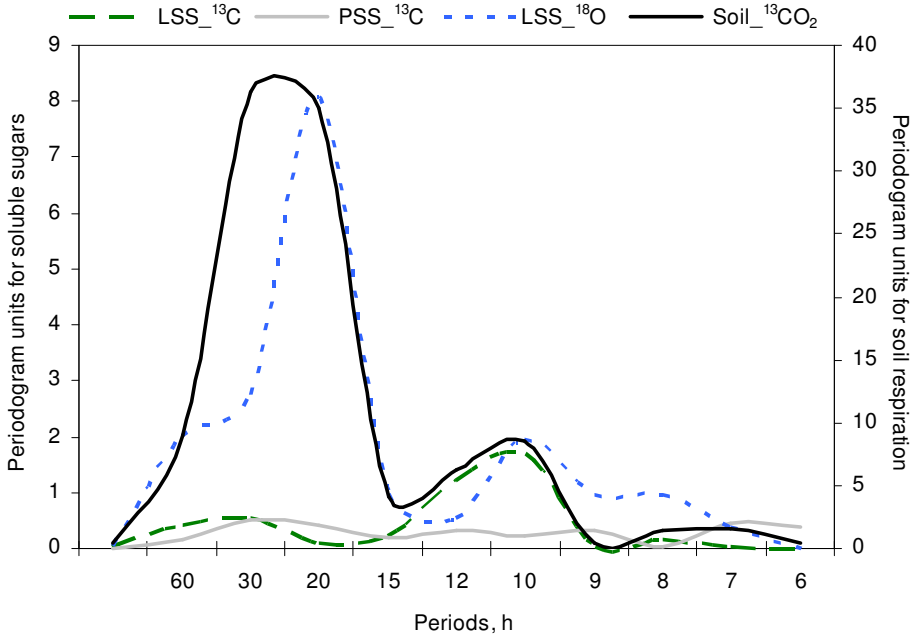

Fig. 6. Periodogram for the time series of ^{13}C and ^{18}O in recently fixed sugars in leaf and phloem, and in ^{13}C of soil respiration. Periods on the *x*-axes correspond to the number of hours necessary to complete one circle. Periodogram units on the *y*-axes are dimensionless.

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