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Experimental evidence of two mechanisms coupling leaf-level C assimilation to rhizosphere CO_2 release

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ABSTRACT

The time span needed for carbon fixed by plants to induce belowground responses of root and rhizosphere microbial metabolic processing is of high importance for quantifying the coupling between plant canopy physiology and soil biogeochemistry, but recent observations of a rapid link cannot be explained by new assimilate transport by phloem mass flow alone. We performed $^{13}CO_2$ labeling experiments designed to test if belowground respiration response to photosynthesis is faster than the arrival of new assimilates and to shed light on potential mechanisms. We provide experimental evidence that at least two mechanisms are employed by plants to couple rhizosphere respiration to canopy assimilation. We observed a fast increase of belowground respiration with the onset of photosynthesis, which we assume is induced by pressure concentration waves travelling through the phloem. A second, much later occurring, peak in respiration is fueled by new assimilates labeled with ^{13}C. Plants and the rhizosphere are thus more tightly coupled than previously thought. Ultimately, the addition of a faster assimilate delivery mechanism to our conceptual framework of ecosystem dynamics will lead to a better understanding of belowground carbon and nutrient cycling and subsequent ecosystem response to disturbance and environmental stress.

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1. Introduction

Fundamental ecosystem processes across different time-scales depend on the connectivity between the plant canopy and rhizosphere biota. These processes are wide in scope and include long term growth and defense strategies which include a range of mechanisms from foraging by roots and mycorrhizae to feedbacks that include microbial effects that impact productivity (Van Der Heijden et al., 2008; Schnitzer et al., 2010; Schnitzer and Klironomos, 2011) and plant fitness (Lau and Lennon, 2011). Over the short term, the belowground carbon sink is supplied by recently fixed assimilates that fluctuate with local climate and phenology (van Hees et al., 2005; Hogberg and Read, 2006), but the connectivity between canopy and soil may be reduced or severed due to drought events (Ruehr et al., 2009; von Rein et al., 2016) or other climate anomalies (Bahn et al., 2014). Rhizosphere respiration is a non-invasive measurement and is often used to assess the connectivity between plant canopy and belowground roots and microorganisms in the rhizosphere, the so-called speed of link (Ekblad and Hogberg, 2001; Kuzyakov and Gavrichkova, 2010; Kayler et al., 2010). The production of CO_2 from belowground signals the delivery of carbon arriving from above ground tissues to roots and rhizosphere biota setting into motion the important biogeochemical cycling and community dynamics necessary for ecosystem function. Thus, accurately quantifying the speed of link will clarify the temporal coupling between above ground canopy physiology and belowground carbon metabolism providing crucial information needed to understand the carbon source-sinks dynamics within the plant-soil carbon continuum.

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A series of reviews have shown that the response of rhizosphere respiration to shifts in canopy physiology resulting from variation in environmental conditions can range in time from on the order of hours to days (Vargas et al., 2011; Kuzyakov and Gavrichkova, 2010; Kayler et al., 2010). Explanations of mechanisms behind the respiratory response rely on the delivery of carbon to biotic sites of respiration (Savage et al., 2016). According to current theory, assimilate transport through plant phloem is considered a function of supply, provided by solute loading in the leaves, and demand, determined by the sink strength of heterotrophic tissues (i.e. the modified dynamic Münch mass flow model (Münch, 1930) reviewed by Van Bel (2003) and Knoblauch and Peters (2010)). In this theory, osmotic gradients within plants result in a pressure differential between source and sink tissues that becomes the driving force of phloem transport. However, according to this model, recent assimilates need to travel the length from sites of assimilation to respiration representing a relatively long delay that cannot account for the observed fast link between canopy and rhizosphere response (Ekblad and Hoegberg, 2001; Gavrichkova and Kuzyakov, 2016 Gavrichkova and Kuzyakov, 2016), especially for tall trees.

Recently, a second hypothesized mechanism, referred to as pressure concentration waves (Thompson, 2006; Mencuccini and Hölttä, 2010a), has been introduced, which predicts a rapid delivery of assimilates to heterotrophic tissues in the plant and organisms within the rhizosphere. These waves are a result of local disturbances in both phloem assimilate concentration and turgor, which are able to distribute changes in concentration and turgor over long distances. Pressure concentration waves enable the delivery of solutes already present in the source-sink continuum to sink tissues faster than phloem solute transport velocity according to the dynamic mass flow model.

Yet, another mechanism attributes biophysical information such as electrical signals propagated by the phloem (Fromm and Lautner, 2007) that might be responsible for the initial fast respiration response. Dark-light transitions can trigger changes in the membrane electric potential of leaf mesophyll cells (Elzenga et al., 1995; Shabala and Newman, 1999) and in roots (Wegner and Zimmermann, 1998; Shabala et al., 2009), which can lead to an apparent decrease in photosynthesis induced by electrical signals that may be partially due to increased respiration rates (Gallé et al., 2015). Moreover, it has been postulated that such light-induced electrical signals in roots play a role in modulating nutrient uptake (Shabala et al., 2009; Gallé et al., 2015).

Thus, the mechanistic framework exists to explain the speed of link, and inferential evidence also exists (Vargas et al., 2011; Kuzyakov and Gavrichkova, 2010); although some information is known about cereals (Gavrichkova and Kuzyakov, 2016), direct experimental evidence of a rapid increase in belowground respiration occurring with the onset or increase of photosynthesis that arrives before the mass flow of new assimilates, as well as detail of the underlying physiology, is notably lacking for trees. We performed a simple experiment designed to detect the different respiratory responses: 1) a slow response via the transport of new assimilates by osmotically driven mass flow (Van Bel, 2003) from the leaves to the rhizosphere, and 2) via a faster mechanism of assimilate delivery to the root-rhizosphere system. To induce a stark contrast in canopy physiology and belowground respiration, we kept juveniles of Douglas fir (*Pseudotsuga menziesii*) in the dark for 8 h during a normal day-night cycle as a control, and a 32 h and 152 h dark treatment, referred to as simply 0, 1 or 6 day- dark treatment for clarity throughout the text. The 32 h and 152 h dark treatments were also performed in order to disturb potential circadian regulation of root/rhizosphere respiration. Such internal changes in respiration might otherwise mimic fast responses to environmental drivers but are otherwise related to the anticipation of regular changes in environmental conditions. Since the circadian memory needs to be entrained, constant dark periods should strongly dampen potential internally controlled variations in respiration.

Before illuminating the leaves, the canopy and soil air-spaces were isolated from one another: the canopy was temporarily enclosed in a plastic cuvette, and labeled $^{13}CO_2$ was introduced into the canopy airspace. The experiment testing for the different coupling mechanisms commenced when the canopy was illuminated, enabling photosynthesis and the subsequent labeling of new assimilates. We hypothesized that if the rate of belowground respiration increases before the arrival of labeled assimilates (indicated by rhizosphere respiration containing labeled $^{13}CO_2$) then a different mechanism must be assumed. If this fast above-to-belowground coupling exists, then we expect that the trees in the carbon starvation treatments (1 and 6 d in the dark) will show a less intensive fast respiration peak. This is because the starvation treatment should lead to decreased sucrose concentrations in the phloem and thus the phloem sugar efflux to the roots triggered by increased root pressure would also be reduced.

2. Materials and methods

We grew 3 year old Douglas fir juveniles in a climate chamber set at 19 °C with a 16 h light/8 h dark period. The trees were periodically watered to field capacity to prevent soil desiccation. A total of four trees (n = 4) were used for each treatment: control (0 d) and dark periods (1, 6 d). Trees were measured for height, and foliage was sampled for isotopic analyses before they were placed individually in a different climate chamber used for labeling. Temperature in the labeling climate chamber was programmed at 19 °C with a maximum light level of 730 μmol m^2 s^1.

To determine belowground gas exchange, we enclosed the whole root-soil system in a soil gas exchange chamber. As a carrier gas we used ambient air drawn from outside the building by an oil-free compressor and regulated the flow through the soil chambers via a mass flow controller (Fig. S1). The two air streams (one originating from the chamber, the other from ambient air) were plumbed into a valve sequencer. A separate pump drew the air from the flow of air passing through the valve sequencer and was dried before being drawn by an internal pump of a cavity ring down spectrometer (G1101-i, Picarro, Santa Clara, USA), which measured the concentrations of $^{12}CO_2$ and $^{13}CO_2$. Flux calculations were based on the 40 min intervals that include 10 min of ambient measurement and 30 min of chamber measurement. Ambient CO_2 concentrations were accounted for according to Bahn et al. (2009) in (Eq. (1)). Flux rates were based on the measured concentrations of $^{13}CO_2$ and $^{12}CO_2$ over time (dC/dt), pressure (P), air temperature (T), gas constant (R), surface area (S), and chamber volume (V) Eq. (2).

$$\delta^{13}C_{Resp} = \frac{\delta^{13}C_{out}[CO_2]_{out} - \delta^{13}C_{in}[CO_2]_{in}}{[CO_2]_{out} - [CO_2]_{in}} \quad (1)$$

$$Resp = \frac{\partial C}{\partial t} \times \frac{PV}{RTS} \quad (2)$$

To label new photosynthates, the plant was fumigated with CO_2 enriched in ^{13}C prior to illumination. In the dark, a plastic cuvette was placed over the entire tree canopy except for one branch that was used for photosynthesis measurement. The cuvette was plumbed with a delivery and exit line leading back to an Erlenmeyer flask that contained 0.3 mg of 99% labeled $Ca^{13}CO_3$. The labeled CO_2 was liberated by adding 85% phosphoric acid in excess; the increase in CO_2 concentration within the cuvette due to

the fumigation was not regulated. The labeled gas was circulated via a pump at a rate of 1 l min^{-1} and then, the light was switched on at 730 μmol m^2 s^1 and left on over the entire time course of the experiment (between 36 and 48 h). The fumigation period differed between treatments. For the 0 and 1 day treatment, the fumigation period was 45 min to 1 h. Due to the low photosynthesis rate, the 6 day treatment proved difficult to label, thus we approximately doubled the amount of labeled $CaCO_3$ and increased the fumigation time to ca. 3 h. CO_2 concentration peaks were determined by recording concentration levels after the initial respiration peak stabilized.

Photosynthesis was measured continuously using an infrared gas analyzer (Walz GFS 3000, Effeltrich, Germany) measuring at 30 s. intervals. Leaves from the branch extruding through the labeling cuvette were placed within the IRGA photosynthesis cuvette. Flux measurements were corrected to account for leaf area that was determined by analyzing photos in ImageJ 1.46 software (imagej.nih.gov/ij/).

Soil temperature was measured in a subset of tree experiments (n = 4). We used a Campbell 107-L probe connected to a data logger (CR 200). Temperature data were recorded every 10 s.

After tracing the label from the canopy to belowground, a series of post experiment measurements were made. To verify that the dark-treatment did not permanently damage the soil-plant hydraulic pathway, we measured leaf water potential using a Scholander Bomb (Skye, Llandrindod Wells, UK). Leaf and root tissue was sampled, ground and analyzed for isotopic composition. 1.5 mg samples were weighed into tin cups and combusted in an elemental analyzer coupled to an isotope ratio mass spectrometer (Delta V Advantage, ThermoFisher, Bremen, Germany) via a Conflo III. Isotopic ratios were converted to per mil scale ($\delta^{13}C$ *vs.* VPDB) and analysis of internal laboratory standards resulted in a precision of 0.1‰.

To provide further evidence that any observed respiration originated from changes in the canopy, we monitored soil respiration from girdled trees (n = 5) after canopy illumination. The light regime was similar to the 0 day treatment. Before canopy light exposure, we removed up to 2 cm of phloem and bark around the tree stem circumference with a razor. In this case, we were only concerned with a rapid flux response similar to that of a pressure concentration wave, thus we did not label the canopy nor did we measure respiration measure beyond 4–5 h.

We used ANOVA and post-hoc Tukey tests to assess statistical differences between CO_2 concentration peaks that occurred after illumination, photosynthesis, labeled organic matter, and leaf water potentials for the different treatments ($\alpha = 0.05$).

3. Results

3.1. Soil respiration

Soil respiration within all treatments increased after 30 min canopy illumination, resulting in respiration peaks of 4.0, 3.7, and 2.7 μmol m^{-2} s^{-1}, for the 0, 1 day, and 6 day treatments respectively (Fig. 1) The peak concentrations were significantly different only at the $\alpha = 0.1$ level and attained the highest respiration peak within 5 h. Based on an average tree height of 46.6 cm, the transfer velocity of this carbon in the first respiratory peak is 0.09 m h^{-1}.

The time to label arrival was different between the 6 day treatment and the other two. The label appeared after approximately 7 h for the 0 day and 1 day treatments, while for the 6 day treatment there was either an absence altogether of a labeled signal or, for two of the trees, the time of label arrival was after approximately 22 h. The corresponding transfer velocities are 0.07 m h^{-1} for the 0 and 1 day treatment and 0.02 for the 6 day m h^{-1}treatment. The rhizosphere in the 1 day dark treatment respired a similar amount of C (10.7 g C m^2) over the time period examined as the 0 day treatment (11.8 g C m^2) ($p > 0.1$), but the plants with a 6 day dark treatment respired about half the amount (6.6 g C m^2, $p < 0.05$). Soil respiration from the girdled plants did not increase after canopy illumination (Fig. 2).

3.2. Photosynthesis

The increase in light and temperature upon illumination resulted in initial peaks in photosynthesis that eventually stabilized (Fig. 3). The standard error of photosynthesis is shown

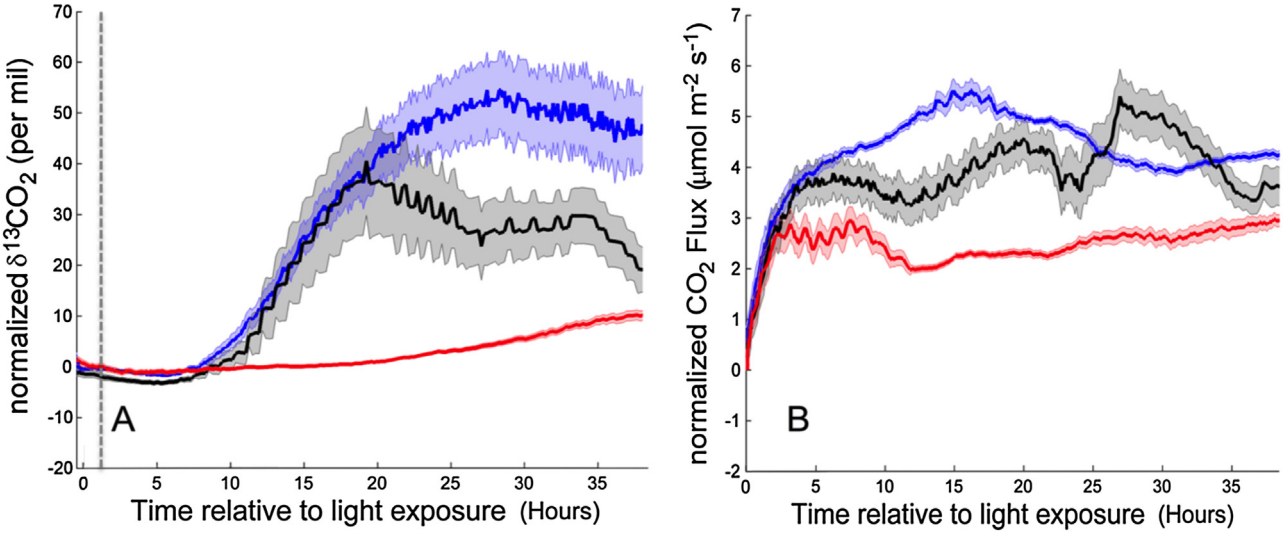

Fig. 1. Normalized isotopic signal (A) and flux rate (B) of belowground respiration. Depicted in the graphs are the means and their standard errors of the 4 trees in the three treatments (blue = 0 days in the dark, black = 1 day in the dark, red = 6 days in the dark). We used a 15 min window to average respiration (n = 4 trees/treatment). Time 0 indicates when the plants were exposed to light. The grey vertical line in panel A refers to the end of the labeling period for the 0 and 1 day treatment, while the 6 day treatment was fumigated up to 3 h. The light remained on for the duration of the experiment (ca. 2 days). We normalized the flux and isotopic signature to the base respiration and isotopic signal measured immediately before light exposure resulting in a starting point of 0 for all fluxes.

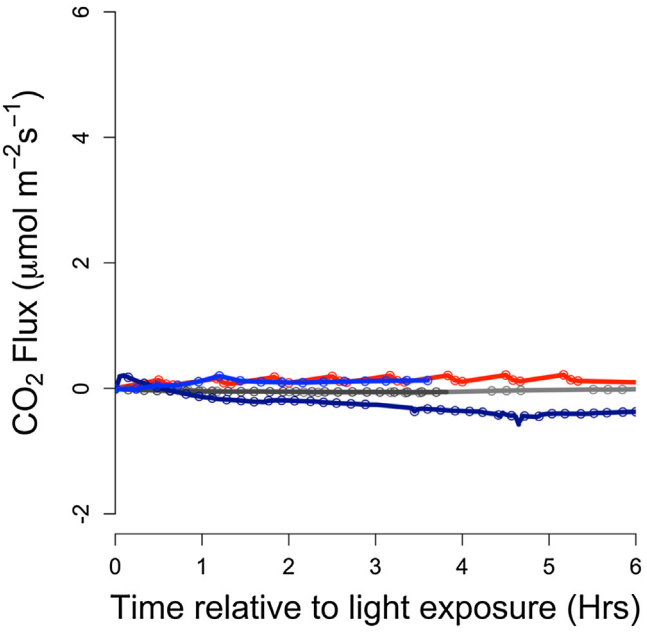

Fig. 2. Soil respiration rates of girdled trees (n = 5). The trees were kept under similar conditions to the 0 day treatment. Girdling was performed prior to illumination, thus eliminating the possibility that an increase of respiration observed after canopy illumination resulted from an artifact due to rhizosphere, rather than canopy, processes. The different colors are used to indicate each tree response.

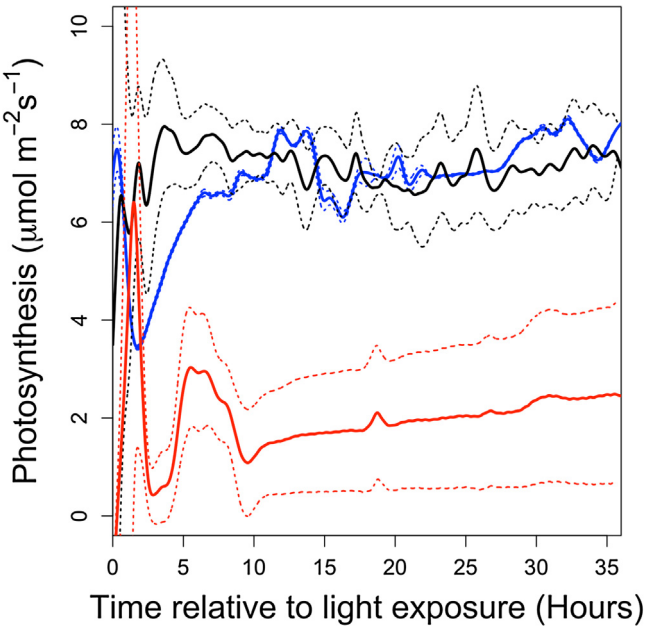

Fig. 3. Photosynthesis rates of juvenile Douglas fir trees recorded after sudden canopy illumination (blue = 0 day, black = 1 day, red = 6 day). Standard errors bound the mean with the corresponding treatment colors.

in a different graph (S3) due to large variability in the measurements of 1 day and 6 day treatment after the growth chamber light was turned on. The photosynthesis rates differed among treatments over time ($P < 0.01$), and while significant, the overall difference between 0 day and 1 day was small (0.2 $\mu molm^{-2} s^{-1}$). The 6 day treatment, however, was consistently low and did not recover to 0 or 1 day photosynthetic rates over the length of the experiment, indicating possible changes to the photosynthetic apparatus of these plants.

3.3. Leaf and roots

The leaf isotopic signature of all treatments was not significantly different at the beginning of the treatment (Table 1) while post-labeling 6 day leaves were significantly different than 0 day and 1 day ($P < 0.01$). Similar patters were found for the post-label root samples ($P < 0.01$). Leaf water potentials of 1 day were significantly different than 0 day and 6 day ($P < 0.05$). Although the growth cabinet controlled the atmospheric temperature, the radiative heating of the pot increased soil temperature. The soil temperatures increased at about a rate of 0.67 °C h^{-1} after the light turned on and then stabilized after 12 h (Fig. 4).

To account for the potential increase in respiration that occurs with temperature, we estimated the expected respiration for each treatment. The basal respiration values ranged from 0.9 to 1.5 $\mu mol^{-1} m^{-2} s^{-1}$ for the 0 day treatment, 0.3–0.5 for the 1 day, and 0.1 to 0.4 for the 6 day treatment. We calculated the estimated flux based on 5 and 12 h light exposure, which corresponds to a 3.4 and 8.0° increase in soil T. The expected respiration increase due to temperature, based on a Q10 of 2.5, results in 0 day = 1.7 ± 0.4, 1 day = 0.6 ± 0.1, 6 day = 0.4 ± 0.1 $\mu mol^{-1} m^{-2} s^{-1}$ for the 3.4° increase and 0 day = 2.4 ± 0.1, 1 day = 0.8 ± 0.3, 6 day = 0.5 ± 0.1 $\mu mol^{-1} m^{-2} s^{-1}$ for the 8.0° increase. In both temperature increase scenarios, the temperature effect could not account for the rapid increase in respiration we observed.

4. Discussion

For all three treatments we observed an immediate increase in belowground respiration upon illumination, providing evidence that supports a carbon transport mechanism that is faster than direct transport of new ^{13}C labeled assimilates from leaves to belowground. The temperature increase in the soil due to the onset of illumination was not large enough to explain this increase of respiration. Additionally, we did not observe such an increase in respiration for a controlled experiment where the saplings were girdled before illumination to eliminate phloem transport (Fig. 2). Since the fast response occurred in the different dark treatments in a similar way, we can also exclude this increase being a result of circadian regulation. Thus, the response is of plant origin, phloem-mediated, and is not an artefact of independent rhizosphere processes. The duration of the respiration increase ranged from 4 to 6 h for the peak of the rapid response. Contrastingly, the isotopically labeled carbon was respired after 7 to 20 h and was accompanied by a second, less pronounced increase of respiration rate. The patterns in the magnitude of C respired by the rhizosphere indicate that only the long-term carbon starved plants were severely affected. Interestingly, the initial rates of respiration increase, coinciding with a rapid delivery mechanism, were similar across treatments, but the first fast respiration peak was clearly lower in magnitude in the 6 day treatment compared to the others, consistent with our hypothesis.

Photosynthesis rates were consistently stratified by treatment. There was a steep increase in photosynthesis that occurred upon illumination that coincided with high variation between the treatments (S3). It is however difficult to determine if the increased rate was a plant response or due to the disturbance of removing the labeling chamber during the experiment. The amount of label present in the leaves and roots was highly variable. Roots were the most highly labeled tissue by the end of the illumination period, lasting typically 1.5 to 2 days. Leaves were also enriched relative to the pre-illuminated leaves, but not to the same degree as roots indicating that newly assimilated carbon was only partially retained in leaves with a larger proportion transported belowground. The low enrichment in all tissues of the 6 day-carbon starved plants was in agreement with the low photosynthesis

Table 1
Tree height (n = 4 trees/treatment), $\delta^{13}C$ of leaves before canopy illumination and labeling, $\delta^{13}C$ of leaves and roots after labeling, and post-illumination leaf water potential.

Treatment	Tree ht [cm]	$\delta^{13}C$ $leaves_{pre}$ [‰]	$\delta^{13}C$ $leaves_{post}$ [‰]	$\delta^{13}C$ $roots_{post}$ [‰]	Ψ $leaves_{post}$ [MPa]
0 Day	47.9 (2.6)	−27.7 (0.3)	32.0 (28.7)	104.4 (68.1)	−1.0 (0.2)
1 Day	45.7 (2.6)	−28.5 (0.3)	42.4 (19.9)	152.6 (81.0)	−1.5 (0.2)
6 Day	41.3 (3.3)	−28.0 (0.3)	−2.5 (10.2)	−4.2 (7.2)	−1.0 (0.1)

Means (standard error), isotopic signatures of leaves were measured from samples removed before and after the illumination period, while root isotopic signatures and leaf water potentials were measured at the end of the illumination period (ca. 2 days).
n = 4.

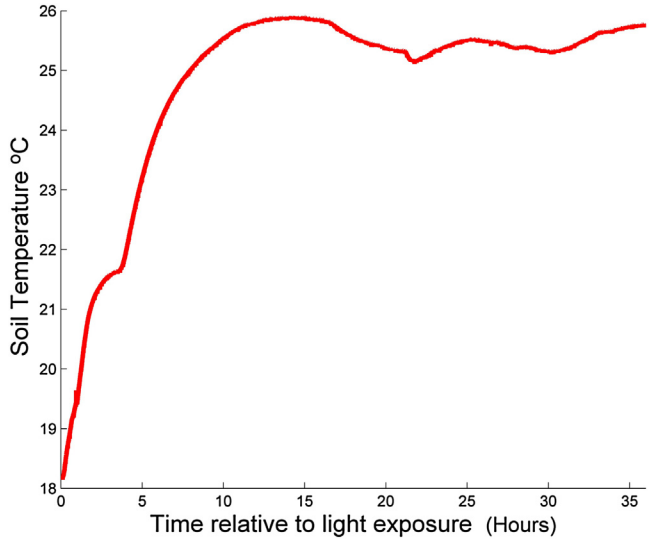

Fig. 4. Soil temperature following canopy illumination (n = 4).

rates. Furthermore, the post-experiment water potential levels were normal, ensuring that the soil-plant water continuum was not damaged, all of which is indicative of a lack of functionality in the photosynthetic apparatus after prolonged darkness. As a consequence of the low assimilation rate of $^{13}CO_2$, we did not see a distinct $^{13}CO_2$ label peak in the belowground respired CO_2 but rather a slow diffuse increase.

Our results show that there are at least two mechanisms coupling above-with belowground processes in trees. We observed both a rapid response of belowground respiration before any label was detected in the emitted CO_2, which is consistent with the pressure-concentration wave hypothesis (Mencuccini and Hölttä, 2010b), and a slower response that coincided with an increase in respiration rate with the arrival of ^{13}C labeled new assimilates. Our results are thus in agreement with recent findings of Gavrichkova and Kuzyakov (2016) for maize, who also observed a fast root respiration response that was attributed to pressure-concentrations waves. In the extreme case of diminished transport of new photosynthates driven by mass flow resulting from 6-days of darkness, we still observed a rapid respiratory response potentially due to pressure concentration waves or other plant-soil carbon coupling mechanism, followed later, by a diffuse and slow increase in respiration associated with the transport of ^{13}C labeled new assimilates to the roots.

Epron et al. (2012) showed that assimilate transport as assessed with isotope labeling techniques was clearly slower in Gymnosperms than in Angiosperms. Liesche et al. (2015) who assessed hydraulic resistance in the phloem transport system attributed these differences to significantly higher resistances in gymnosperms than in angiosperms. They also concluded that the higher phloem hydraulic resistance was only partially offset by the longer sieve elements of gymnosperms. A mechanism that allows faster coupling between assimilation and the energy demanding processes belowground would thus be of particular advantage for gymnosperms. Still, such a fast coupling as observed with Douglas-fir in our study was also detected in maize (Gavrichkova and Kuzyakov, 2016) and, thus, we need to assume that the underlying mechanism is common to both, angiosperms and gymnosperms.

Our experimental results of different mechanisms coupling photosynthesis to belowground respiration with different velocities are consistent with time series (wavelet coherence) analyses of photosynthesis and soil CO_2 efflux in different forest ecosystems (Vargas et al., 2011). These authors frequently observed a fast coupling of plant photosynthetic response to soil respiration on a one-day time scale, which is faster than the transport time of new assimilates given the height of the trees in the systems analyzed. In addition, the authors observed a slower response of soil respiration to temporal patterns in photosynthesis, though the number of these observations were fewer.

Given that techniques to measure phloem turgor are difficult and still in experimental development, we cannot confirm that the fast respiration response is due to a pressure concentration wave or another mechanism, such as electric signaling. However, since there was an absence of an increase in soil respiration observed in the girdling experiment, we can state that the respiration increase we observed is primarily related to the onset of photosynthesis and phloem dynamics. As we did not measure phloem pressure and mass flow directly, we cannot rule out that other biophysical information such as electrical signals propagated by the phloem (Fromm and Lautner, 2007) that might be responsible for the initial fast respiration response. We also acknowledge that we created an artificial environment for plants by placing them in the dark disrupting circadian cycles of many enzymes and processes that might regulate respiration (de Dios et al., 2012). Yet, this provided an environment where we could test if the initial fast respiration response was associated with pressure concentration waves, rather than potential internally controlled variations in respiration due to circadian rhythms. Since the pressure concentration wave hypothesis fully explains our observations, including the supply of the respiratory substrate, we consider this mechanism to be most likely responsible for the fast coupling.

Clearly, the fast initial pulse of respiration indicates a response by the canopy that is transmitted belowground; however, it remains to be seen how this response emerges at a stand or ecosystem scale. For larger trees or mixed age and mixed species stands we might expect to see a variable rapid response of belowground respiration with the onset of photosynthesis (Kuzyakov and Gavrichkova, 2010; Mencuccini and Hölttä, 2010b). There are a myriad of ways in which the initial respiration response may manifest (Vargas et al., 2011). In our experimental study, the fast respiration peak also occurred in carbon-starved trees indicating that this signaling between canopy and belowground sites of respiration is maintained. This clearly is intriguing and may have important ecophysiological implications, but it

remains to be seen what the respiration response means in the sense of carbon supply or status of the tree or ecosystem. Our results show that the initial increase in respiration is only sustained – or even increased – when subsequently newly fixed assimilates are transported belowground.

The way forward relies on method development to directly characterize flow and turgor along the phloem pathway (Peuke et al., 2001; Mencuccini et al., 2013), until then, research and modeling of the plant-soil continuum must consider both mechanisms of assimilate delivery to sink tissues and points of heterotrophic respiration. Furthermore, detailed experimental and field data addressing the impact of this rapid response in rhizosphere respiration are sorely lacking. Future field and laboratory research is needed to elucidate the significance of this rapid response and how this new addition to our understanding of plant-soil interactions influences belowground biogeochemical cycles in the face of changing climate.

Contribution statement

All authors contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and composition of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2016.12.002.

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