

Two distinct plant respiratory physiotypes might exist which correspond to fast-growing and slow-growing species

Salvador Nogués ^{1,*}, Salvador Aljazairi ¹, Claudia Arias ¹, Elena Sánchez ¹, Iker Aranjuelo ^{1,2}

¹ Unitat de Fisiologia Vegetal, Departament de Biologia Vegetal, Universitat de Barcelona, E-08028 Barcelona, Spain

² Current address: Instituto de Agrobiotecnología (IdAB), Universidad Pública de Navarra-CSIC-Gobierno de Navarra, Campus de Arrosadía, E-31192-Mutilva Baja, Spain

* Corresponding author; fax +34-934021465; e-mail salvador.nogues@ub.edu

Running title: Two distinct respiratory physiotypes in plants

SUMMARY

The origin of the carbon atoms in CO₂ respired by leaves in the dark of several plant species has been studied using ¹³C/¹²C stable isotopes. This study was conducted using an open gas exchange system for isotope labeling that was coupled to an elemental analyser and further linked to an isotope ratio mass spectrometer (EA-IRMS) or coupled to a gas chromatography-combustion-isotope ratio mass spectrometer (GC-C-IRMS). We demonstrate here that the carbon, which is recently assimilated during photosynthesis, accounts for nearly ca. 50% of the carbon in the CO₂ lost through dark respiration after illumination in fast-growing and cultivated plants and trees and, accounts for only ca. 10% in slow-growing plants. Moreover, our study shows that fast-growing plants, which had the largest percentages of newly fixed carbon of leaf-respired CO₂, were also those with the largest shoot/root ratios, whereas slow-growing plants showed the lowest shoot/root values.

Key words: carbon stable isotopes; fast- and slow-growing plants; photosynthesis; respiration.

Abbreviations: $\delta^{13}\text{C}$, carbon isotope composition; $\Delta^{13}\text{C}$, carbon isotope discrimination; A_n , net photosynthetic rate; C_{new} , labeled C in respired CO₂ ; IRMS, isotope ratio mass spectrometry; PPFD, photosynthetic active photon flux density; R_d , dark respiration; RQ, respiration quotient.

INTRODUCTION

In leaves, the CO₂ fixed by the Calvin cycle is subsequently converted to triose phosphates which are partitioned between i) glycolysis and mitochondrial respiration, ii) processes leading to sucrose synthesis and export, and iii) starch synthesis and temporary storage. The reducing power provided by photosynthesis drives C and N assimilation, whereas in tissues which remain unexposed to light and in non-green tissues such as roots, the necessary reducing power is mainly supplied by the oxidative pentose phosphate pathway (Foyer et al., 2011). Previous studies (Atkin et al., 1996; Loveys et al., 2002) showed that 30-70 % of CO₂ fixed by photosynthesis during the day is respired by the plants and is therefore lost to the atmosphere. Moreover, according to Atkin et al. (2007), 50-70% of whole plant respiration takes place in the leaves. The assimilation of N by plants involves the reduction of nitrate to nitrite by nitrite reductase using NADH produced by the malate shuttled from the mitochondria or chloroplasts (Foyer and Noctor 2002). Estimates of C partitioning in roots suggest that about 5 % of root C catabolism is coupled to soil nitrate absorption, 15 % to nitrate assimilation and 3 % to ammonium assimilation (Bloom et al., 2002).

Whereas plants use photosynthesis to produce the carbohydrate substrate on which they depend, glycolysis and respiration are the processes whereby the energy stored in these carbohydrates is released. Respiration is a key physiological process in sustaining growth and biomass production of plants and ecosystems (Tcherkez et al., 2012). Respiratory metabolism provides ATP, reducing equivalents and metabolic intermediates used in biosynthesis elsewhere in the cell (Araújo et al., 2014). As observed by Araújo et al. (2014), although historically photosynthesis and respiration have been considered to be independent pathways, over the last decade, the functioning of chloroplasts and mitochondria has been described to be coordinated and tightly interact through intracellular metabolite pools. The metabolic interaction of respiratory metabolism with other pathways is being determined, particularly its relationship to Calvin cycle reactions in photosynthesis, photorespiration and nitrate assimilation (Araújo et al. 2014 and references therein).

Plant respiration has been described as differing from one species to another (Wright et al. 2001, Turnbull et al. 2003). It was hypothesized that slow-growing plants (with lower growth and ion transport rates) would have lower respiration rates than fast-growing plants. However, as observed by previous studies (Poorter et al., 1991; Scheurwater et al., 1998), differences in respiration between fast- and slow-growing plants were much smaller than theoretically expected. According to Scheurwater et al. (1998) such differences could be explained by the fact that in fast-growing species (species with 3-fold higher relative growth rates, RGR, than slow-growing species; Poorter et al. 1990) respiratory costs are 70 % lower than in slow-growing plants (Scheurwater et al., 1998). However, according to other authors (Atkin and Tjoelker 2003), alpine plants, for example, show higher respiratory rates than lowland species. Those studies suggest that a greater amount of photoassimilates are invested in maintenance than growth processes in slow-growing plants when compared to fast-growing plants. Respiration can be broken down into three components, i.e. the respiratory cost of root growth and of ion uptake, and maintenance respiration. Protein turnover and the maintenance of ion gradients are regarded as the two most important maintenance processes in terms of energy requirements (Bouma et al., 1994; Scheurwater et al., 2000). With respect to the maintenance costs, a previous study conducted with the fast-growing *Dactylis glomerata* and the slow-growing *Festuca ovina* showed that both plant species spent between 22-30 % of their daily ATP production dedicated to maintenance on protein turnover, which corresponds to 11-15 % of total root ATP production per day (Scheurwater et al., 2000).

The origin of C used in respiratory processes is another matter of controversy. Even if respired C is derived from compounds such as malate, pyruvate, isocitrate and α -ketoglutarate, such C may proceed from recently fixed photoassimilates and/or remobilization of C storage forms such as starch, sugar and fructans (Lehmeier et al., 2008; 2010). The residence time of respired C varies from compounds that are rapidly (seconds to minutes) transferred to respiration centres to long lived compounds (such as proteins or storage carbohydrates) with long residence times (days to months) (Lehmeier et al., 2008). Lehmeier et al. (2008) showed that 43 % of respiration in perennial ryegrass was supported by recently fixed photosynthates whereas the 57 % was supported by the remobilization of storage compounds. Those studies highlighted the fact that carbohydrate respiration is supported by a heterogeneous mixture of

molecules that cycle, more or less extensively, through a network of biochemical compounds and compartments.

Carbon residence time has been previously analyzed through the use of stable isotopes (Schnyder et al., 2003; Nogués et al., 2004; Lötscher and Gayler 2005; Lehmeier et al., 2008; 2010; Aranjuelo et al., 2009). $^{13}\text{CO}_2$ labeling carried out by Lötscher and Gayler (2005) in *Medicago sativa* plants showed two phases in the appearance of enriched $^{13}\text{CO}_2$ in respiration. The largest amount of labeled ^{13}C (corresponding to current photosynthate activity) appeared in the first phase, whereas in the second phase the contribution of non-labeled C exceeded the labeled ^{13}C . The second respiration phase was fuelled by one (or more) C stores.

Plants have several contrasting and complementary strategies for optimizing C and N uptake. However, these strategies do not occur randomly across terrestrial environments. Slow-growing plants usually grow in harsh and constraining conditions that might favor the recycling of reserves. In contrast, fast-growing species have to sustain rapid growth that requires higher respiration and metabolic activity (compared with slow-growing species). Therefore, for a given amount of assimilated carbon, it would be expected that slow-growing plants would invest a lower amount of recently fixed carbon in respiration processes than fast-growing plants (Baptist et al., 2009; Aranjuelo et al., 2011).

Although respiration metabolism has been extensively characterized over the last decade, important gaps remain to be elucidated. To date, the different contributions of C pools to leaf respiration in fast- and slow-growing plants is still unknown. Here, the $^{13}\text{C}/^{12}\text{C}$ isotope labeling technique was used to study the respiratory metabolism of recently fixed carbon in the leaves of ten different plant species. We used a system that consists of an LI-6400 open gas-exchange system coupled to an elemental analyzer (EA) and to an isotope ratio mass spectrometer (IRMS, for a recent review see Ghashghaie and Tcherkez, 2013). This system takes advantage of the difference in $\delta^{13}\text{C}$ between atmospheric CO_2 (ca. -9.5‰ , see Material and Methods) and commercially available CO_2 (^{12}C -enriched e.g. ca. -51.2‰). This allows one to calculate the contribution of stored carbon vs. current photoassimilates to the production of CO_2 through respiration (Schnyder et al., 2003). It is noteworthy that this would not have

been possible if heavily labeled carbon had been used (i.e. several percent more of ^{13}C would have blurred the contribution made by non-labeled carbon). The abundance of ^{13}C in the CO_2 used for labeling is in the same order of magnitude as that found in nature, thereby allowing us to calculate the proportion of ‘new’ (i.e. recently fixed) carbon in CO_2 respired in the dark (Nogués et al., 2004). Furthermore, this system also allowed us to estimate leaf metabolic fluxes *in vivo*.

The aim of this study was to determine the origin of the carbon atoms in the CO_2 respired by leaves of several C3 and one C4 plant species. Some data presented in this paper is in part a re-analysis of previously published data (Nogués et al. , 2004; 2006a; 2006b; Aranjuelo et al., 2009), such that this study brings together this previously published data along with new data for a more complete and in-depth analysis of plant respiration.

MATERIAL AND METHODS

Plant material

In the present study, a large number of plants grown in different environments were used (i.e. from wild-grown to greenhouse-grown plants):

(i) Alpine *Ranunculus glacialis* (L.) plants were collected from the Galibier Pass in the French Alps, at an elevation of 2700 m, as previously described (Nogués et al., 2006a). For the measurements, the petiole was cut and maintained under water throughout the experiments, otherwise leaves were floated on water.

(ii) Two slow-growing Mediterranean plants (*Chamaerops humilis* L. and *Cycas revolute* Thunb., gymnosperm) were grown in pots for 20 months in a greenhouse at the Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Cabrils, Barcelona, as previously described (Pardo et al., 2009; Aranjuelo et al., 2009). The plants were grown in 4-L pots (one plant per pot) containing a mixture of peat and perlite in a 2:1 ratio (v:v) and they were continuously watered with ca. 1-L day⁻¹ of Hoagland complete nutrient solution (pH 6.5) through a drip irrigation system (Hoagland and Arnon, 1950).

(iii) Sunflower (*Helianthus annuus* L.) plants were grown for 5 weeks in four growth chambers (E15, Conviron, Winnipeg, Canada). These four growth chambers formed

part of the mesocosm $^{13}\text{CO}_2/^{12}\text{CO}_2$ open gas exchange system as described in detail by Schnyder et al. (2003). The plants were sown individually in plastic pots (5 cm diameter – 35 cm depth) filled with washed quartz sand. Modified Hoagland nutrient solution (7.5 mol N m^{-3}) was supplied by an automatic irrigation system throughout the experiment. Irradiance during the 16 h photoperiod was supplied by cool white fluorescent tubes (16 x 160 W; Sylvania Germany GmbH, Erlangen, Germany) and incandescent lamps (12 x 100 W; General Electric Germany, München, Germany), and was maintained at *ca.* $500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ PPFD at the top of the canopy by adjusting the height of the lamps following plant development. Air temperature was controlled at 20/16 °C and relative humidity at 75/80% during the photo and dark periods, respectively.

(iv) French bean (*Phaseolus vulgaris* L.) plants were grown from seeds in 1-L pots of potting mix for 5 weeks in a greenhouse at Université Paris Sud as described by Nogués et al. (2004). Minimum PPFD during a 16-h photoperiod was maintained at approximately $500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ by supplementary lighting. Temperature and vapor pressure deficit were maintained at *ca.* 25.5/18.5°C and 1.4/1.2 kPa day/night, respectively.

(v) Three isolated 20-year-old beech (*Fagus sylvatica* L.) trees were grown at the campus of the Université Paris Sud (Orsay, France) as previously described (Nogués et al., 2006b). Mean annual precipitation, and minimum and maximum temperatures of the site are 685 mm, 7°C and 16°C, respectively.

(vi) *Medicago sativa*, *Triticum turgidum*, *Arundo donax* and the C4 *Zea mays* L. plants were grown in pots in a greenhouse at Servei de Camps Experimentals of the University of Barcelona. They were planted in pots filled with sand and watered daily with a complete Hoagland solution. Details of the growing conditions at the University of Barcelona can be found in Cabrera-Bosquet et al. (2007). Plants were grown in the greenhouse under mean day/night temperatures of *ca.* 25/15°C and a maximum PPFD of *ca.* $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$.

All experiments were carried out on the fully expanded mature leaves for (at least) three plants for each species studied.

Gas exchange determinations

Plants were removed from the greenhouse or the field after a light period of about 8-10 h and the leaf was placed for *ca.* 50 min in a respiration chamber to take measurements of dark-respired CO₂ (unlabeled). After the initial measurement of dark-respired CO₂, the leaf was removed from the respiration chamber and placed in a specially designed gas-exchange labeling chamber built within the laboratory for online isotope labeling (see below; Nogués et al., 2004). After labeling, the leaf was removed from the labeling chamber, and the leaf was returned to the respiration chamber system for measurements of dark-respired CO₂ (labeled).

Gas-exchange measurements were carried out with the Li-6400 gas exchange portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA) on healthy and fully expanded leaves under conditions similar to growth conditions (500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, 25°C, 380 $\mu\text{mol mol}^{-1}$ CO₂, 21% O₂). Plants were dark-adapted for 50 minutes before dark respiration (R_d) measurements were taken (Nogués et al., 2004; Florez-Serasa et al., 2012).

The respiration quotient (RQ) was calculated from the ratio of carbon production [$v(\text{CO}_2)$] to oxygen consumption [$v(\text{O}_2)$] according Nogués et al. (2004): $\text{RQ} = v(\text{CO}_2)/v(\text{O}_2)$. CO₂ production in darkness was measured using the Li-6400 described above. Oxygen consumption of leaf discs was measured with an oxygen electrode (Hansatech, Norfolk, England). RQ values close to 1 indicate highly oxygenated substrates (e.g. carbohydrates) and RQ values close to 0.6 indicate weakly oxygenated substrates (e.g. fatty acids) which are used during respiratory processes. RQ values higher than 1 may be observed when metabolites such as malate or citrate are oxidized.

Isotope labeling measurements

As mentioned above, after the initial measurement of dark-respired CO₂, leaves were placed in a custom designed and built gas-exchange chamber for isotope labeling, as described by Nogués et al. (2004). The chamber was connected in parallel to the sample air hose of the LI-6400. This aluminium chamber (20x12x6 cm³), fitted with a clear plastic lid, can hold two to four leaves (total leaf surface *ca.* 50 cm²). Two fans were placed in the chamber which generated a boundary layer conductance to water of *ca.* 6.7

mol m⁻² s⁻¹. Leaf temperature in the chamber was maintained at *ca.* 20°C by water cooling the jacket around the leaf chamber, and was measured with a copper-constantan thermocouple plugged into the thermocouple sensor connector of the LI-6400 chamber/IRGA. Ingoing air was passed through the chamber at a rate of 1-L min⁻¹ and monitored by the LI-6400. Molar fractions of CO₂ and humidity were measured with the LI-6400 infrared gas analyzer (IRGA). The PPFD inside the chamber was maintained at 500 μmol m⁻² s⁻¹ during the labeling period. Light was supplied by a 500-W halogen lamp (Massive N.V., Kontich, Belgium). The lamp was placed at approximately 30 cm above the chamber and 5 cm of deionized water and 1 cm of glass in the container were used to filter the radiation. The PPFD at the leaf level inside the chamber was maintained at 500 μmol m⁻² s⁻¹ during the labeling period. For labeling, CO₂ was supplied from a bottle (Air Liquide, Grigny, France) with a δ¹³C value depleted in ¹³C relative to the air (for example -51.2‰ ± 0.1‰; see Table 1).

All species assimilated the same amount of labeled CO₂ (i.e. *ca.* 350 mmol C m⁻²) but the assimilation time varied between the different plant species studied according to their net assimilation rates (Table 2). For example, *Phaseolus vulgaris* had an A_n of *ca.* 12.4 μmol C m⁻² s⁻¹ (Table 2), so it was labeled as *ca.* 8 h which is the time needed to reach *ca.* 350 mmol C m⁻² (12.4 μmol C m⁻² s⁻¹ x 8 h x 3.6); and so on.

After photosynthetic measurements, the outgoing air of the chamber was shunted and the air with a CO₂ concentration of *ca.* 300 μL L⁻¹ was collected in 50-ml glass balloons or in 10-ml vacutainers and analyzed in order to measure photosynthetic carbon isotopic discrimination (Δ¹³C). The Δ¹³C measured varied between 20.4‰ and 5.3‰ for C3 and C4 plants, respectively.

After labeling, leaves were removed from the labeling chamber and replaced in the respiration chamber for measurements of dark-respired CO₂ (labeled). Gas samples containing the air with a CO₂ concentration of *ca.* 300 μL L⁻¹ were collected for analysis in 50-ml glass balloons (Scott Glass, Mainz, Germany) or in 10-ml vacutainers (BD vacutainers, Plymouth, UK). The gas inside the balloon was then introduced into an elemental analyzer (EA) NA-1500 (Carlo-Erba, Milan) and pumped through a 15-mL loop as described by Tcherkez et al. (2003). On the other hand, the gas inside the

vacutainers was directly injected into a gas chromatography-combustion-isotope ratio mass spectrometer (GC-C-IRMS) as previously described (Nogués et al., 2008). It is worth noting that the CO₂ respired in the dark after labeling comes from old carbon that was already in the leaf before labeling and new carbon that was recently fixed during labeling.

C isotope composition

Leaves samples were dried in an oven at 60° C for 48 hours and ground to a fine powder. Then 1 mg samples were weighed in tin capsules to determine the carbon isotope composition using an Elemental Analyzer Flash 112 (Carbo Erba, Milan) coupled to an isotope ratio mass spectrometry IRMS Delta C Conflo III Interface (Termo Finnigan, Germany). Results of carbon isotope ratio analyses are reported per mil (‰) on the relative δ-scale as δ¹³C and are referred to the international standard V-PDB (Vienna Pee Dee Belemnite) according to the following equation:

$$\delta^{13}\text{C} = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \quad (\text{Eq. 1})$$

where R is the ¹³C/¹²C ratio. The standard PDB is a belemnite fossil coming from geological formation Pee Dee in South Carolina, USA. The PDB is slightly ¹³C-enriched ($R_{\text{PDB}} = 0.0112372$) compared to almost all organic and inorganic materials but remains close to them so that δ¹³C values of nearly all biological samples are very small and are thus expressed in per mil (‰, i.e. 10⁻³) and are negative (Ghashghaie and Tcherkez, 2013).

Calculation of the percentage of new carbon in respired CO₂

The proportion of new carbon (derived from the labeling) in CO₂ respired in darkness after illumination was calculated following Nogués et al. (2004).

$$x = 100 \times \frac{\delta Y' - \delta Y}{(\delta_o - \Delta Y - \delta Y)} \quad (\text{Eq. 2})$$

where $\delta Y'$ and δY are the isotope compositions from labeled and non-labeled, respectively; δ_o is the isotope composition of the outlet air and ΔY is the isotopic discrimination.

Plant biomass analysis

At the end of the experiment, plants were harvested and oven dried at 60° C for 48 hours, and analyses of biomass of shoots and roots were carried out. Relative growth rate (RGR) was calculated as described by Wolfe et al. (1998). Total plant leaf area was estimated prior to drying using a flat-bed scanner.

RESULTS AND DISCUSSION

As is shown in table 1, in the analyses of the labeled C percentage in respired CO₂ (C_{new}) two different groups were detected. Carbon which had been recently assimilated during photosynthesis accounted for ca. 50% of the carbon in CO₂ lost through dark respiration after illumination in *Helianthus annuus*, *Phaseolus vulgaris*, *Fagus sylvatica*, *Zea mays*, *Medicago sativa*, *Triticum turgidum* and *Arundo donax*; and ca. 10% in *Ranunculus glacialis*, *Chamaerops humilis* and *Cycas revoluta* (Table 1; Nogués et al., 2004, 2006a,b; Aranjuelo et al., 2009). The analyses of both groups highlighted the fact that the first group was composed of fast-growing plants with C3 and C4 photosynthetic machinery while the second group was made up of slow-growing ones. These differing values indicate that in nature there might be two distinct “respiratory physiotypes”. Therefore, for a given amount of assimilated carbon, parsimonious plants have a slow turnover in the respiratory pool, perhaps favoring the recycling of reserves (i.e. *Ranunculus glacialis* and *Chamaerops humilis*) and as a result, have a small proportion of ‘new’ carbon after illumination (Table 1, Nogués et al., 2006b). In contrast, other plants such as *Helianthus annuus*, *Phaseolus vulgaris*, *Fagus sylvatica*, *Zea mays*, *Medicago sativa*, *Triticum turgidum* and *Arundo donax* invest more recently assimilated carbon in respiration. Presumably, this is due to the fact that plant respiratory strategies are dictated by the biological constraints of their environment. In general, Q₁₀ (the proportional change in respiration per 10°C rise in temperature) values are lower in tissues where respiratory flux is limited by substrate availability, and as a result, Atkin and Tjoelker (2003) have proposed that overall Q₁₀

values will be lower in plants where photosynthesis and the subsequent synthesis of sugars is limited. On the other hand, increases in the availability of respiratory substrate that are produced as a consequence of sustained higher rates of photosynthesis might result in an increase in the Q_{10} of respiration. Interestingly, Atkin and Tjoelker (2003) identified two types of respiratory acclimation to temperature in plants, one of which is underpinned by adjustments in the Q_{10} (called Type I) and the other by changes in the enzymatic capacity of the respiratory system (called Type II); Type I acclimation allows for rapid changes to occur in the respiratory flux at high temperatures following changes in the thermal environment, whereas Type II acclimation is likely to be maximal upon the development of new leaves and roots following a change in temperature.

Although in previous studies it has been observed that plants invest a large amount of recently fixed C in respiration (Day et al., 1985; Scheurwater et al., 2000; Lehmeier et al., 2010), it has also been noted that depending on the plant which is being analyzed, this amount can be lower (Nogués et al., 2006a,b; Aranjuelo et al., 2009; Baptist et al., 2009). As mentioned above, respiration is fuelled by different compounds derived from recently fixed photoassimilates and/or remobilization of storage compounds such as starch, sucrose and fructans (Lehmeier et al. 2008; 2010). The fact that C_{new} did not reach 100 % demonstrated that carbohydrate respiration was supported by a heterogeneous mixture of molecules with different residency times.

For plants such as *Ranunculus glacialis*, *Chamaerops humilis* and *Cycas revoluta* which grow slowly due to the harsh environments in which they live, i.e. alpine conditions and, hot and dry conditions respectively, most of the newly assimilated carbon is not respired and these species could be denominated as “low respiratory turn-over plants” (Nogués et al., 2006a). In fast-growing and cultivated plant species (such as *Helianthus annuus*, *Phaseolus vulgaris*, *Zea mays*, *Medicago sativa*, *Triticum turgidum* and *Arundo donax*); and trees (like *Fagus sylvatica*) nearly 50% of the respiratory CO_2 comes from recently assimilated carbon; these could be denominated as “high respiratory turn-over plants” (Fig. 1). However, further studies into the biodiversity of the leaf respiratory response is required to verify the validity of this hypothesis and to relate it to the Atkin and Tjoelker model. It is worth noting that the mean relative growth rate (RGR) ranged from *ca.* 4 $\text{mg g}^{-1} \text{d}^{-1}$ for slow-growing plants like *Chamaerops humilis* (Aranjuelo et

al., 2009) to ca. 200 mg g⁻¹ d⁻¹ for fast-growing plants like *Helianthus annuus* (data not shown).

The increase in the respiratory rate as a function of assimilated C (Table 2) makes it clear that there was a tight link between photosynthesis and respiration. The correlation between these two parameters (A_n versus R_d) was significant (Fig. 2). Even if *Chamaerops humilis* and *Cycas revoluta* were also sensitive, for *Ranunculus glacialis*, changes in A_n had the closest correlation with increases in R_d . This could be due to the different growth strategies of both plants. Given the limited time that *Ranunculus glacialis* has for growth under natural conditions, the high photosynthetic and respiration activity could be explained by the growth strategy of these plants. Their high metabolic activity could be due to the fact that they maximize resource acquisition when growth conditions are optimal. Gas exchange measurements were conducted in July, a period of maximum activity for *Ranunculus glacialis* plants. The lower R_d rates detected in *Chamaerops humilis* and *Cycas revolute* might be explained by the fact that those plants showed a conservative strategy that maximizes resource conservation. On the other hand, the low correlation observed in A_n versus R_d of fast-growing plants demonstrated that the respiration of these plants was less sensitive to their photosynthetic performance.

Bowling et al. (2008) suggested that high respiration rates could be correlated to natural ¹³C-abundance in evolved CO₂. Our study did not detect any significant correlation between respiration rate and $\delta^{13}\text{C}$ in fast- and slow-growing plants. However, Tcherkez (2010) suggested that fast-growing plants with high respiration rates produce more ¹³C-depleted CO₂ than slow-growing plants. It is possible that in slow-growing plants, a higher proportion of carbon could be diverted from the tricarboxylic acid cycle into secondary metabolism (Bowling et al. 2008; Werner et al. 2009). Using ¹³CO₂ labeling, Priault et al. (2009) found that (slow-growing) aromatic plants with low tricarboxylic acid cycle activity (i.e. they invest more in secondary metabolites like isoprene) resulted in a pronounced increase in $\delta^{13}\text{C}$ of respired CO₂, but in fast-growing herbs with a high respiratory demand no changes were exhibited in respired $\delta^{13}\text{CO}_2$ since C was fully respired. Again, Tcherkez (2010) argued that this would have two consequences: i) the metabolic flux throughout the pyruvate dehydrogenase would have to be very large, and ii) the metabolic commitment within the tricarboxylic acid cycle would be very small.

Other explanations for these variations in isotopic patterns in respired CO₂ need to be investigated such as the involvement of other respiratory substrates (e.g. lipids which are ¹³C-depleted; Tcherkez et al. 2003) or other metabolic pathways (e.g. the pentose-phosphate pathway as shown in roots; Bathellier et al., 2009; Ghashghaie and Badeck, 2014). The observation that all the plants studied had a respiratory quotient close to 1.0 highlighted the fact that they were mostly respiring carbohydrates.

Although our study showed that, with the exception of *Ranunculus glacialis*, the respiration rate of slow-growing plants was lower than that of fast-growing plants (Table 2), it should also be pointed out that respiration costs may not necessarily follow the same pattern. Respiration provides metabolic energy for growth, maintenance and ion transport (Mata et al., 1996). Scheurwater et al. (2000) found 3-fold higher specific costs for ion transport, expressed as mol O₂ per net mol NO₃⁻ taken up, in slow-growing plants. According to those authors, the higher costs were partly due to the higher efflux of N (per net NO₃⁻ uptake) and the larger maintenance costs for slow-growing plants than for fast-growing plants. According to Scheurwater et al. (2002), under optimal nutrient availability conditions, shoots are the main site of whole plant NO₃⁻ reduction in both fast- and slow-growing grasses. However, the mechanisms underlying such a shoot versus root pattern of N reduction may indicate large interspecific variability in non-optimal environmental conditions. As described by Baptist et al. (2009) in a study conducted with fast-growing (*Carex foetida*) and slow-growing (*Kobresia myosuroides*) alpine plants, the fast-growing species displayed improved photosynthetic capacity and decreased N reduction capacity in leaves, which was compensated by the preferential C allocation to root growth and/or storage. These plants increased the percentage of reduced N translocated to above-ground organs so as to compensate for the lower N assimilation capacity. The high C flux allocated to the below-ground compartment in *Carex foetida* promoted significant levels of NO₃⁻ reduction in the roots. Interestingly, in this sense, our study shows that fast-growing plants, which had the largest percentages of newly fixed carbon of leaf-respired CO₂, were also those with the largest shoot/root ratios, whereas slow-growing plants showed the lowest shoot/root values (Fig. 3). These results suggest that fast-growing plants invested most of recently fixed C in shoot respiration, while in slow-growing plants, more C_{new} was partitioned toward roots to sustain N assimilation and transport.

In conclusion, data presented in this study suggest that the two distinct respiratory phenotypes of plants exist and correspond to fast-growing (including trees) and slow-growing species. Slow-growing plants are likely invest more recently assimilated C in storage compounds than fast-growing plants. More specifically, the study revealed that in slow-growing plants, 90 % of respired carbon was derived from “old” carbon which was remobilized for its respiration. The fact that those plants had low shoot/root ratio suggest that a large part of this C was partitioned toward roots. On the other hand, respiration in fast-growing plants (with large shoot/root) was composed of *ca.* 50 % of recently assimilated C and of *ca.* 50 % “old” C. However, more experiments are now needed in order to identify the nature of this both phenotypes and the role of roots in C partitioning.

ACKNOWLEDGMENTS

This study was supported in part by *Ministerio de Educación y Ciencia* research project AGL2009-13539-C02-01 and the EC research project OPTIMA. The authors wish to thank Guillaume Tcherkez, Gabriel Cornic and Jaleh Ghashghaie for their comments at the initial stages of this study.

REFERENCES

- Aranjuelo I, Pardo A, Biel C, Savé R, Azcón-Bieto J, Nogués S. Leaf carbon management in slow-growing plants exposed to elevated CO₂. *Glob Chan Biol* 2009; 2: 300-09
- Aranjuelo I, Ebbets AL, Evans RD, Tissue DT, Nogués S, van Gestel N, Payton P, Ebbert V, Adams III WW, Nowak RS, Smith SD. Maintenance of C sinks sustains enhanced C assimilation during long-term exposure to elevated [CO₂] in Mojave Desert shrubs. *Oecologia* 2011; 167: 339-54
- Araújo WL, Nunes-Nesi A, Fernie AR. On the role of plant mitochondrial metabolism and its impact on photosynthesis in both optimal and sub-optimal growth conditions. *Photosynth Res* 2014; 119, 141-56
- Atkin OK, Botman B, Lambers H. The causes of inherently slow growth in alpine plants: an analyses based on the underlying carbon economies of alpine and lowland *Poa* species. *Funct Ecol* 1996; 10, 698-707

- Atkin OK, Tjoelker MG. Thermal acclimation and the dynamics response of plant respiration to temperature. *Trends Plant Sci* 2003; 8: 343-51
- Atkin OK, Scheurwater I, Pons TL. Respiration as a percentage of daily photosynthesis in homeostatic at moderate, but not high, growth temperatures. *New Phytol* 2007; 174: 367-80
- Baptist F, Tcherkez G, Aubert S, Pontailier JY, Choler P, Nogués S. ^{13}C and ^{15}N allocations of two alpine species from early and late snowmelt locations reflect their different growth strategies. *J Exp Bot* 2009; 60: 2725-35
- Bathellier C, Tcherkez G, Bligny R, Gout E, Cornic G, Ghashghaie J. Metabolic origin of the $\delta^{13}\text{C}$ of respired CO_2 in roots of *Phaseolus vulgaris*. *New Phytol* 2009; 181: 387-99
- Bloom AJ, Smart DR, Nguyen DT, Searles PS. Nitrogen assimilation and growth of wheat under elevated carbon dioxide. *Proc Natl Acad Sci USA* 2002; 99, 1730-35
- Bouma TJ, De Visser R, Janssen JHJA, De Kock MJ, Van Leeuwen PH, Lambers H. Respiratory energy requirements and rate of protein turnover *in vivo* determined by the use of an inhibitor of protein synthesis and a probe to assess its effect. *Physiol Plant* 1994; 92, 585-94
- Bowling DR, Pataki DE, Randerson JT. Carbon isotopes in terrestrial ecosystem pools and CO_2 fluxes. *New Phytol* 2008; 178: 24-40
- Cabrera-Bosquet LI, Molero G, Bort J, Nogués S, Araus JL. The combined effect of constant water deficit and nitrogen supply on WUE, NUE and ^{13}C in durum wheat potted plants. *Ann Appl Biol* 2007; 151: 277-89
- Day DA, Oene C de Vos, Wilson D, Lambers H. Regulation of respiration in the leaves and roots of two *Lolium perenne* populations with contrasting mature leaf respiration rates and crop yields. *Plant Physiol* 1985; 78: 678-83.
- Florez-Sarasa I, Araújo WL, Wallström SV, Rasmusson AG, Fernie AR, Ribas-Carbo M. Light-responsive metabolite and transcript levels are maintained following a dark-adaptation period in leaves of *Arabidopsis thaliana*. *New Phytol* 2012; 195: 136-48
- Foyer CH, Noctor G. Photosynthetic nitrogen assimilation: inter-pathway control and signalling. In: Foyer CH, Noctor G, eds. *Photosynthetic nitrogen assimilation*

- and associated carbon respiratory metabolism. Amsterdam: Kluwer Academic Publishers 2002; 1-22.
- Foyer CH, Noctor G, Hodges M. Respiration and nitrogen assimilation: targeting mitochondria-associated metabolism as means to enhance nitrogen use efficiency. *J Exp Bot* 2011; 62: 1467-82
- Ghashghaie J, Tcherkez G. Isotope Ratio Mass Spectrometry Technique to Follow Plant Metabolism: Principles and Applications of $^{12}\text{C}/^{13}\text{C}$ Isotopes. *Adv Bot Res* 2013; 67: 377-408
- Ghashghaie J, Badeck FW. Opposite carbon isotope discrimination during dark respiration in leaves versus roots – a review. *New Phytol* 2014; 201: 751-69
- Hoagland DR, Arnon DJ. The water-culture method for growing plants without soil. *California Agric. Exp. Station Circ.* 1950; 347: 1-32
- Lehmeier CA, Lattanzi FA, Schäufele R, Wild M, Schnyder H. Root and shoot respiration of perennial ryegrass are supplied by the same substrate pools: Assessment by dynamic ^{13}C labeling and compartmental analysis of tracer kinetics. *Plant Physiol* 2008; 148: 1148-58
- Lehmeier CA, Lattanzi FA, Schäufele R, Schnyder H. Nitrogen deficiency increases the residence time of respiratory carbon in the respiratory substrate supply system of perennial ryegrass. *Plant Cell Environ* 2010; 33: 76-87
- Lötscher M, Gayler S. Contribution of current photosynthate to root respiration of non-nodulated *Medicago sativa*: effects of light and nitrogen supply. *Plant Biol* 2005; 7: 301-10
- Loveys BR, Scheurwater I, Pons TL, Fitter AH, Atkin OK Growth temperature influences the underlying components of relative growth rate: an investigation using inherently fast- and slow-growing plant species. *Plant Cell Environ* 2002; 25, 975-87
- Mata C, Scheurwater I, Martins-Loucao MA, Lambers H. Root respiration, growth and nitrogen uptake of *Quercus suber* seedlings. *Plant Physiol Biochem* 1996; 34: 727-34

- Nogués S, Tcherkez G, Streb P, Pardo A, Baptist F, Bligny R, Ghashghaie J, Cornic G. Respiratory carbon metabolism in the high mountain plant species *Ranunculus glacialis*. *J Exp Bot* 2006a; 57: 3837–45
- Nogués S, Tcherkez G, Cornic G, Ghashghaie J. Respiratory carbon metabolism following illumination in intact French bean leaves using $^{13}\text{C}/^{12}\text{C}$ isotope labeling. *Plant Physiol* 2004; 136: 3245-54
- Nogués S, Damesin C, Tcherkez G, Maunoury F, Cornic G, Ghashghaie J. ^{13}C isotope labeling to study leaf carbon respiration and allocation in twigs of field-grown beech trees. *Rapid Commun Mass Spectrom* 2006b; 20: 219-26
- Nogués S, Aranjuelo I, Pardo A, Azcón-Bieto J. Assessing the stable carbon isotopic composition of intercellular CO_2 in a CAM plant using gas chromatography-combustion-isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2008; 22: 1017-22
- Pardo A, Aranjuelo I, Biel C, Savé R, Azcón-Bieto J, Nogués S. Effects of long-term exposure to elevated CO_2 conditions in slow-growing plants using a ^{12}C -enriched CO_2 -labelling technique. *Rapid Commun Mass Spectrom* 2009: 207-16
- Priault P, Wegener F, Werner C. Pronounced differences in diurnal variation of carbon isotope composition of leaf respired CO_2 among functional groups. *New Phytol* 2009; 181: 400-12.
- Poorter H, Remkes C, Lambers H Carbon and nitrogen economy of 24 wild species differing in relative growth rate. *Plant Physiol* 1990; 94: 621-27
- Poorter H, Van der Werf A, Atkin O K and Lambers H. Respiratory energy requirements of roots vary with the potential growth rate of a plant species. *Physiol Plant* 1991; 83, 469–75
- Scheurwater I, Cornelissen C, Dictus F, Welschen R and Lambers H. Why do fast- and slow-growing grass species differ so little in their rate of root respiration, considering the large differences in rate of growth and ion uptake? *Plant Cell Environ* 1998; 21: 995– 1005
- Scheurwater I, Dünnebacke M, Eising R, Lambers H. Respiratory cost and rate of protein turnover in the roots of a fast-growing (*Dactylis glomerata* L.) and a slow-growing (*Festuca ovina* L.) grass species. *J Exp Bot* 2000; 51: 1089-97

- Scheurwater I, Koren M, Lambers H, Atkin OK The contribution of roots and shoots to whole plant nitrate reduction in fast- and slow-growing grass species. *J Exp Bot* 2002; 53: 1635-42
- Schnyder H, Schäufele R, Lötscher M, Gebbing T. Disentangling CO₂ fluxes: direct measurements of mesocosm-scale natural abundance ¹³CO₂/¹²CO₂ gas exchange, ¹³C discrimination, and labelling of CO₂ exchange flux components in controlled environments. *Plant Cell Environ* 2003; 26: 1863-74
- Tcherkez G, Nogués S, Bleton J, Cornic G, Badeck F, Ghashghaie J Metabolic origin of carbon isotope composition of leaf dark respired CO₂ in French bean. *Plant Physiol* 2003; 131: 237-44
- Tcherkez G. Do metabolic fluxes matter for interpreting isotopic respiratory signals? *New Phytol* 2010; 186: 566-68
- Tcherkez G, Boex-Fontvieille E, Mahe A, Hodges M. Respiratory carbon fluxes in leaves. *Curr Opin Plant Biol* 2012; 15:308-14
- Turnbull MH, Whitehead D, Tissue DT, Schuster WSF, Brown KJ, Griffin KL. Scaling foliar respiration in two contrasting forest canopies. *Funct. Ecol.* 2003; 17: 101-14.
- Werner C, Wegener F, Unger S, Nogués S, Priault P. Short term dynamics of isotopic composition of leaf respired CO₂ upon darkening: measurements and implications. *Rapid Commun Mass Spectrom* 2009; 23: 2428-38.
- Wolfe DW, Gifford RM, Hilbert D, Luo Y. Integration of photosynthetic acclimation to CO₂ at the whole-plant level. *Glob Change Biol* 1998; 4: 879-93.
- Wright IJ, Reich PB, Westoby M. Strategy-shift in leaf physiology, structure and nutrient content between species of high and low rainfall and high and low nutrient habitats. *Funct. Ecol.* 2001; 15: 423-34.

Tables

Table 1. $\delta^{13}\text{C}$ of the CO_2 respired before and after labeling (‰), $\delta^{13}\text{C}$ of the CO_2 used during labeling (‰), $\Delta^{13}\text{C}$ measured during the labeling period (‰) and the % of new carbon in the CO_2 respired after labeling are shown for several C3 and one C4 (*Zea mays*) plant species. All species assimilated the same amount of labeled CO_2 (i.e. ca. 350 mmol C m⁻²). Data is the mean of (at least) three replicates \pm SE. *Ranunculus glacialis* data is from Nogués et al. (2006b). *Chamaerops humilis* and *Cycas revoluta* (gymnosperm) data is from Aranjuelo et al. (2009). *Phaseolus vulgaris* data is from Nogués et al. (2004). *Fagus sylvatica* data is from Nogués et al. (2006a).

Plant species	$\delta^{13}\text{C}$ (‰)			$\Delta^{13}\text{C}$ during labeling(‰)	New C in CO_2 (%)
	Before	After	Used during labeling		
<i>Ranunculus glacialis</i>	-23.4 \pm 0.7	-28.0 \pm 0.8	-51.2 \pm 0.1	20.4 \pm 0.7	9.6 \pm 1.1
<i>Chamaerops humilis</i>	-21.8 \pm 0.5	-23.2 \pm 0.3	-22.9 \pm 0.1	15.5 \pm 0.9	6.7 \pm 0.7
<i>Cycas revoluta</i>	-19.2 \pm 0.3	-23.3 \pm 0.2	-22.9 \pm 0.1	13.1 \pm 1.1	17.6 \pm 0.6
<i>Helianthus annuus</i>	-15.6 \pm 0.7	-38.1 \pm 1.0	-45.4 \pm 0.1	17.9 \pm 1.0	47.0 \pm 0.8
<i>Phaseolus vulgaris</i>	-22.0 \pm 0.6	-44.8 \pm 0.8	-51.2 \pm 0.1	20.1 \pm 0.2	50.3 \pm 0.9
<i>Fagus sylvatica</i>	-18.8 \pm 0.7	-44.5 \pm 0.9	-51.2 \pm 0.1	17.0 \pm 0.6	56.8 \pm 1.0
<i>Zea mays</i>	-15.4 \pm 0.5	-25.9 \pm 0.4	-37.9 \pm 0.1	5.3 \pm 0.5	46.7 \pm 0.9
<i>Medicago sativa</i>	-24.8 \pm 0.5	-31.3 \pm 1.3	-37.9 \pm 0.1	17.2 \pm 1.1	49.6 \pm 0.9
<i>Triticum turgidum</i>	-18.8 \pm 0.3	-27.0 \pm 1.3	-37.9 \pm 0.1	18.1 \pm 0.9	43.0 \pm 1.8
<i>Arundo donax</i>	-20.9 \pm 0.8	-28.5 \pm 1.1	-37.9 \pm 0.1	18.9 \pm 1.2	44.7 \pm 1.5

Table 2. Net assimilation rates (A_n , $\mu\text{mol m}^{-2} \text{s}^{-1}$), respiration rates in the dark (R_d , $\mu\text{mol m}^{-2} \text{s}^{-1}$), R_d/A_n ratios (%) and respiration quotients (RQ) are shown for mature leaves of the studied plant species. Measurements were done under atmospheric conditions at PPFD 1000-1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ or in the dark. Data is the mean of (at least) three replicates \pm SE. *Ranunculus glacialis* data is from Nogués et al. (2006b). *Chamaerops humilis* and *Cycas revoluta* (gymnosperm) data is from Aranjuelo et al. (2009). *Phaseolus vulgaris* data is from Nogués et al. (2004). *Fagus sylvatica* data is from Nogués et al. (2006a).

Plant species	A_n ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	R_d ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	R_d/A_n (%)	RQ
<i>Ranunculus glacialis</i>	12.5 \pm 0.9	2.2 \pm 0.3	17.0 \pm 1.3	1.1 \pm 0.1
<i>Chamaerops humilis</i>	3.12 \pm 0.2	0.3 \pm 0.1	9.6 \pm 0.3	0.9 \pm 0.1
<i>Cycas revoluta</i>	1.33 \pm 0.1	0.2 \pm 0.1	15.1 \pm 0.2	1.0 \pm 0.1
<i>Helianthus annuus</i>	17.9 \pm 3.4	1.0 \pm 0.2	5.6 \pm 1.5	1.0 \pm 0.1
<i>Phaseolus vulgaris</i>	12.4 \pm 0.2	0.9 \pm 0.1	7.3 \pm 0.2	1.0 \pm 0.1
<i>Fagus sylvatica</i>	7.2 \pm 1.5	0.9 \pm 0.5	13.0 \pm 1.7	n.d.
<i>Zea mays</i>	24.4 \pm 0.3	3.0 \pm 0.4	12.3 \pm 0.5	1.0 \pm 0.1
<i>Medicago sativa</i>	25.8 \pm 3.0	2.3 \pm 0.3	8.9 \pm 1.5	1.0 \pm 0.1
<i>Triticum turgidum</i>	18.8 \pm 2.4	1.7 \pm 0.9	9.0 \pm 1.6	1.0 \pm 0.1
<i>Arundo donax</i>	23.4 \pm 1.0	1.8 \pm 0.8	7.7 \pm 1.2	1.2 \pm 0.1

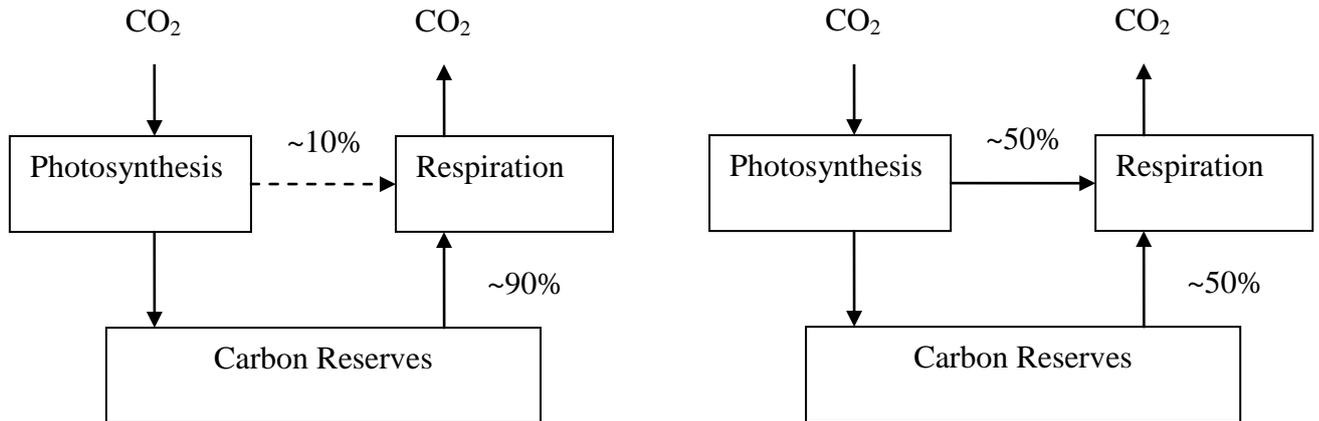
Figure Legends

Figure 1. Schematic representation of the contribution of new photoassimilates to respiration and to carbon reserves in mature leaves of several plant species. All the species assimilated the same amount of labeled CO₂ (i.e. ca. 350 mmol C m⁻²).

Figure 2. The relationship between the dark respiration (R_d) and photosynthetic rates (A_n) of the studied plant species. Each point represents a single plant species (they are the means of three replicate plants for each species and the standard errors are lower than 10% in all cases). Closed circles are slow-growing and open circles are fast-growing plants, respectively. The line represents regression ($r^2 = 0.76$; $P < 0.05$). Data from Table 2.

Figure 3. The relationship between the percentage of new carbon in the δ¹³C of leaf-respired CO₂ vs. plant shoot/root ratios of the studied plant species. Each point represents a single plant species (they are the means of three replicate plants for each species and the standard errors are lower than 10% in all cases). Closed circles are slow-growing and open circles are fast-growing plants, respectively.

Figure 1



R. glacialis
Ch. humilis
C. revoluta

H. annuus
P. vulgaris
F. sylvatica
Z. mays
M. sativa
T. turgidum
A. donax

Figure 2

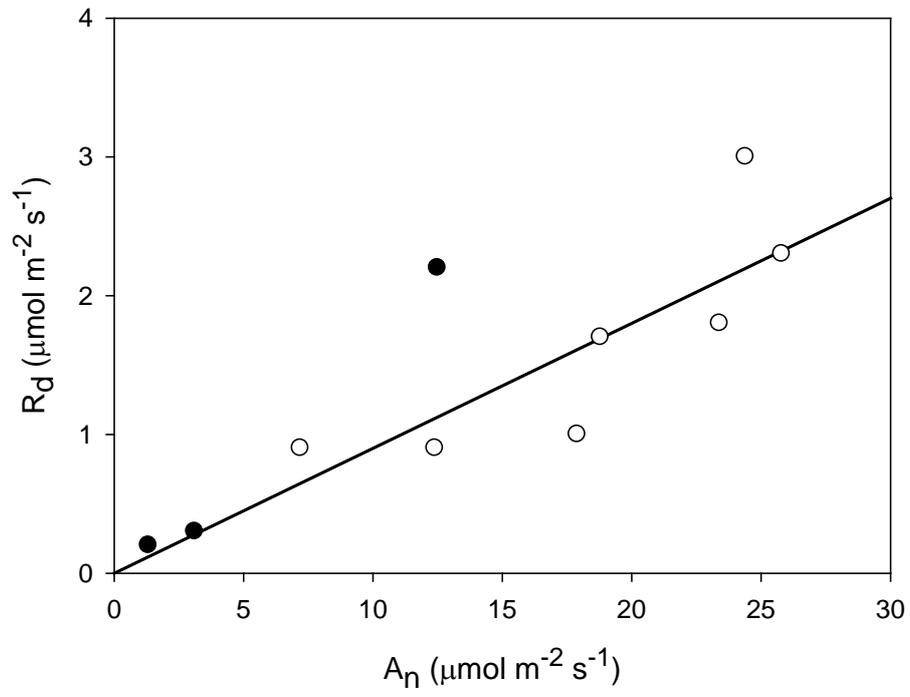
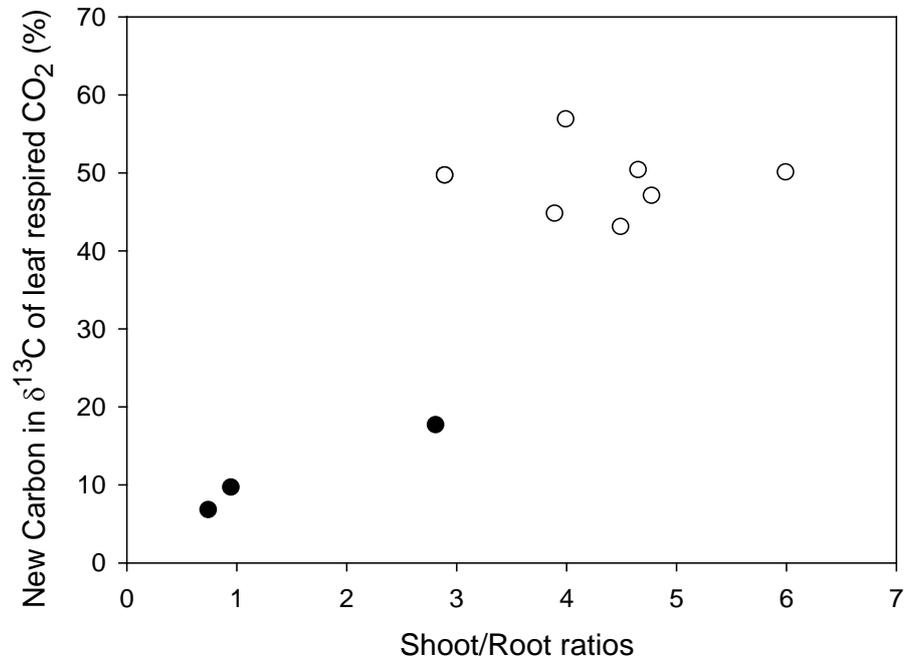


Figure 3



Supporting Information

Table S1. Sucrose and starch contents (%) and $\delta^{13}\text{C}$ (‰) are shown for leaves of some plant species before isotope labeling. Data is the means of (at least) three replicates \pm SE. *Ranunculus glacialis* data is from Nogués et al. (2006b). *Phaseolus vulgaris* data is from Nogués et al. (2004). *Fagus sylvatica* data is from Nogués et al. (2006a).

Plant species	Suc (%)	$\delta^{13}\text{C}$ Suc (‰)	Starch (%)	$\delta^{13}\text{C}$ starch (‰)
<i>Ranunculus glacialis</i>	1.0 \pm 0.2	-24.0 \pm 0.4	2.8 \pm 0.7	-25.2 \pm 0.5
<i>Helianthus annuus</i>	n.d.	-26.8 \pm 0.9	n.d.	-26.4 \pm 0.8
<i>Phaseolus vulgaris</i>	0.3 \pm 0.1	-27.2 \pm 0.5	1.9 \pm 0.5	-28.4 \pm 0.2
<i>Fagus sylvatica</i>	4.6 \pm 1.0	-23.7 \pm 0.6	3.7 \pm 0.9	-24.8 \pm 0.7