Phenotyping

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Introduction

This chapter deals with protocols for phenotyping that is the describing expressed characteristics of an organism, here *Medicago truncatula*, as model species. In this first version the chapter should be better named **shoot and root phenotyping** because one paragraph deals with shoot and the importance of a standard framework of description for a better understanding and three paragraphs deal with roots (description, nodulation, nitrogenase activity, carbon and nitrogen flux determination). Nothing odds as *Medicago truncatula* has been chosen as model species for symbiotic nitrogen acquisition by legume plant. Latter version will complete this chapter with other characteristics and notably with morphologic ones. At present this morphologic characterization is based on guidelines edited by IBPGR: the Descriptors for annual *Medicago.* These guidelines encourage collection of four categories of data: accession, collection, characterization and preliminary evaluation. This document is used by all curators of gene bank and allows a common language on accession description. Additional characteristics are under discussion and will be presented in second version.

1 A standard framework for facilitating the phenotyping of the shoot part

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A phenology key for characterising phenotypes

Having a standard phenology key, both used and understood by the whole *M. truncatula* community, is essential for facilitating and making more reproducible phenotyping approaches. In Chapter "Morphology, development and plant architecture of *M. truncatula*", a terminology for identifying organs and a system for the notation of the plant developmental stages have been described. These tools can be of practical use for characterizing and comparing *M. truncatula* phenotypes (Moreau et al. 2006).

A model of vegetative development for characterizing the temporal development pattern

Being able to identify phenotypic differences in the timing of establishment of the vegetative organs requires a framework that takes into account the temporal development pattern. In order to complete the phenology key, an ecophysiological model (Moreau et al. 2006) describing the temporal developmental pattern during the vegetative cycle, is presented Chapter "Mutant screening / phenology key".

Suggestion of simplified notations for phenotyping a large number of plants

Using these tools allows a precise characterization of plant development. However, when phenotyping a large number of plants, simplified notations can be performed in order to highlight genotypic traits:

- Only the rate of leaf appearance on the two first primary branches (B0 and B1) was shown to be a genotypic trait that was independent of environment (Moreau et al. 2006). So, the plant developmental stages and the development timing can be determined using observations performed only on B0 and B1 which are the most representative for characterizing genotypes;
- Repeated and regular observations can be performed throughout vegetative cycle. However, due to the linearity of these relationships for the primary branches, only 3 or 4 observation dates are sufficient to study development rates.

Practical applications of the framework

The use of these simplified notations should be helpful for:

- Standardizing how the plant material should be characterized to be used for functional genomics analyses: both the position of the organs and the developmental stage of the plants that have been used for biochemical or gene expression analysis (Cf. "Morphology, development and plant architecture of *M. truncatula* – Vegetative development");
- Predicting vegetative development (Cf. Chapter "Mutant screening / phenology key")

- Characterizing mutants for which the development of specific organs or of the whole plant is affected, allowing a more accurate phenotyping. (Cf. Chapter “Mutant screening / phenology key”)

Reference

Moreau D., Salon C. and Munier-Jolain N. (2006) Using a standard framework for the phenotypic analysis of *Medicago truncatula*: an effective method for characterizing the plant material used for functional genomics approaches. *Plant, Cell Env* **29**, 1087-1098.

2 - Acetylene reduction assay (ARA): protocol for estimating nitrogenase activity in *M. truncatula* root nodules

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Nitrogenase is an enzyme capable of catalyzing the reduction of N_2 to NH_3. It also has the capacity of reducing acetylene to ethylene. In the presence of 10% of acetylene, almost all electron flow through nitrogenase is used to reduce acetylene to ethylene. So, measuring the quantity of ethylene that is produced provides a way of estimating nitrogenase activity. Based on this principle, the Acetylene Reduction Assay (ARA) is a fast, inexpensive and easy to perform method (Hardy et al. 1968) that has extensively been used for measuring nitrogenase activity in root nodules of many legume species.

However, this technique has been disapproved because nitrogenase activity could decrease during the assay, due to the presence of acetylene. This "acetylene-induced decline" can occur within 8 minutes of exposure of the roots to acetylene and can decrease nitrogenase activity of more than 50% within 30 minutes (Minchin et al. 1983). This decline results from an O_2 limitation of nitrogenase activity, originating from an increase in the resistance to O_2 diffusion in the nodule cortex (Witty et al. 1984). Moreover, an increase in the resistance to O_2 diffusion in the nodule cortex can also originate from different practices performed during the assay, such as shoot removal or shaking root parts, resulting in an underestimation of the nitrogenase activity. In the case of detopped and shaken roots, the decrease due to disturbance can be effective within 15 minutes (Minchin et al. 1986).

The results of Minchin et al. clearly demonstrate that ARA does not allow quantifying total nitrogenase activity. However, if performed properly, it can be of practical interest for qualitative purposes, especially for identifying relative differences in nitrogenase activity. This method can be used for classifying treatments for which ARA measurements have been performed at a given date and in given environmental conditions (Vessey 1994); and also for detecting the presence of nitrogenase for instance for looking at time to onset of fixation or screening for effective/ineffective rhizobial strains (Minchin et al. 1994).

Here, we describe a protocol for ARA measurements that we have adapted to *M. truncatula*. This protocol deals with the traditional, closed acetylene reduction assay firstly described by Hardy et al. (1968): it is performed in closed vessels containing detopped nodulated root systems. This protocol has been successfully tested to compare nitrogenase activity between different treatments for A17 plants. It can be used to compare nitrogenase activity between genotypes or rhizobial strains and between biotic or abiotic treatments.

Practical recommendations for limiting errors of measurements

✓ Nitrogenase activity depends upon the environmental conditions in which measurements are made. As a consequence, it is necessary (1) to measure the environmental conditions during the ARA, and (2) that all measurements are made under constant environmental conditions in order to be able to compare different treatments. So:

- Measurements in glasshouse are not recommended because environmental conditions, such as temperature and radiation, can strongly fluctuate in short lapses of time;
- Changing the environmental conditions just before measurements have to be avoided: if plants have to be moved for instance from the glasshouse to the lab in which ARA is made, it is advised to move them the day preceding ARA.

✓ Nitrogenase activity is subjected to nyctemeral variations. So, measurements made on different treatments have to be performed at the same period of the day.

✓ In order to obtain reproducible data, our protocol requires 4 repetitions per treatments, corresponding to 4 vessels per treatment. In each vessel, 4 root systems are used. So, 16 plants per treatment are necessary. However, this is an "optimal" protocol that can be lightened according to the quantity of plant material available.

✓ In order to avoid the "acetylene induced decline" and the decline due to plant disturbance:

- It is necessary to minimize the time from sampling to initiating the measurements (Vessey 1994). In our protocol, all the measurements are achieved within 7 minutes after the injection of acetylene for each treatment (Table1); they are rigorously timed to have a perfectly identical procedure for all treatments;
- Shacking plants for suppressing the root medium is avoided: only the root medium that can be easily removed is suppressed.

Example of experimental design for comparing nitrogenase activity between 6 treatments

In this example, nitrogenase activity is compared for six different treatments. Four vessels containing four root systems are used for each treatment. Four series of six vessels are successively performed (Table1). In each series, one vessel corresponds to one treatment, and the vessels are randomized. For each vessel, 7 steps are necessary:

(a) Removing the plant from its pot;

(b) Gently removing the major part of the root medium without shaking roots;

(c) Cutting the shoot part at the level of the neck;

(d) Introducing the root part into the vessel and closing the vessel with a stopper;

(e) Drawing 10% of the air that is contained in the vessel;

(f) Injecting acetylene with a volume equal to 10% of the vessel volume;

(g) Regularly collecting gaseous samples from the vessel.

Each series of measurements lengthens 15 minutes and requires 5 operators whose tasks are presented in Table 1.

Table 1: Example of experimental design for a series of 6 vessels. This protocol requires 5 operators. Each of them is represented by one color. The operator in red also coordinates the different steps by indicating the time at each minute to all the operators.

Time (min)		Vessel 1	Vessel 2	Vessel 3	Vessel 4	Vessel 5	Vessel 6
From	To						
00:00	00:01	(a), (b), (c), (d)					
00:01	00:02	(e)	(a), (b), (c), (d)				
00:02	00:03	(f)	(e)	(a), (b), (c), (d)			
00:03	00:04		(f)	(e)	(a), (b), (c), (d)		
00:04	00:05			(f)	(e)	(a), (b), (c), (d)	
00:05	00:06	1ST sample			(f)	(e)	(a), (b), (c), (d)
00:06	00:07	2ND sample	1ST sample			(f)	(e)
00:07	00:08	2ND sample	2ND sample	1ST sample			(f)
00:08	00:09	3RD sample	2ND sample	2ND sample	1ST sample		
00:09	00:10	4TH sample	3RD sample	2ND sample	2ND sample	1ST sample	
00:10	00:11		4TH sample	3RD sample	2ND sample	2ND sample	1ST sample
00:11	00:12			4TH sample	3RD sample	2ND sample	2ND sample
00:12	00:13				4TH sample	3RD sample	2ND sample
00:13	00:14					4TH sample	3RD sample
00:14	00:15						4TH sample

Specific material for a series of 6 vessels

- ✓ 1 chronometer;
- ✓ 1 container for collecting the root medium;
- ✓ scissors for separating shoot and root parts;
- ✓ 6 containers for collecting shoot parts;
- ✓ 6 numbered vessels whose size can vary according to the size of the root parts: 600 mL is sufficient at the beginning of the vegetative cycle but at the end of the vegetative cycle vessels with a minimum volume of 1 L can be necessary;
- ✓ 6 stoppers with rubber septum;
- ✓ 30 numbered vacuum blood serum collection tubes (6 vessels* 5 tubes per vessel) (*Venoject* from Terumo®: www.terumo-europe.com);
- ✓ 5 tube holders (one per operator) (Terumo®: www.terumo-europe.com);

- ✓ 1 container with acetylene;
- ✓ 2 syringes for drawing air and injecting acetylene.

Data analysis

The quantity of ethylene that is contained into the vacuum blood serum collection tubes is estimated using gas chromatography. The treatments are then compared according to the rate of production of ethylene which is an indicator of the intensity of nitrogenase activity.

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3 Techniques for phenotyping root architecture and nodulation development

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This protocol of plant growth has been devised in order to perform rapid screening of numerous plants in mutant screens to characterize their nodulation and root phenotypes.

Seed disinfection and germination:
Prior to germination seeds were scarified with abrasive paper and sterilized for 15 min in 3.5% calcium hypochlorite. They were then sown in the dark at 4°C for 24h on filter-paper added with sterile distilled water in closed Petri dishes. Swollen seeds were then planted.

Plant growth conditions:
Substrate of growth was sand of a 2mm mean granulometry s sterilized by steam or a 1:1 attapulgite clay: clay pellet mixture. These substrates were put in plastic pots allowing a 16 cm depth of substrate. Plants were grown either isolated (1 plant / 1 liter pot) or in populations (100 plants/16 liters pot). Within 24h after planting, they were inoculated with a compatible *Sinorhizobium meliloti* strain. Plants were grown in a glasshouse with a 16h day photoperiod at a 15-22°C night-day mean temperature. Ventilation was assured for temperatures higher than 24°C. Plants were irrigated daily with N free solution (Sagan et al 1995, Morandi et al 2000) or with a solution of very low level of mineral nitrogen (0.625 meq of N). At these levels, nodulation is not inhibited.

Visual nodulation scoring

After 35 days of growth, roots were gently separated from the substrate, washed under running water and their nodulation was visually scored as

- Nod –- phenotype when no nodule could be observed
- Nod + Fix – phenotype with white nodules usually shorter than 2 mm
- Nod + Fix + phenotype (wild phenotype) with pink nodules usually longer than 2mm
- Nod ++ Fix+ phenotype for plants having at least 5 times more numerous nodules than the control

Image analysis for root and nodule characterization
After 35 days of growth, roots were gently separated from the substrate and washed under running water. Each root system was carefully spread out in a transparency film (310 by 425 mm), 24-bit color scanned at 300 dpi with a reflective lighting system, with a large format scanner (Epson Expression 1640XL). Root and nodule surface areas and lengths were

determined from this color image using WinRhizo version Pro2004a (Régent Insrument Inc., Québec) from differential determination of colors.

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4 C and N flux determination

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4.1 Overview on methods to measure symbiotic N acquisition by legume plant such as *Medicago truncatula*

Apart from the classical root absorption of soil mineral nitrogen resources legume plants have the particularity to acquire nitrogen (N) from the atmospheric dinitrogen reservoir via a symbiosis with *Rhizobia*. There are two groups of methods to measure the amount of N arising from symbiotic fixation. The first one consists in measuring the products of nitrogen fixation by the nitrogenase enzyme such as either i) the measurement of hydrogen evolution (Layzell et al; 1984), or ii) the reducing capacity of the nitrogenase in the case of the Acetylene Reduction Assay (ARA, Minchin et al., 1983; Witty et al., 1984), or iii) the N compounds assimilated and then vehiculated by saps as in the case of the measurement of ureides in tropical legumes (Hunt and Layzell, 1993). ARA is still very popular, and provides indication of total nitrogenase activity on detached roots; The measurement of H_2 evolution is non-invasive, non destructive but restricted to symbioses which have an uptake hydrogenase enzyme (HUP+); The measurement of ureides in xylem sap is not possible in the case of temperate legumes such as peas or *medicago* which export amides (Peoples et al., 1989). These methods are tedious, and only furnish snap shots concerning symbiotic fixation activity. Moreover, these are only semi-quantitative and only allow comparison between treatments.

Isotopic methods belong to the second group. They are the only quantitative methods to measure and relative contributions of symbiotic N fixation and mineral N assimilation. They rely on the use of differences in ^{15}N enrichment[1] between the sources of N potentially available for leguminous crops (atmospheric dinitrogen versus soil mineral nitrogen). The ^{15}N enrichment of a legume plant reflects the relative contribution of each of the N source. These methods do not disturb plant functioning and allow yield independent long term experiments. These methods can be used both in controlled conditions and in the field. Besides, isotopic methods can be also be used over short periods to measure C or N accumulation from one single source in the plant and to follow the fate of assimilates in the plant.

[1] The proportion of stable isotope ^{15}N in a plant part relative to the total amount of N atoms (^{14}N + ^{15}N) is called ^{15}N enrichment.

4.2 Isotopic methods to measure over a long time the relative contribution of Symbiotic Nitrogen Fixation and root assimilation to overall N acquisition by legume plants

Isotopic methods can be used either in the field or in the greenhouse to measure time-integrated relative contributions of symbiotic N fixation and mineral N assimilation. These methods require precise measurement of ^{15}N enrichment of the two sources of N available to the plant. The ^{15}N enrichment of atmospheric dinitrogen is rather stable and similar among locations. In the greenhouse, ^{15}N enrichment of mineral N is artificially enriched through the known composition of nutrient solution. In the field, if ^{15}N enrichment of the soil is not different enough from that of the air (Mariotti et al, 1983) it is artificially enriched through the application of labeled fertilizer. Because the ^{15}N enrichment of the soil is variable in space and time in the field (Rennie and Rennie, 1983), a non-fixing reference plant is used to provide realistic estimates of the ^{15}N abundance resulting from mineral N uptake by the fixing plant: its ^{15}N enrichment is assumed to be similar to that of soil mineral N, as it is its only N source. The non fixing reference plant therefore should have access during its growth cycle to the same soil N pools. As such it should have similar phenology, growth and root exploration as the legume, it should also be grown close to the legume, be subjected to the same labeled fertilizer additions if applied, and have similar water availability. The contribution of symbiotic fixation to overall N acquisition by the crop is calculated by the extent of dilution by the atmosphere N (reflecting symbiotic fixation in the legume) of the ^{15}N enrichment of soil mineral nitrogen (measured by the ^{15}N enrichment of the reference plant). Two main indirect methods are employed according to the extent of ^{15}N enrichment differential between the soil and the atmosphere.

Indirect Method	***Natural abundance (NA)***	***Isotopic Dilution method (ID)***
Principle	Exploits the natural differences in ^{15}N enrichment existing between the soil and the atmosphere (Amarger et al., 1979).	Involves the application of ^{15}N labeled fertilizer to enhance the difference in ^{15}N enrichment between the soil and atmosphere (Rennie and Rennie, 1983).
Applicability	Field	Field and greenhouse
Main hypothesis	Reference plant ^{15}N enrichment is supposed to be similar to that of mineral nitrogen assimilated by the legume plant.	^{15}N enrichment of the reference plant (Eref) grown with labeled ^{15}N fertilizer is assumed to represent that of mineral nitrogen arising from both soil and fertilizer. In the field, either soil is assumed to be uniformly labeled, or the fixing and reference plants are supposed to retrieve in similar proportions soil mineral N and N fertilizer In the greenhouse mineral N arises from the nutrient solution, the enrichment of which has to be constant throughout the experiment.
Limits and Constraints	Precision becomes critical when the ^{15}N differential between soil and atmospheric dinitrogen is lower than 0.0015 % or when the contribution of symbiotic fixation to overall nitrogen acquisition is low (Unkovich et al., 1994).	In the field, most of the cases soil is not uniformly labeled with the added fertilizer and, then because of different root exploration and retrieval activities, the main hypothesis of ID may not be satisfied. No constraints in the greenhouse

Specific treatments	none	In the field; ^{15}N-labelled fertilizer is applied at sowing as a solution both on the non fixing plant and legume plant (about 60 $mL.m^{-2}$). In the greenhouse, nutrient solution supplied to the plants is uniformly labeled throughout the whole experiment
Measurements and calculation before the experiment	Measurement of the amount and ^{15}N enrichment of N left over at sowing to determine if it will be sufficiently high.	In the field, measurement of the amount and ^{15}N enrichment of N left over at sowing to calculate the ^{15}N enrichment of the fertilizer to add. In the greenhouse, calculation of the enrichment of the nutrient solution as a function of duration of the experiment, presumed biomass accumulation and frequency of harvests.
Measurements at harvest	Measurement of ^{15}N enrichment of plant material	
Calculation	$$\%Ndfa = \frac{E_{\min eralN} - E_{leg}}{E_{\min eralN} - \varepsilon_{fix}} * 100$$ *% Ndf (Soil, fertilizer) = 100 - % Ndfa* % Ndfa : percentage of air derived nitrogen (symbtiotic N fixation) % Ndf (Soil, fertilizer) = percentage of soil (and fertilizer) derived N in the legume plant E_{leg} = ^{15}N-enrichment of the legume plant respectively. EMineral N: ^{15}N-enrichment of mineral N ie of the nutrient solution in the greenhouse or of the non fixing reference plant in the field. ε_{fix} is the isotopic fractionation (^{15}N discrimination relative to ^{14}N) due to symbiotic N fixation. It is measured as the ^{15}N-enrichment of the legume plant relying solely on symbiotic nitrogen fixation, usually in the range of - 1 ‰ to - 2 ‰. This parameter as to be determined with high precision when the differential between 15N enrichment of the air and mineral N is low (case of the NA method).	

4.3 Direct isotopic methods to measure over short time nitrogen or carbon acquisition and the fate of assimilates arising from a single labeled source

In controlled conditions when a legume plant rely exclusively either on symbiotic fixation of atmospheric nitrogen or root absorption of soil mineral nitrogen it becomes possible to measure nitrogen acquisition only by measuring biomass increment and nitrogen content. However as this method relies on destructive sampling, it is too imprecise on a short time interval due to inter plant heterogeneity because

The ^{15}N-label of the combined nitrogen (NO_3-, NH_4) in the nutritive solution allows measuring its absorption by the plants. Conversely, when root atmosphere is enriched with $^{15}N_2$ it allows to measure directly and with great efficiency symbiotic nitrogen fixation. To maintain a constant ^{15}N enrichment of the atmosphere surrounding the nodulated roots, the root system has to be confined within specifically designed container and the root atmosphere has to be either confined (closed circuit) or continuously flushed by a $^{15}N_2$- enriched flow (open circuit).

In the same way, special equipment with confined and regulated aerial atmosphere allows measuring C acquisition by photosynthesis and the fate of photo assimilates to the different organs, including the amount of C respired (Voisin et al, 2001). Relationship between C and N

metabolism can be achieved combining C and N labeling (Warembourg et al, 1989; Voisin et al, 2003).

Direct Method	$^{15}N_2$	$^{15}NO_3$
Principle	A known amount of $^{15}N_2$ is introduced in the root atmosphere. The plant assimilates through symbiosis the mixture of $^{15}N_2/^{14}N_2$ contained in the air surrounding nodulated roots.	The known and constant enrichment of $^{15}NO_3$ added in the nutritive solution allows to measure nitrate absorption by the plant.
Advantages	The only quantitative methods that allow labeling unambiguously N being reduced through symbiosis ($^{15}N_2$) or root absorption ($^{15}NO_3$). The ^{15}N enrichment of the labeled source (air or mineral N) has to be constant. When it is sufficiently high there is no need of an unlabeled plant as a control.	
Constraints	Experimental setup is complex because it needs to be gas proof. When the root atmosphere is closed, the O_2 and CO_2 concentrations have to be maintained through injection or withdrawal respectively. When the root atmosphere is continuously flushed with ^{15}N enriched mixture, the cost becomes detrimental for long term studies. Such labeling experiments are difficult to conduct on a long time scale.	None as the experimental setup only relies on the addition of labeled N compounds to the nutrient solution.
Harvest and measurements	Measurement of the ^{15}N enrichment in the root atmosphere during the labeling period.	Measurement of the ^{15}N enrichment in the nutrient solution supplied during the labeling period.
	At the end of the labeling experiment, measurement of the ^{15}N enrichment of the plant parts of labeled plants and unlabelled control plants.	
Calculation	$$\%Ndfs = \frac{E_{control} - E_{leg}}{E_{control} - E_{source}} * 100$$ % Ndfs = percentage of N in the legume plant derived from the labeled source ($^{15}N_2$ of the atmosphere or $^{15}NO_3$ for the nutrient solution) E_{leg} and $E_{control}$ = ^{15}N-enrichment of the legume plant and the non labeled control plant respectively. E_{source} = ^{15}N-enrichment of labeled source I;e; either the root atmosphere (labeling with $^{15}N_2$) or the nutritive solution respectively.	

4.4 Measuring Carbon flux

Direct Method	***$^{13}CO_2$***	***$^{14}CO_2$***
Principle	Label carbon dioxide to quantify the amount of C entering the plant through photosynthesis and track its fate (allocation within plant parts, respiration, exudation…).	
Main hypothesis	The stable (^{13}C) or radio labeled (^{14}C) isotope behaves like the natural (^{12}C).	
Advantages	Not dangerous Allows labeling from some minutes up to several days or weeks. No constraints	Inexpensive method No need of controlled plant Very sensitive method allowing very short time experiments (ie less than a minute).
Constraints	Necessitates a specific plant enclosure where to perform the labeling experiment and to maintain a stable ^{13}C (or ^{14}C) enrichment. Atmosphere has to be homogenous and similar for the various plants being labeled.	
	Expensive analysis and associated apparatus (mass spectrometer) Need of a control plant to measure natural ^{13}C enrichment as natural air ^{13}C enrichment is not neglect able Uneasy detection	Ionisation dangers Uses (and evacuation) are associated to very strict rules and regulations
Specific treatments	Before labeling, it may be necessary to flush the entire labeling chamber to get rid of all the unlabelled atmospheric CO_2 and then to inject a known mixture of $^{13}CO_2/^{12}CO_2$.	
Harvest and measurements	Measurement of the $^{13}C/^{14}C$ enrichment in the atmosphere during the labeling period	
	a gas sample is taken for measurement in mass spectrometry of the ^{13}C enrichment.	a gas sample is taken from the plant enclosure and bubbled in a KOH solution to trap carbon dioxide and count its radioactivity by scintillation counting.
	At the end of the labeling experiment, measurement of the $^{13}C/^{14}C$ enrichment of the plant parts.	
Calculation	$$\%Cdfp = \frac{E_{control} - E_{leg}}{E_{control} - E_{source}} * 100$$ % Cdfp = percentage of C in the legume plant derived from photosynthesis during the labeling period, relative to total carbon in the plants E_{leg} and $E_{control}$ = ^{13}C or ^{14}C-enrichment of the labeled legume plant and an unlabeled control plant. E_{source} = ^{13}C or ^{14}C-enrichment of the labeled plant atmosphere.	

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