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Dinitrogen Fixation

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Biological N_2 fixation is the conversion of atmospheric N_2 to NH_3 by bacteria. The reduction of the triple bond of N_2 to form NH_3 is performed under anaerobic conditions by the nitrogenase enzyme complex at the expense of 12–16 ATP with H_2 as a by-product. The NH_3 produced by dinitrogen fixation is subsequently assimilated into organic forms of nitrogen by standard metabolic pathways. This input of organic nitrogen from N_2 fixation is often the largest input to the nitrogen cycle of nonfertilized soil ecosystems.

The bacteria that fix N_2 are taxonomically diverse. They differ in their source of energy (some are phototrophs, but most are chemotrophs), carbon (some are autotrophs, but most are heterotrophs), their tolerance for oxygen, and the degree of their association with other organisms. This latter characteristic is the most important with respect to the measurement of N_2 fixation because it largely determines the magnitude of fixation. Free-living N_2-fixing bacteria, such as *Azotobacter* and the cyanobacteria, typically fix <5 kg N ha^{-1} y^{-1}; N_2-fixing bacteria intimately associated with plant roots, such as *Azospirillum,* may fix up to 50 kg N ha^{-1} y^{-1}, although <10 kg N ha^{-1} y^{-1} is more typical; whereas the root nodule–forming symbioses of *Rhizobium* and *Frankia* can fix several hundred kg N ha^{-1} y^{-1}.

Nitrogen fixation is often measured as part of an ecosystem's nitrogen cycle, in which case an annual rate of N_2 fixation is desired. Estimates of N_2 fixation are also made over shorter periods to assess the effects of environmental factors or treatment manipulations. These short-term measurements are often focused on understanding the controls and regulation of N_2 fixation.

Table 12.1. Major Characteristics of Methods Commonly Used to Measure N_2 Fixation

Method	Features
N accretion	Integrative, requires a control or baseline, requires accounting of other imputs and outputs, insensitive
Acetylene reduction	Short-term rate, requires conversion factor, sensitive, susceptible to interferences
^{15}N isotope dilution	Integrative, requires a control, moderately sensitive
^{15}N natural abundance	Integrative, requires a control, insensitive, sometimes only qualitative
$^{15}N_2$ incorporation	Short-term rate, moderately sensitive

Available Methods

Several methods have been devised to measure N_2 fixation (Tab. 12.1). As with all methods, each has advantages and disadvantages. The major considerations for selection are sensitivity, duration, sample type, and whether relative or absolute rates are required.

Nitrogen Balance

This method is also known as the nitrogen accretion or nitrogen difference method. In unmanaged ecosystems the approach has been either to measure the accumulation of nitrogen in ecosystem components over time, often decades (e.g., Youngberg and Wollum 1976), or to measure nitrogen accumulation along a chronosequence (e.g., Newton et al. 1968). Although any accumulation of nitrogen is obviously the net difference between all gains and losses of N, in undisturbed systems the net accumulation is often attributed exclusively to N_2 fixation because other gains and losses are assumed to balance each other.

The nitrogen difference approach has also been used in managed, particularly agricultural, ecosystems over shorter periods, usually one growing season. Most commonly this approach has been used to estimate symbiotic or associative N_2 fixation by comparing the accumulation of plant nitrogen between the fixing plant and a nonfixing control. Rates of N_2 fixation must be more than 20 kg N ha^{-1} y^{-1} to be determined by this approach (Weaver 1986).

A more detailed description of the nitrogen accretion method will not be given because it is relatively insensitive and its inherent assumptions may be unjustified. It should be noted, however, that periodic monitoring of ecosystem nitrogen pools may be useful at many sites for measuring long-term patterns of nitrogen accumulation, particularly sites that contain leguminous or actinorhizal N_2 fixing symbioses. See Chapter 5, this volume, for methods of soil nitrogen analysis.

Acetylene Reduction

The discovery 30 years ago that the nitrogenase enzyme will reduce acetylene (C_2H_2) to ethylene (C_2H_4) quickly led to the widespread use of the acetylene re-

duction assay as a measure of N_2 fixation. This assay has been used extensively because it has great sensitivity and is inexpensive. Its sensitivity allows short-term (minutes to days) measurements at even low levels of activity. It is less useful as an integrative measure, however, and has been found to have many shortcomings, including the variability of the factor to convert from ethylene production to N_2 fixation and the sensitivity of activity to sample handling. Disturbance effects are especially important when measuring associative or symbiotic N_2 fixation, which limits the utility of this method under field conditions. Despite these limitations, however, it is useful for comparative purposes and for qualitative assessment of N_2 fixation, and it can be used quantitatively under some circumstances if carefully used and calibrated.

^{15}N-Based Methods

Although it has long been recognized that ^{15}N can be used in various ways to assess N_2 fixation, the use of ^{15}N-based methods has expanded in recent years. This is partly because of dissatisfaction with nitrogen balance and acetylene reduction methods but probably more because of the declining cost of ^{15}N-labeled materials, the increased availability and sensitivity of mass spectrometers, and the lower cost of ^{15}N analysis. There are several ^{15}N-based methods, including

- labeling soils with ^{15}N and applying the principles of isotope dilution;
- taking advantage of variations in the natural abundance of ^{15}N in the atmosphere and soils; and
- using $^{15}N_2$ to measure directly ^{15}N incorporation by N_2 fixation.

^{15}N Isotope Dilution

Over the past decade, the ^{15}N isotope dilution method has become increasingly common in field studies of N_2 fixation. It has most often been applied to studies of symbiotic or associative N_2 fixation and is the most sensitive way to measure long-term rates of N_2 fixation.

The principle behind this method is to label soil with sufficient ^{15}N to raise it significantly above ^{15}N natural abundance and subsequently to compare the ^{15}N labeling of a putative N_2-fixing plant-microbe association with that of a non-N_2-fixing reference plant. The ^{15}N abundance of the reference plant is assumed to reflect the ^{15}N labeling of the plant-available soil N. The ^{15}N abundance of the putative N_2-fixing plant-microbe association should then lie somewhere between that of the control plant and that of the atmosphere.

In practice, there are numerous pitfalls to the ^{15}N isotope dilution technique. These include the uniformity with which soil is labeled with ^{15}N, whether the reference plant and N_2-fixing plant are similar in their spatial and temporal nitrogen uptake patterns, and what plant tissues should be sampled. These potential problems can be controlled and minimized, and are normally less severe than the shortcomings of other N_2 fixation methods. A greater difficulty to overcome, particularly with woody perennial plants, is scaling the fraction of nitrogen derived from N_2 fixation to an areal estimate.

^{15}N Natural Abundance

Many soils are enriched in ^{15}N relative to the N_2 in the atmosphere. Thus, plants with associative or symbiotic N_2-fixing bacteria will have a ^{15}N abundance intermediate between that of the atmosphere and that of reference plants taking up nitrogen from the soil, one that reflects the proportions of nitrogen from N_2 fixation and from nitrogen uptake. Multiplying the proportion that came from N_2 fixation by the total nitrogen content of the plant will yield an estimate of N_2 fixed.

This approach is straightforward in principle (Shearer and Kohl 1986) and has the advantage that soils do not have to be disturbed by the addition of ^{15}N-labeled materials. However, it also has many potential drawbacks, the major one being its sensitivity. Although the instrument precision of modern mass spectrometers has improved, replicate plant or soil samples seldom have a precision better than $\pm$ 0.2‰. For a soil that differs from the atmosphere by 4.0‰, this means that the proportion of nitrogen coming from N_2 fixation can be determined within only $\pm 5\%$ (e.g., Unkovich et al. 1994). Because many soils with native vegetation have ^{15}N abundances closer to the atmosphere than this (Hansen and Pate 1987), the use of variations in ^{15}N natural abundance may be limited to agricultural soils. It is also true that soils, and their associated vegetation, can show a high degree of spatial variability; the ^{15}N abundance of plants also varies from tissue to tissue and temporally over the growing season (Shearer et al. 1983; Selles et al. 1986; Bremer and van Kessel 1990). Several recent field studies (Bremer and van Kessel 1990; Stevenson et al. 1995) have shown that, despite this natural variability, the ^{15}N natural abundance method gave similar mean estimates of N_2 fixation as nitrogen balance or ^{15}N isotope dilution studies. It should be noted, however, that these same studies found no significant correlation in the percentage of nitrogen derived from the atmosphere when comparing the ^{15}N natural abundance method with other approaches based on individual measurements.

At the current time, it would appear that the ^{15}N natural abundance method could be used to qualitatively test plant tissue to assess for the presence of symbiotic N_2 fixation and as a semiquantitative measure of N_2 fixation, provided there is at least a 2–4‰ difference between the ^{15}N abundance of the soil and the atmosphere. Greater differences may allow this technique to be used quantitatively.

Because the basic principles for choosing reference plants and performing the isotope dilution calculations are the same for those of the ^{15}N isotope dilution method, a detailed protocol will not be presented for the ^{15}N natural abundance method.

$^{15}N_2$ Incorporation

The most direct way to demonstrate the presence of N_2 fixation is to measure the incorporation of ^{15}N into a sample exposed to $^{15}N_2$ (Warembourg 1993). Like the acetylene reduction assay, $^{15}N_2$ incorporation provides a short-term rate and requires a closed incubation system. Because of the need for a gastight assay system, $^{15}N_2$ incorporation is better suited for laboratory than field studies. Compared with the acetylene reduction assay, $^{15}N_2$ incorporation does not suffer from interferences

Table 12.2. Decision Matrix for Selecting the Most Appropriate Method for Measuring N_2 Fixation

		Type and Rate of Fixation		
Sample Duration	Sample Location	Nonsymbiotic (2 kg N · $ha^{-1} \cdot y^{-1}$ or 0.5 nmol $N_2 \cdot g^{-1} \cdot d^{-1}$)	Associative (20 kg N · $ha^{-1} \cdot y^{-1}$ or 5 nmol $N_2 \cdot g^{-1} \cdot d^{-1}$)	Symbiotic (200 kg N · $ha^{-1} \cdot y^{-1}$ or 50 nmol $N_2 \cdot g^{-1} \cdot d^{-1}$)
Short-term (<4 days)	Laboratory	Acetylene reduction	$^{15}N_2$ incorporation, acetylene reduction	$^{15}N_2$ incorporation, acetylene reduction
	Field	Acetylene reduction	Acetylene reduction	Acetylene reduction
Long-term (>28 days)	Laboratory	—	^{15}N isotope dilution	^{15}N isotope dilution
	Field	—	^{15}N isotope dilution	^{15}N isotope dilution

Notes: To select a method, first decide on the type of sample you will be using, followed by the desired duration of the measurement period, and whether the measurement is to be made in the laboratory or in the field. The approximate sensitivity represents the typical activity over an annual or daily period for the type of sample chosen. When more that two methods are given, they are listed in order of preference.

and, of course, does not require a conversion factor. It is slightly less sensitive than the acetylene reduction assay, however. A major use of $^{15}N_2$ incorporation has been to determine appropriate conversion factors for the acetylene reduction assay.

Recommended Protocols

As indicated by the previous discussion, there is no single, universal protocol for measuring N_2 fixation. Table 12.2 has been constructed to assist in the selection of the most appropriate method for a given sample type, duration, and location. For example, if one were interested in assessing N_2 fixation of bacteria associated with the rhizosphere of a Douglas fir seedling, one could perform a short-term assay in the lab using either acetylene reduction or $^{15}N_2$ incorporation. If only relative rates were needed, acetylene reduction would probably be the method of choice because it is less expensive. However, if actual N_2 fixation rates are needed, then $^{15}N_2$ incorporation might be more appropriate because no conversion factor is required. As suggested by Table 12.2, estimates of N_2 fixation in the field are generally best done by using ^{15}N isotope dilution. We will describe all three methods.

N_2 Fixation by Acetylene Reduction

Measurement of ethylene production is easy, inexpensive, and sensitive. It is ideally suited for short-term assays, although the results of several short-term assays made sequentially can be integrated to provide a long-term estimate of N_2 fixation. The acetylene reduction assay is best suited for litter, soil, or samples of woody debris, but it can be adapted for use with root systems or root nodules. As mentioned previously, the lack of a universal factor to convert from ethylene production to N_2 fixed is a drawback; a conversion factor must always be determined empirically for a given system using $^{15}N_2$ incorporation.

The following protocol is designed for use with samples of litter, soil, or woody debris, although modifications are noted for applying acetylene reduction to systems that include plants. This method is also described by Bergersen (1980), Silvester et al. (1989), and Weaver and Danso (1994).

Materials

1. Gas chromatograph. Acetylene and ethylene are measured using a gas chromatograph equipped with a flame ionization detector. A 1.5 m long × 3.2 mm diameter column packed with Porapak Q is commonly used to separate the gases of interest. Nitrogen is used as a carrier gas, and compressed air and H_2 are used to fuel the flame. A strip-chart recorder or integrator is used to measure the signal of the gas chromatograph. Ethylene standards are needed for calibration.
2. Acetylene. Acetylene can be purchased commercially or generated by reacting calcium carbide (CaC_2) with water (Weaver and Danso 1994). Generation from CaC_2 is preferred because the acetylene produced in this manner has

fewer contaminants. In either case, however, it is necessary to purify the acetylene by passing it through traps of concentrated sulfuric acid and water to remove residual contaminants. Mylar balloons or PVC beach balls are convenient for storage of purified acetylene. Remember that acetylene is a flammable gas and a potential explosion hazard.

3. Incubation vessel. Gastight incubation containers are needed for the incubations. These could consist of metal or plastic pipes used to take the sample and subsequently capped at each end with rubber stoppers or septa. Another convenient incubation vessel is a canning jar sealed with a canning lid fitted with a rubber septum. Other more sophisticated cuvettes have been developed for use with plant root systems and flow-through systems (Warembourg 1993; Silvester et al. 1989). The important point is that the system is gastight and sufficiently large so that there is an adequate supply of O_2 for the duration of the experiment but small enough to enhance sensitivity.
4. Miscellaneous equipment. Plastic syringes of various sizes for the addition of acetylene and collection of gas samples, and needles for the syringes are needed. Gas samples can be stored for a short period (<1 hour) in 1 mL plastic syringes fitted with stopcocks or with the needles stuck into a rubber stopper. For longer storage, evacuated, leak-proof vials can be used (see Chapter 10, this volume). With any type of storage container it is important to also store reference standards handled in the same manner as samples to account for contamination, leaks, and absorption of target gases.

Procedure

1. Five to ten replicate samples are normally taken for analysis of litter, soil, or woody debris. To avoid diffusion problems with samples of woody debris, it is best to sample by layer (e.g., bark, sapwood, or decay class) and to cut the wood from each layer into matchstick-sized pieces (Jurgensen et al. 1987; Griffiths et al. 1993). The volume or mass of each layer must also be known to calculate an overall rate.
2. The samples are placed into the incubation vessels, which are sealed gastight.
3. Acetylene is added to a final concentration of 10 kPa (10% v/v), and the headspace is mixed well. An internal standard, such as propane, can be added to estimate total gas volume if sample volume and water content varies. Two types of controls are needed:
   (a) one without a sample and with acetylene to check for contaminating levels of ethylene in the acetylene, and
   (b) one with a sample but without acetylene to measure background ethylene production.
4. Gas samples are taken periodically throughout the incubation for analysis of acetylene and ethylene. Concentrations of acetylene and ethylene are determined from standard curves. Because the ethylene produced is insignificant compared with the acetylene in the system, any decrease in acetylene concentration indicates a gas leak from the incubation vessel.

5. The volume of the headspace and sample dry weight are determined at the end of the incubation. Volume can be calculated:
   (a) by difference from the total volume of the container if the volume of the dry solid sample and water are known,
   (b) from dilution of an internal gas standard, e.g., propane, or
   (c) using a pressure transducer (see Chapter 14, this volume).

Calculations

Ethylene production rates (i.e., acetylene reduction rates) are obtained from the ethylene concentration versus time data. This can be done in four steps:

1. Correct for any contaminating ethylene contained in the acetylene by subtracting the ethylene contaminant concentration (C_c) from the ethylene concentration of each sample incubated in the presence of acetylene (C) to obtain the contaminant-corrected ethylene concentration (C_a) for each sample:

$$C_a = C - C_c$$

2. Calculate the rate of contaminant-corrected ethylene production by linear regression of contaminant-corrected ethylene concentrations with time of sampling. The slope (P_a) is the rate of increase in contaminant-corrected ethylene production. Ideally this should be a linear increase with an r^2 value greater than 0.9.
3. Adjust the contaminant-corrected ethylene production rate for background production of ethylene in the absence of acetylene. This is done by calculating the slope (P_b) of background ethylene production from the linear regression of background ethylene concentration with time of sampling and then subtracting this rate of background ethylene production from the rate of ethylene production in the presence of acetylene to give the background-corrected rate of ethylene production (P):

$$P = P_a - P_b$$

4. The acetylene reduction activity (ARA) is then calculated on a unit weight (e.g., $nmol \cdot g^{-1} \cdot d^{-1}$) or area basis as follows:

$$ARA = P \times H/D$$

where

ARA = acetylene reduction activity
P = ethylene production rate
H = headspace volume
D = sample dry weight or surface area

Conversion to amount of N_2 fixed can be done by dividing ARA by the empirical (using $^{15}N_2$) or theoretical ratios of moles of acetylene reduced per

mole of N_2 fixed. Because of the questionable nature of the theoretical stoichiometric conversion factor, it has become standard to simply report *ARA* when the $^{15}N_2$-derived factor is unavailable.

Special Considerations

Although sophisticated flow-through systems have been developed for the measurement of N_2 fixation associated with plant roots or nodules, a cuvette that encloses the root system and is sealed about the stem is probably the most practical (e.g., Warembourg 1993). In field situations, excised root segments containing nodules have been used for short-term (<3 minutes) in situ incubations.

The use of the acetylene reduction method with symbiotic root nodules or root-associated N_2-fixing bacteria has several unique difficulties, however. The most important problems are associated with the negative impact that disturbance of root systems (or shoots, for that matter) have on N_2 fixation (Boddey 1987; Giller 1987) or that acetylene may have on nodule physiology—the so-called acetylene-induced decline (Minchin and Witty 1989). Thus, when applying the acetylene reduction method for plant systems, it is important to minimize physical disturbance. The acetylene-induced decline is more difficult to overcome, although several studies have shown that the initial, high rate of ethylene production before the onset of the decline may give a reasonable estimate of N_2 fixation activity (Minchin and Witty 1989; Schwintzer and Tjepkema 1994). Thus, for excised root/nodule systems, 150 second incubations are used.

Rates of ethylene production in root nodules are often expressed on a per gram nodule basis and must therefore be multiplied by total nodule biomass per unit area when extrapolating to an area basis. Frequent temporal measurements must be made to accurately assess seasonal or annual fixation rates because symbiotic N_2 fixation is sensitive to plant phenology and fluctuations in temperature, moisture, and photoperiod. These extrapolations in space and time can be associated with significant error.

N_2 Fixation by $^{15}N_2$ Incorporation

The use of $^{15}N_2$ gas to directly determine the amount of N_2 fixed is most conveniently performed in the laboratory. As for the acetylene reduction method, the $^{15}N_2$ incorporation method is a short-term assay that requires a closed incubation system. Although it could be applied to samples of litter, soil, or woody debris, it is about one-tenth as sensitive as the acetylene reduction assay. Thus, the details of $^{15}N_2$ incorporation will be described for plant root systems. Additional details can be found in Warembourg (1993) and Weaver and Danso (1994).

Materials

1. Mass spectrometer. ^{15}N abundance is most accurately and precisely measured with a mass spectrometer (Hauck 1982; Mulvaney 1993; Hauck et al. 1994).

If a mass spectrometer is not available locally, a number of laboratories will perform ^{15}N analysis on a fee basis. The most critical aspect of preparing plant or soil samples for ^{15}N analysis is to make certain that the sample is very finely ground (40-mesh) to ensure sample homogeneity.

2. $^{15}N_2$. $^{15}N_2$ is available from several commercial sources, usually at 99 atom % ^{15}N. It can be purchased in lecture bottles or in sealed glass ampules. Although these are the most convenient forms in which to acquire $^{15}N_2$, it can also be made by oxidizing $^{15}NH_4^+$ salts with alkaline hypobromite (Warembourg 1993; Weaver and Danso 1994).
3. Incubation vessel. A closed, gastight system is needed. For litter, soil, and woody debris the same type of incubation system as described earlier for the acetylene reduction assay can be used. More elaborate systems may be needed for plant systems (e.g., Warembourg 1993). Small plants can be enclosed entirely, although care must be taken to minimize any excess heat load from exposure to growth lights. For larger plants simply seal root systems from the external atmosphere. As with the acetylene reduction assay, the gas volume of the incubation system is a compromise between minimizing the gas volume to reduce the amount of $^{15}N_2$ that must be used and having a sufficiently large volume for adequate aeration and dilution of any gaseous products that might interfere with the assay. Weaver and Danso (1994) suggest a ratio of 0.3 L gas phase volume per 1 g of plant tissue.
4. Miscellaneous equipment. Adequate conditions must be available for plant growth (e.g., environmental chamber, greenhouse) because N_2 fixation rates of plant-associated bacteria are dependent on photosynthetic rates. Plastic syringes and needles of various sizes are convenient for transferring and sampling gases. An oven for drying plant tissue and a device for finely grinding the dried tissue are needed. For small quantities, a mortar and pestle will work. As in all ^{15}N methods, care must be taken to avoid cross-contamination of samples.

Procedure

The protocol described is for use with plant-associated N_2 fixation, but it can easily be modified for litter, soil, or woody debris, which will likely require longer incubation times because of lower rates of N_2 fixation.

1. The plant may be grown in water culture, a soilless mix, or soil. It is important to maintain good plant growth conditions. Five replicates are often sufficient.
2. The plant's root system is placed in the gastight incubation vessel and sealed with a nontoxic substance, e.g., Terostat putty or adhesive mastic (Winship and Tjepkema 1990).
3. $^{15}N_2$ is added to the gastight system and the headspace mixed well. A nonlabeled control should also be used to determine the background ^{15}N abundance. Often 10 atom % $^{15}N_2$ is an adequate working concentration, however, this can be adjusted depending on the expected N_2 fixation rate, duration of the experiment, and mass spectrometer precision (Weaver and Danso 1994).

It may also be a good idea to add a small amount of an inert gas, such as He, to serve as an internal standard to check for gas leakage.

4. Shortly after the addition of $^{15}N_2$, a sample should be taken to determine the actual ^{15}N abundance of the headspace atmosphere. As stated earlier, the incubation length depends on the N_2-fixing activity and the $^{15}N_2$ abundance, although when using plants it is probably best to incubate through one or more diurnal cycle because N_2 fixation rates are dependent on photosynthetic activity. It is important that the O_2 concentration not be depleted during the incubation.
5. At the end of the incubation another gas sample should be taken for ^{15}N analysis and averaged with that of the initial gas sample to determine the mean atom % ^{15}N of the N_2 in the headspace.
6. The plant is then sampled for ^{15}N and total N analysis. Plant tissue is dried (70 °C) and ground (40-mesh). Drying and grinding the entire plant for analysis is simplest, although plant parts can be sampled, analyzed separately, and a dry mass weighted average based on nitrogen content used for calculations.

Calculations

It is most convenient to work in terms of atom % ^{15}N excess (AE). For plant tissue (AE_p), this is the difference in atom % ^{15}N between the labeled and control plant tissues. For the ^{15}N-labeled atmosphere (AE_a) this is the difference in the mean atom % ^{15}N of the N_2 in the headspace and that of the unlabeled atmosphere, or natural abundance (0.3663 atom % ^{15}N).

The fraction of nitrogen the plant derived from N_2 fixation (*FNA*) is calculated by dividing the atom % ^{15}N excess of the plant tissue (either whole plant or weighted average based on nitrogen content of various plant parts) by the average atom % ^{15}N excess of the labeled atmosphere:

$$FNA = AE_p/AE_a$$

where

FNA = fraction of plant nitrogen derived from N_2 fixation, which can be expressed as a percentage by dividing by 100 to give the percent nitrogen derived from the atmosphere (commonly known as % Ndfa)
AE_p = atom % ^{15}N excess of the plant tissue
AE_a = mean atom % ^{15}N excess of the N_2 in headspace

If plant parts are analyzed separately, then a weighted atom % ^{15}N excess must be used in place of AE_p.

$$WAE_p = \Sigma AE_{p,i} \times TN_i/\Sigma TN_i$$

where:

WAE_p = total nitrogen weighted average atom % ^{15}N excess of the plant
$AE_{p,i}$ = atom % ^{15}N excess of the ith plant tissue
TN_i = total nitrogen content of the ith plant tissue

Multiplying *FNA* by the total plant nitrogen content gives the total amount of N_2 fixed, which can be expressed as a N_2 fixation rate by dividing by the incubation time and normalizing to the desired plant metric, e.g., nodule dry weight, total plant mass, leaf area index.

N_2 Fixation by ^{15}N Isotope Dilution

The ^{15}N isotope dilution approach labels the soil, or nutrient solution, with ^{15}N instead of the N_2 in the atmosphere. This is usually much simpler than labeling atmosphere, which makes this method amenable to the field as well as the laboratory. It also provides an integrative measure of N_2 fixation, which is difficult with either the $^{15}N_2$ incorporation or the acetylene reduction methods. Although ^{15}N isotope dilution has become the method of choice for field studies of N_2 fixation, there are limitations that must be evaluated carefully (Chalk 1985; Danso 1986; Vose and Victoria 1986; Winship and Tjepkema 1990; Danso et al. 1992; Warembourg 1993; Weaver and Danso 1994).

The following protocol describes the application of ^{15}N isotope dilution to the field measurement of N_2 fixation by associations of N_2-fixing bacteria and nonwoody plants, annuals in particular. Modifications of the method for use with perennial woody plants are also given.

Materials

1. ^{15}N -enriched fertilizer. Most commonly ^{15}N-enriched urea, ammonium salts, or nitrate salts are used to enrich the soil solution, with ammonium sulfate often being the least expensive. Many companies sell ^{15}N-labeled materials.
2. Reference plants. The selection of appropriate reference plants as a control for determining N_2 fixation by ^{15}N isotope dilution is critical. The ideal reference plant should not fix N_2 and should have a root system that exploits about the same volume of soil, that grows and take up soil nitrogen in the same temporal pattern, and that shows similar response to environmental factors and cultural manipulations. Nonnodulating varieties of some grain legumes (e.g., soybeans) exist and may be a good choice. If a soil is devoid of the nodulating N_2-fixing bacteria, then inoculated plants can be compared with noninoculated plants. Sudan grass has been suggested as a good reference plant for grain legumes and perennial ryegrass for pasture legumes. There is no standard reference plant for native plant species, and it is often impractical to introduce a reference plant. Thus, non-N_2-fixing plants already occupying the site are commonly used as reference plants. Careful selection of preexistent reference plants is required with the goals of selecting species with similar rooting habit and temporal patterns of nitrogen uptake.
3. Mass spectrometer. ^{15}N abundance is most accurately and precisely measured with a mass spectrometer (Hauck 1982; Mulvaney 1993; Hauck et al. 1994). If a mass spectrometer is not available locally, several laboratories will perform ^{15}N analysis on a fee basis. The most critical aspect of preparing plant

or soil samples for ^{15}N analysis is to make certain that the sample is very finely ground (40-mesh) to ensure sample homogeneity.

4. Miscellaneous equipment. An oven for drying plant tissue and a device for finely grinding the dried tissue are needed. For small quantities, a mortar and pestle will work. As in all ^{15}N methods, care must be taken to avoid cross-contamination of samples.

Procedure

1. Field plots need to be selected and designed. A randomized complete plot design with six replicates is normally sufficient. The size of the plots is dependent on the type of plant. For grain legumes, 1–5 m^2 plots that encompass four to five rows are adequate; 1–2 m^2 plots are adequate for pasture legumes. Often ^{15}N-fertilized subplots are located within each plot to reduce the cost of ^{15}N fertilizer. The nonfixing reference plants should be located as close as possible to the N_2-fixing plants and can be intermixed in pasture systems.
2. The ^{15}N fertilizer is applied to plots of the fixing and nonfixing plants at the same time and rate. Unlabeled plots containing fixing and nonfixing plants should also be established to determine the background ^{15}N abundance of the fixing and nonfixing plants. For most situations, adding about 1 kg ^{15}N/ha is adequate for a study lasting 1 year (e.g., an enrichment of 5–10 atom % ^{15}N applied at rates of 5–10 kg N/ha). It is important that the total amount of nitrogen added is not high enough to adversely affect N_2 fixation rates. Sometimes ^{15}N-enriched organic materials are used (e.g., plant residues or animal manures). When using organic materials, the suggested 1 kg ^{15}N/ha is probably still a good guide. The ^{15}N-labeled material can be added in many different ways, although application as a solution using a sprayer is often the most convenient and provides for uniform labeling of soil. There may be some advantages to labeling the soil in advance to allow for the added ^{15}N to equilibrate with the native soil nitrogen or for adding the ^{15}N in multiple additions, particularly for perennial plants (Baker et al. 1995). Care should be taken that ^{15}N is applied only where desired to minimize potential contamination between plots.
3. The ^{15}N isotope dilution approach is integrative, although it is possible to sample plants periodically over the growing season to determine N_2 fixation over different times. More commonly, however, plants are harvested at the end of the growing season. Because there are usually some differences in the ^{15}N abundance of different plant parts, it is important to either harvest and process the entire plant or to carefully sample the different tissues to obtain a weighted average.
4. Plant tissues are oven dried at 70 °C and then finely ground (40-mesh) for analysis.

Calculations

It is most convenient to work in terms of atom % ^{15}N excess. The atom % ^{15}N excess of the fixing plant (AE_f) and reference plant (AE_r) are defined as

$$AE_f = A_{fl} - A_{fu}$$
$$AE_r = A_{rl} - A_{ru}$$

where

A_{fl} = atom % ^{15}N of the fixing plant grown on the ^{15}N-labeled plot
A_{fu} = atom % ^{15}N of the fixing plant grown on the unlabeled plot
A_{rl} = atom % ^{15}N of the reference plant grown on the ^{15}N-labeled plot
A_{ru} = atom % ^{15}N of the reference plant grown on the unlabeled plot

The fraction of nitrogen coming from (*FNA*) is calculated as

$$FNA = 1 - AE_f/AE_r$$

If plant tissues are analyzed separately, then *WAE* (calculation shown earlier) can be used in place of *AE*.

Converting *FNA* to an areal N_2 fixation rate requires information about the biomass and N content of the N_2-fixing plant. Typically this is performed by multiplying *FNA* by the total N concentration of the plant and by the biomass of the N_2-fixing plant on an areal basis (e.g., kg/m).

Special Considerations

Adapting the ^{15}N isotope dilution method for use with woody perennial plants requires several modifications (Danso et al. 1992; Parrotta et al. 1994; Baker et al. 1995).

1. Plot size. For N_2-fixing trees, larger plots are needed, with crown size being a reasonable guide to the size of the ^{15}N-labeled area. It is common to use paired-tree plots to conserve the amount of ^{15}N fertilizer needed. This can be facilitated by using paired fixing and reference plants or having N_2-fixing and reference plants located in a checkerboard pattern.
2. ^{15}N labeling. Several (two or three) applications over the growing season will better label the entire rooting volume.
3. There is no standard reference plant for trees or shrubs; therefore, one often has to use the nonfixing plants that are present on the site. This has obvious disadvantages, but if the fixing plant gets most of its nitrogen from N_2 fixation, any deviations from the "ideal" reference plant may not be very important (e.g., Unkovich et al. 1994).
4. An additional problem with perennial plants is that these plants also contain a reserve pool of nitrogen in addition to nitrogen taken up from the soil or N_2 fixed. Baker et al. (1995) have developed an equation that accounts for the influence of plant nitrogen reserves. This calculation requires the measurement of total nitrogen at each time interval as well as ^{15}N abundance.
5. When calculating the amount of N_2 fixed on an areal basis for woody perennials, it is necessary to multiply the fraction of nitrogen derived from N_2 fixation by the nitrogen increment of above- and belowground tissues rather than

the total biomass of these tissues. Accurate estimates of plant nitrogen increment are not easy to obtain and make scaling to an areal basis difficult at best.

As mentioned previously, using differences in the natural ^{15}N abundance of N_2-fixing and nonfixing reference plants to calculate *FNA* has many similarities to the isotope dilution method, except that no ^{15}N fertilizer is used. Reference plants must be chosen with care, and the native soil must be sufficiently different in ^{15}N abundance (at least 2‰) than that of the atmosphere. Additional details can be found in Danso et al. (1992), Shearer and Kohl (1993), and Weaver and Danso (1994).

Conclusions

There are many approaches to measuring N_2 fixation, none of which is universally applicable. It is possible, however, to wisely choose the most appropriate method for a given system and thereby obtain reasonable estimates of N_2 fixation rates. For most field studies, particularly those measuring annual N_2 fixation rates, the ^{15}N isotope dilution method is recommended. For shorter-term assays or measurements of free-living N_2 fixation, the acetylene reduction method calibrated with the $^{15}N_2$ incorporation method, or just the $^{15}N_2$ incorporation method itself, is recommended.

Although a significant amount of research has been performed on N_2 fixation methodology, there are still relatively unexplored areas that need further research. It would be useful to reevaluate whether the acetylene reduction assay can be adapted for use with root nodule symbioses in the field to give similar estimates of N_2 fixation compared with the ^{15}N isotope dilution method. More work is also needed on questions regarding the measurement of N_2 fixation of root-nodulated woody perennials, e.g., differentiating the importance of the reserve pool of plant nitrogen and ways of estimating yearly increments of root and nodule biomass. Further evaluation of the use of variations in ^{15}N natural abundance to quantitatively measure N_2 fixation rates is also warranted because of the approach's potential as a relatively nondisruptive means of estimating annual rates of N_2 fixation.

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STANDARD SOIL METHODS

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