Field application of the $^{15}$N isotope dilution technique for the reliable quantification of plant-associated biological nitrogen fixation

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Abstract

To apply the isotope dilution (ID) technique, it is necessary to grow the "$N_2$-fixing" crop in a soil where the mineral N is labelled with $^{15}$N. Normally the "$N_2$-fixing" crop and a suitable non-$N_2$-fixing control crop are grown in the same labelled soil and the $^{15}$N enrichment of the control crop is assumed to be equal to the $^{15}$N enrichment of the nitrogen (N) derived from the soil in the "$N_2$-fixing" crop. In this case the proportion of unlabelled N being derived from the air via biological N$_2$ fixation (BNF) in the "$N_2$-fixing" crop will be proportional to the dilution of the enrichment of the N derived from the labelled soil.

To label the soil, the technique most often used is to add a single addition of $^{15}$N-labelled N fertilizer shortly before, at, or shortly after, the planting of the crops. Data in the literature clearly show that this technique results in a rapid fall in the $^{15}$N enrichment of soil mineral N with time. Under these conditions, if the control and the "$N_2$-fixing" crops have different patterns of N uptake from the soil they will inevitably obtain different $^{15}$N enrichments in the soil-derived N. In this case the isotope dilution technique cannot be applied, or if it is, there will be an error introduced into, the estimate of the contribution of N derived from BNF.

Several experiments are described which explore different strategies of application of the ID technique to attenuate the errors involved. The results suggest that it is wise to use slow-release forms of labelled N, or in some cases, multiple additions, to diminish temporal changes in the $^{15}$N enrichment of soil mineral N. The use of several control crops produces a range of different estimates of the BNF contributions to the "$N_2$-fixing" crops, and the extent of this range gives a measure of the accuracy of the estimates. Likewise the use of more than one $^{15}$N enrichment technique in the same experiment will also give a range of estimates which can be treated similarly. The potential of other techniques, such as sequential harvesting of both control and test crops, are also discussed.

Introduction

Of the techniques available to quantify the contribution of biological N$_2$ fixation (BNF) to nodulated legumes and other "$N_2$-fixing" plants, only techniques which utilize the stable isotope $^{15}$N can provide direct estimates of the quantity of biologically-fixed N incorporated into plant tissue. The use of $^{15}$N-labelled N$_2$ gas is usually only feasible for short-term experiments under controlled conditions, but the $^{15}$N isotope dilution (ID) technique can be applied to field studies over the whole plant growth cycle.

To apply the ID technique it is necessary to grow the "$N_2$-fixing" legume in a soil where the mineral N is labelled with $^{15}$N. If the $^{15}$N enrichment of the N being absorbed from the soil by this plant is known, then the amount of unlabelled N being derived from the air via BNF will be proportional to the dilution of the enrichment of the N derived from the labelled soil.

Normally the "$N_2$-fixing" crop and a suitable non-$N_2$-fixing control crop are grown in the same labelled soil and the $^{15}$N enrichment of the control crop is regarded as the $^{15}$N enrichment of the N in the legume derived from the soil. In fact, if the $^{15}$N enrichment of the labelled soil N can be determined by direct analysis of the $^{15}$N enrichment of the soil mineral N [12,19,38], then the use of control crops can be dispensed with.
In the case where a non-N\textsubscript{2}-fixing control crop is utilized, the basic assumption made in applying the technique is that the \textsuperscript{15}N enrichment of the N derived from the soil by the "N\textsubscript{2}-fixing" crop is equal to the \textsuperscript{15}N enrichment of the N in the control crop. Alternatively this can be expressed as: The ratio (R) of labelled fertilizer N to unlabelled soil N accumulated by the plants is the same for the "N\textsubscript{2}-fixing" crop and the control crop.

**Labelling the soil N with \textsuperscript{15}N**

**Addition of soluble labelled N fertilizer**

The technique most often used is to add a single addition of soluble \textsuperscript{15}N-labelled N fertilizer (eg. ammonium sulphate or urea) to the surface of the soil shortly before, at, or shortly after, the planting of the crops. This form of \textsuperscript{15}N addition results in a rapid fall in the \textsuperscript{15}N enrichment of soil mineral N, as the \textsuperscript{15}N-labelled N is added to the soil mineral N pool which is continuously being replenished by unlabelled N from the mineralization of soil organic matter \cite{17,38,49}. This is illustrated by data from a recent field experiment where \textsuperscript{15}N-labelled ammonium sulphate (10 kg N ha\textsuperscript{-1}) was added 1 day after planting of *Phaseolus vulgaris* (Fig. 1 - Boddey et al., unpubl. data). Under these conditions if the control and the legume crops have different patterns of uptake of N from the soil they will inevitably obtain different \textsuperscript{15}N enrichments in the soil-derived N. As is evident from the basic assumption of the technique this mean that the isotope dilution technique cannot be applied, or if it is, there will be an error introduced into the estimate of the contribution of N derived from BNF \cite{3,11,14,35,45,50,51,52}.

The obvious solution to this problem would appear to be to select a non-N\textsubscript{2}-fixing control crop which has the same soil-N uptake pattern as the "N\textsubscript{2}-fixing" crop. While the uptake of soil N by a non-N\textsubscript{2}-fixing crop can be studied by sequential harvests, this is not possible in the case of the "N\textsubscript{2}-fixing" crop as there is no way to distinguish between unlabelled N derived from soil and that derived from BNF.

Ledgard et al. \cite{28} developed a technique to compare the ratio (R) of fertilizer N to soil N in "N\textsubscript{2}-fixing" and control crops which depended on the use of increasing additions of labelled N and the measurement of the natural \textsuperscript{15}N abundance in both crops in a treatment where no N fertilizer was added. The technique requires great care to be taken to avoid contamination of the unfertilized plots with enriched N and the use of a sensitive (double inlet) mass spectrometer. While the technique can theoretically be used to select appropriate control crops for any particular legume crop (any control crop which attained a \textsuperscript{15}N enrichment similar to the enrichment of the N derived from the soil by the legume), the extra work involved and its low sensitivity in soils with low natural \textsuperscript{15}N abundance \cite{3}, has not encouraged its application by other workers.

A further technique was explored by Wagner and Zapata \cite{46} based on the interesting idea that if the ratio of added labelled to native unlabelled sulphur taken up from the soil by the two crops is equal, then the ratio of labelled to unlabelled N should also be equal. As both S and N are controlled in the soil by similar microbiological processes the idea was logical. However, in two experiments designed to test this technique, Hamilton et al. \cite{20,21} found that there was no fixed relationship between the ratios of labelled to unlabelled S and labelled and unlabelled N among the different control crops and legume crops and thus the ratio of labelled to unlabelled S could not be used as evidence for equal (or unequal) ratios of labelled to unlabelled N derived from soil sources by the different crops.

Rennie and Thomas \cite{36} suggested that it was possible to test if the \textsuperscript{15}N enrichment of the soil N taken up by the "N\textsubscript{2}-fixing" and control was equal, by calculating the soil 'A-value' for each of the crops from the % Ndff and % Ndfs (%N derived from fertilizer and soil, respectively). They quite correctly stated that if the 'A\textsubscript{s}-value' was equal for the "N\textsubscript{2}-fixing" and control crops then the two crops did remove N from the soil...