Plant and Soil **144**: 23–29, 1992.

PLSO 9127

An indirect method for estimating ^{15}N isotope fractionation during nitrogen fixation by a legume under field conditions

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Received 14 March 1991. Revised March 1992

Key words: biological nitrogen fixation, isotope fractionation, isotope discrimination, natural abundance, ^{15}N

Abstract

A new technique is proposed for measuring ^{15}N isotope fractionation during N fixation that obviates some of the possible disadvantages of existing methods. Accurate calculation of N fixation by legumes using the ^{15}N natural abundance technique requires a value for the isotopic composition of fixed N as an input. Isotopic fractionation in fixed N in legumes has usually been measured using N- free solution culture but results can vary with *Rhizobium* strain and growth conditions. The proposed method avoids these problems and can be used as an integral part of a field experiment for evaluating N fixation.

The technique is essentially a process of adjusting values of $\delta^{15}N^{a}$ for fixed N until % N fixation calculated by the ^{15}N natural abundance method best matches % N fixation estimated by the ^{15}N enrichment method. The use of high % N fixation values improves the sensitivity and reliability of the method.

A field evaluation of this comparison technique using chickpea (*Cicer arietinum* L.) provided a ^{15}N isotope fractionation factor (−2.37‰) for fixed N close to that obtained by N-free solution culture methods (−2.10‰). The availability of these two independent techniques allowed mutual corroboration of estimates of ^{15}N isotope fractionation during N fixation.

Introduction

The ^{15}N isotope dilution technique has become a popular method for estimating N fixation in legumes principally because it provides fixation estimates integrated over time. The principles of this technique are well-known (La Rue and Patterson, 1981) and are not described here.

There are two variations of the ^{15}N isotope dilution technique; one involves enrichment of available soil N by additions of ^{15}N enriched fertilisers (the ^{15}N enrichment method) and the other makes use of natural ^{15}N enrichment of available soil N (the ^{15}N natural abundance method).

Although the latter method was originally thought to give only qualitative or semi-quantitative information, it has given results similar to those from the ^{15}N enrichment method and with similar precision (Ledgard and Peoples, 1988; Shearer and Kohl, 1986).

Estimating N fixation by the ^{15}N natural

[a] In our calculations one $\delta^{15}N$ unit is an enrichment above the natural abundance value equal to 1/1000 of the natural abundance value for atmospheric N. Atmospheric N is taken as 0.3663 atom % ^{15}N. Therefore 1 $\delta^{15}N$ unit expressed in parts per thousand (‰) equals 0.3663×10^{-3} atom % ^{15}N enrichment.

abundance technique requires an adjustment for fractionation between ^{15}N and ^{14}N isotopes during the process of N fixation. During N fixation, the heavier ^{15}N isotope is usually discriminated against (La Rue and Patterson, 1981) so that atmospheric N fixed by the plant in depleted in ^{15}N relative to the natural abundance level in atmospheric N. Shearer and Kohl (1988) have shown that ^{15}N enrichment of fixed N may also occur indicating that N fixation by legumes can lead to either ^{15}N enrichment or depletion.

The equation below, which is essentially that shown in Ledgard and Peoples (1988), may be used for calculating the percent of total legume N derived from fixation.

$$\% \text{ N fixed} = 100\left[\frac{\delta^{15}\text{N control} - \delta^{15}\text{N legume}}{\delta^{15}\text{N control} - \text{B}}\right] \quad (1)$$

where 'δ^{15}N control' and 'δ^{15}N legume' are the per mil ^{15}N enrichment of N in the control crop and legume crop and 'B' is the $\delta^{15}N$ of fixed N for the legume, all with reference to the natural abundance standard.

Equation 1 can be used to estimate % N fixed with both variations of the ^{15}N isotope dilution technique, however, B becomes an important component of the equation only when the ^{15}N isotope dilution technique is used with ^{15}N enrichment approximating natural abundance levels (Ledgard and Peoples, 1988; Shearer and Kohl, 1986).

Importance of using a correct B value

Accurate estimation of % N fixation using the ^{15}N natural abundance technique requires an

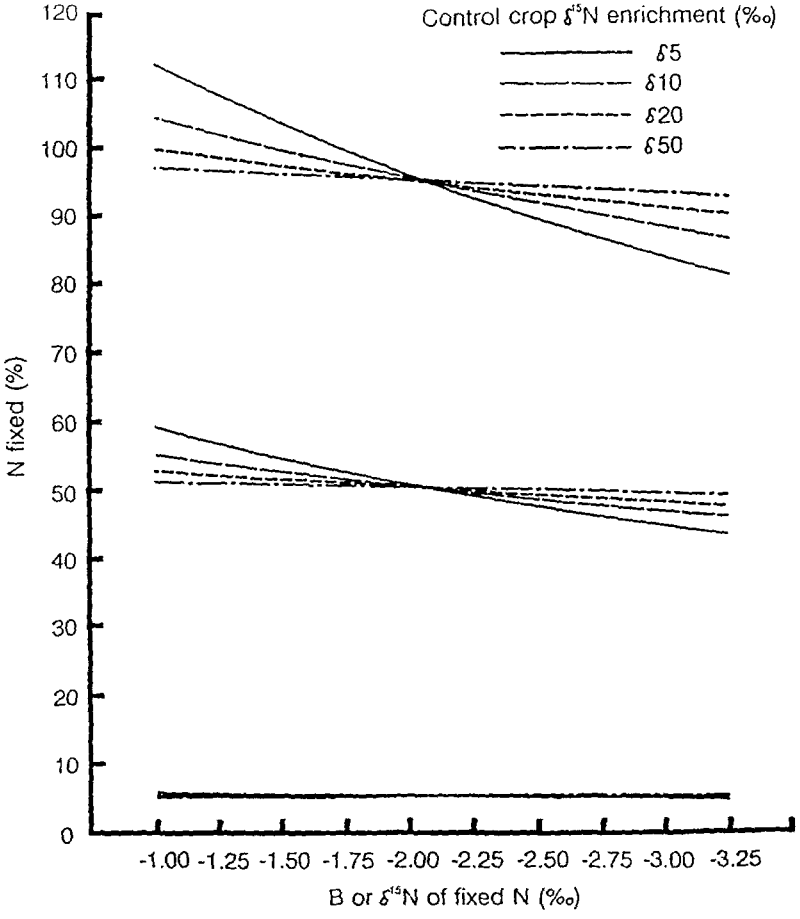

Fig. 1. The influence of ^{15}N enrichment of control crop and various incorrect B values on *apparent* % N fixation in chickpea tops for real values of 5%, 50% and 95% N fixation. This figure assumes a correct B value of $-\delta 2.10$ for chickpea tops.

accurate B value for the legume species being studied. The relative importance of this B value being correct in different situations is detailed below.

Figure 1 demonstrates the calculated influence of ^{15}N enrichment of the control crop and various B values on apparent % N fixed using the ^{15}N isotope dilution technique. The calculations were done for chickpea (*Cicer arietinum* L. cv. Reselected Tyson) tops, for which we measured a $\delta^{15}N$ value for B of −2.10‰ for plants grown in N-free solution culture (authors' unpublished data). This value was assumed to be the true value of B in Figure 1.

This figure indicates that an error in B value is only of practical importance at $\delta^{15}N$ enrichment levels in the control crop below about 50‰ when % N fixation is high. The relative error in estimated % N fixation due to an incorrect B value is constant for varying values of % N fixation. For low % N fixation values the absolute error is therefore small and control crop $\delta^{15}N$ values considerably less than 50‰ would provide acceptable estimates of fixation even if B was not accurately known.

As natural ^{15}N enrichment of total N in soils is generally less than 15‰, usually 5–12‰, (Hauck and Bremner, 1976; Shearer and Kohl, 1986) and with derived mineral N being of approximately similar enrichment it can be appreciated from Figure 1 that a correct or nearly correct estimate of B must be obtained in order to calculate accurately % N fixed using the ^{15}N natural abundance method, particularly when % N fixation is high.

The present method for estimating B

Isotopic fractionation during N fixation is commonly estimated by determining the ^{15}N content of N fixed by a legume inoculated with Rhizobium growing in a N-free solution. Hydroponic culture is used to minimise the legume's access to extraneous sources of N that may lead to errors. Seed N must also be accounted for both in terms of quantity and ^{15}N abundance, particularly with large seeds. Commonly, uninoculated legume plants are grown in isolation from, but in parallel with, inoculated plants using similar equipment and nutrient solutions.

Under N-free hydroponic culture uninoculated plants are dependent entirely on seed sources for their N supply and their growth is severely limited by comparison with inoculated plants.

Both inoculated and uninoculated plants are analysed for total N content and ^{15}N abundance. Subtraction of total N and total ^{15}N of uninoculated plants from values obtained for inoculated plants provides a dual adjustment for seed N and any extraneous N that may have been assimilated by inoculated plants during their culture. Remaining N and ^{15}N in inoculated plants should reflect the quantity of N fixed and any isotope fractionation during the fixation process.

Various authors, including Kohl and Shearer (1980), Bergersen and Turner (1983) and Ledgard (1989) have shown differences in ^{15}N abundance between plant parts. Shearer et al. (1982) found that root nodules actively fixing N in ureide-transporting legumes were enriched in ^{15}N compared to other plant organs whereas with amide-transporting legumes they were not. This ^{15}N fractionation between plant parts has led to isotope fractionation factors often being quoted for both tops and whole plants.

The technique described above for estimating the B value of a legume suffers from some disadvantages. It generally requires a separate glasshouse or laboratory experiment to that where N fixation is being measured and requires the paraphernalia, precision and attention of hydroponic or solution culture techniques.

Various authors e.g. Steele et al. (1983), Yoneyama et al. (1986) and Ledgard (1989) showed that ^{15}N abundance in plant tops varied compared to that in plant roots and nodules depending on rhizobium strain. If a B value is used in the ^{15}N natural abundance method that does not account for the effects of all rhizobium strains and their proportionate influence on B, then estimates of N fixation in particular plant parts, such as tops, may be inaccurate.

In field situations, various known or unknown strains of rhizobia may infect a particular legume to a variable extent. It would be difficult to simulate such variable rhizobia infections in solution culture and therefore difficult to arrive at an appropriate B value for estimating N fixation in various plant parts.

Ledgard (1989) has also shown that growing conditions can influence the B value obtained in solution culture.

B values obtained from solution culture should continue to be useful in many N fixation studies, however under the conditions outlined above they may not always be appropriate. A new method to overcome these problems is proposed below. It allows B to be determined as an integral part of a field experiment designed to estimate legume N fixation.

Theory and mechanics of the technique

This technique uses both the ^{15}N enrichment and ^{15}N natural abundance methods in parallel under the same conditions.

Estimates of % N fixation by the ^{15}N enrichment method are made and data are also taken to estimate % N fixation by the ^{15}N natural abundance method. Various values of B are tested in Equation 1 for estimating % N fixation by ^{15}N natural abundance. The B value that gives the least sum of squares for differences between % N fixation estimated by ^{15}N enrichment and ^{15}N natural abundance for a range of % N fixation values will be the best estimate of B for those conditions.

This is essentially a process of adjusting B values until % N fixation as measured by ^{15}N natural abundance best matches ^{15}N enrichment data. The technique requires very reliable ^{15}N enrichment data that are used as a reference for estimating B. Errors in ^{15}N enrichment data would cause errors in estimates of B.

For maximum sensitivity, B should be estimated from high % N fixation data as change in B causes the greatest differences in % N fixation when fixation is high (Fig. 1). Using high % N fixation data allows a more sensitive match between ^{15}N enrichment and natural abundance data sets. Also ^{15}N enrichment data are generally more reliable at high values of % N fixation (Fried et al., 1983; Reichardt et al., 1987).

Field evaluation of the comparison technique

The comparison technique for estimating B was evaluated using data from an unpublished field experiment carried out on a Vertisol near Toowoomba, Queensland. In this experiment % N fixation in chickpea tops (cv. Reselected Tyson) was measured by both ^{15}N enrichment and natural abundance techniques for a range of % N fixation values generated by varying soil nitrate levels at planting. Barley was used as a control in estimating % N fixation for both techniques. Only values exceeding 50% N fixation were used, thus providing adequate sensitivity in the comparison and sufficient reliability of ^{15}N enrichment data.

Analytical procedures for estimating ^{15}N natural abundance of N in chickpea and barley are described in Doughton et al. (1991). Similar procedures were used for ^{15}N enriched samples with the exception that samples prepared for mass spectrometry were completely dried rather than left in minimum volume solution as required for ^{15}N natural abundance samples (Turner and Bergersen 1983).

For each treatment, the sum of squares of differences between the ^{15}N enrichment estimate of % N fixation and estimates by the ^{15}N natural abundance method for various B values were calculated. These data are shown in Table 1. B values and their corresponding sums of squares of differences from Table 1 were then related through a quadratic equation: sum of squares of differences $= 4589.44 + 3510.85B + 740.08B^2$ ($R^2 = 0.99$).

This equation was differentiated to determine the minimum sum of squares which was 425.7 with a corresponding B value of −2.37‰. This was taken as the best estimate of the true value of B.

An approximation of the B value (−2.30‰) can be extracted directly from Table 1 by matching a B value to the smallest sum of squares shown.

The range of B values in Table 1 was selected to span the B value that gave the minimum sum of squares found during preliminary analysis of these data.

Table 1 shows little change in the sum of squares of differences between means (^{15}N enriched-^{15}N natural abundance) for values of B between −2.10‰ and −2.60‰. Errors in estimating B in this range would have minor significance in estimating % N fixed by the ^{15}N

Table 1. Mean % N fixation in chickpea tops estimated by the ^{15}N enrichment method and by the ^{15}N natural abundance method for a range of B values together with sums of squares of differences between the ^{15}N enrichment estimates and the various ^{15}N abundance estimates

% N fixed[a] (^{15}N enriched)	% N fixed[a] (^{15}N natural abundance) for B values of										
	−1.80	−1.90	−2.00	−2.10	−2.20	−2.30	−2.40	−2.50	−2.60	−2.70	−2.90
92.9	89.9	86.9	85.9	84.8	83.8	82.9	81.9	81.0	80.1	79.2	77.5
89.0	93.3	92.2	91.0	89.9	88.9	87.8	86.8	85.8	84.8	83.8	82.0
73.3	80.6	79.6	78.6	77.7	76.8	75.9	74.9	74.1	73.2	72.4	70.8
69.3	64.7	63.5	62.4	61.3	60.2	59.2	58.2	57.2	56.3	55.3	53.6
56.9	73.6	72.6	71.6	70.6	69.7	68.7	67.8	67.0	66.1	65.3	63.7
55.5	65.3	63.7	62.3	60.9	59.5	58.3	57.0	55.9	54.8	53.7	51.7
55.0	59.7	58.8	58.0	57.2	56.4	55.6	54.9	54.1	53.4	52.7	51.4
53.4	66.6	65.5	64.4	63.4	62.5	61.5	60.6	59.7	58.8	58.0	56.4
Sum of squares of differences	627.9	594.7	523.4	473.0	441.3	426.7	427.8	443.1	471.3	511.6	623.6

[a] means of four replicates. Values are rounded to the first decimal place.

natural abundance method for this data set. Reference to Figure 1 confirms this.

The minimum number of comparisons of means required to provide a reliable estimate of B would depend greatly on the reliability and variability of data compared and would therefore vary between experiments. Comparisons need not come from plots with variable % N fixation rates. They could be made from one, preferably high % N fixation treatment with sufficient samples taken to provide a reliable B value.

Discussion

The optimum B value obtained by the comparison method of −2.37‰ was close to a value of −2.10‰ obtained using the N free solution culture technique. The proximity of these values gives confidence that the techniques are comparable for this particular chickpea-rhizobium symbiosis. A useful feature of having another independent technique for estimating B is that it allows mutual corroboration of both techniques.

Agreement between these two estimates may have been assisted by a very specific chickpea-rhizobium symbiosis both in the field and in solution culture. Without inoculation, nodulation of chickpea did not occur on the field site. Hence both the solution culture and comparison technique were estimating a B value for a similar chickpea-rhizobium (Strain CC1192) symbiosis, uncontaminated by other rhizobium strains.

A disadvantage of the comparison technique is that it requires accurate data sets for % N fixation measured by both ^{15}N enrichment and natural abundance methods. These cannot always be guaranteed considering the multiplicity of potential errors affecting these methods (Ledgard and Peoples, 1988; Shearer and Kohl, 1986; Witty, 1983). Selective use of high % N fixation data for estimating B should reduce such errors because of the greater reliability of these data (Fried et al., 1983; Reichardt et al., 1987) and the resultant discarding of less reliable, low fixation data. Unreliable estimates of % N fixation would lead to inaccurate estimates of B. Where % N fixation data are unreliable they should not be used not should B values be derived from such data.

The N-free solution culture technique is suitable for obtaining estimates of B where legumes have a specific rhizobium requirement. However, the comparison technique may be more useful in field situations where strains of rhizobia infecting the host legume are varied or unknown and where N fixation measurements are required in particular plant parts such as plant tops.

Multiple or unknown rhizobium strain effects should be accounted for in field estimates of B by the comparison technique whereas this may be difficult to achieve in N-free solution culture.

The comparison technique may provide a further advantage not available from solution culture techniques for estimating B. Ledgard (1989) using hydroponically grown white and red clover (*Trifolium repens* L. and *Trifolium pratense* L., respectively) showed small variations within species in isotope fractionation of fixed N related to molybdenun nutrition and frequency of watering. These were sufficient to cause small (5%) differences in N fixation estimated by ^{15}N natural abundance.

He further suggested that similar variations in B resulting from other unspecified factors may have led to overestimates of the precision of N fixation estimated by ^{15}N natural abundance in published field studies. Differences in published estimates of B for a particular legume species (La Rue and Patterson, 1981; Ledgard, 1989; Shearer and Kohl 1986) suggests that this is so.

This indicates that solution culture estimates of B may not be valid for all situations involving a particular legume species or cultivar. This being the case, the best estimate of B for a field estimate of N fixation would be one measured in that particular environment. Such an estimate is possible with the comparison technique.

The comparison method should account for most site and environmental variations to B except possibly variations induced by particular experimental treatments. Any such treatment-induced variations to B could be further individually established using the comparison technique.

The technique would be particularly suitable where a large number of N fixation measurements are required for a single legume cultivar. The number of ^{15}N enriched comparisons required need only be sufficient to establish a reliable B value with the bulk of remaining N fixation estimates being done using the less expensive ^{15}N natural abundance method. This of course assumes that soil at the site has sufficient natural ^{15}N enrichment of mineral N with low enough variability to be suitable for use of the ^{15}N natural abundance method (Shearer and Kohl, 1988).

The comparison method is not the answer to all the problems of measuring B but could be a useful additional check on B values obtained from N-free solution culture and provides a further option when solution culture methods are less appropriate as in the examples outlined above.

Acknowledgements

Thanks are extended to M J Hughes and C C Barry for technical assistance with this study and to A J King and C W McEwan for able assistance with ^{15}N analyses. Financial support provided by the Wheat Industry Research Committee for Queensland is gratefully acknowledged.

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