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Nitrogen Fixation in Chickpea. II*
Comparison of ^{15}N Enrichment and ^{15}N Natural Abundance Methods for Estimating Nitrogen Fixation

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Abstract

The ^{15}N enrichment and ^{15}N natural abundance methods for estimating N_2 fixation in chickpea were compared over a range of soil NO_3-N levels at crop establishment varying from 10 to 326 kg N/ha (0–120 cm depth). Barley was used as a non–N_2 fixing control crop.

Both methods estimated reduced N_2 fixation as soil NO_3-N levels at crop establishment increased. Similar estimates of % N_2 fixation were obtained at high values, but at low values the ^{15}N enrichment method gave lower estimates, some of which were negative. The ^{15}N natural abundance method provided realistic estimates of % N_2 fixation across all soil NO_3-N levels at crop establishment. An asymptotic curve described a close ($R^2 = 0{\cdot}95$) relationship between these factors.

Standard errors of estimates of means for the ^{15}N natural abundance method remained acceptable and relatively stable over the full range of measurements; however, with the ^{15}N enrichment method they became unacceptably large at low values of % N_2 fixation. These large errors may have been partly due to legume and control plants assimilating mineral N of differing ^{15}N enrichment. High mineral N levels associated with low values of % N_2 fixation were also shown to reduce reliability of N_2 fixation values estimated by the ^{15}N enrichment method. These errors caused potentially greater inaccuracy at low values of % N_2 fixation than at high values. To compare N_2 fixation means statistically, transformations were necessary to stabilize variance and to impart lower weightings to plots with low values of % N_2 fixation.

Keywords: biological nitrogen fixation, ^{15}N natural abundance, ^{15}N enrichment, chickpea, ^{15}N methodology, legumes.

Introduction

The ^{15}N isotope dilution technique has become a popular method for estimating N_2 fixation in legumes, principally because it provides estimates integrated over time (Peoples and Herridge 1990; Danso *et al.* 1993). The method, first described by McAuliffe *et al.* (1958), is based on both a legume and a non–N_2 fixing control plant assimilating soil mineral N of identical $^{15}N/^{14}N$ ratio, with ^{15}N in the legume being diluted by fixed N_2 of lower $^{15}N/^{14}N$ ratio derived from the

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atmosphere. The extent of ^{15}N dilution in the legume is measured against the ^{15}N concentration in the control plant, allowing the proportion of N_2 fixed in the legume to be calculated.

There are two main variations of the ^{15}N dilution technique; one involves enrichment of available soil N by additions of ^{15}N-enriched fertilizers (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). Detailed descriptions of these two methods and the principles of ^{15}N dilution in estimating N_2 fixation may be found in reviews by Chalk (1985), Shearer and Kohl (1986) and Peoples and Herridge (1990).

The ^{15}N enrichment method has been used extensively for estimating N_2 fixation (Chalk 1985); however, in recent years the ^{15}N natural abundance method has also gained in popularity (Danso *et al.* 1993). It does not require expensive ^{15}N-enriched fertilizer and has given results of similar precision to the ^{15}N enrichment method (Shearer and Kohl 1986; Ofori *et al.* 1987; Ledgard and Peoples 1988; Bremner and van Kessel 1990).

In their review, Peoples and Herridge (1990) state that there is no single correct way of measuring N_2 fixation and that each of the many techniques have unique advantages and limitations. Certainly there are several potential errors, both common and differing, that can affect the ^{15}N enrichment and ^{15}N natural abundance methods (Fried *et al.* 1983; Witty 1983; Shearer and Kohl 1986; Ledgard and Peoples 1988; Peoples and Herridge 1990).

In this paper we compare both methods across a wide range of % N_2 fixation values obtained during a field study with chickpea (*Cicer arietinum* L.) on the Darling Downs in Queensland. Several of the problems of ^{15}N dilution techniques are detailed along with their effects at different values of % N_2 fixation.

Materials and Methods

Characteristics of this site are described in Doughton *et al.* (1991) while other trial site details, cultural practices and the experimental design, together with methods for soil and plant sampling are all described in Doughton *et al.* (1993).

The two methods of measuring N_2 fixation were carried out on the same field plots which were 30 m long with 9 rows per plot at 25 cm spacing. Immediately after field plots were machine sown to either chickpea or barley, ^{15}N microplots were randomly sited over one of the three central rows in each plot. Each microplot was a single 80 cm length of row. ^{15}N was applied by pipetting 20 mL of aqueous solution containing 17·784 mg of 65 atom % ^{15}N potassium nitrate in a narrow band directly onto the row. The rate of labelled N was 1·3 kg/ha. It was assumed that this would have minimal effect on chickpea growth and N_2 fixation. A spray irrigation of 37 mm was applied over the entire experiment to wash applied commercial and ^{15}N fertilizers into the soil.

Plants were sampled 130 days after sowing. Whole chickpea and barley tops were taken from the centre 40 cm of the microplots. Plants for measurement of dry matter yield, total N yield and ^{15}N natural abundance were taken from 2 m of row located at least 15 m from the ^{15}N microplot in each field plot, with care taken to avoid contamination from ^{15}N enriched microplots. Total N_2 fixed per hectare was calculated using this total N yield and % N_2 fixation for each method.

Percent N_2 fixation (^{15}N enrichment) was calculated as described by La Rue and Patterson (1981):

$$\% \ N_2 \text{ fixed} = 100\left(1 - \frac{\text{atom \% excess } ^{15}\text{N chickpea}}{\text{atom \% excess } ^{15}\text{N barley}}\right). \tag{1}$$

Percent N_2 fixation (^{15}N natural abundance) was calculated by the equation of Ledgard and Peoples (1988):

$$\% \ N_2 \text{ fixed} = 100\left(\frac{\delta^{15}\text{N barley} - \delta^{15}\text{N chickpea}}{\delta^{15}\text{N barley} - B}\right), \qquad (2)$$

where in our case δ^{15}N barley and δ^{15}N chickpea are the parts per 1000 ^{15}N enrichment of N in barley and chickpea tops and B is the δ^{15}N of fixed N_2 for chickpea, all with reference to the natural abundance of atmospheric N. Therefore one δ^{15}N unit expressed in parts per thousand (‰) equals $0 \cdot 3663 \times 10^{-3}$ atom % ^{15}N enrichment. The use of atom % ^{15}N instead of the mass 29/mass 28 ratio has a negligible effect on the calculation of natural levels of δ^{15}N (Mariotti *et al.* 1981).

A δ^{15}N value of $-2 \cdot 10$ for B in chickpea tops was used in Eqn 2 and was derived from N-free solution culture of chickpea as described in Doughton *et al.* (1992).

Percent N_2 fixation was calculated individually for each plot of chickpea using its companion barley plot.

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

Results and Discussion

Figs 1 and 2 show mean ^{15}N concentrations of N contained in chickpea and barley tops for different soil NO_3-N levels at establishment using ^{15}N enrichment and ^{15}N natural abundance methods respectively. Different units are used to express these concentrations in accordance with current convention.

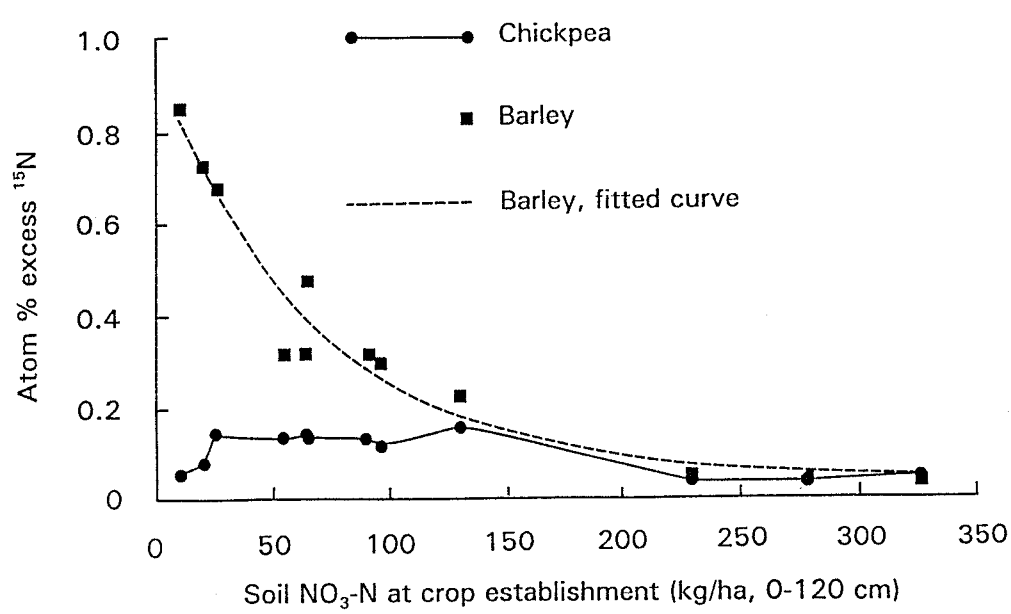

Fig. 1. Atom % excess ^{15}N of N in chickpea and barley tops 130 days from planting for various levels of soil NO_3-N at crop establishment (^{15}N enrichment method). For barley fitted curve, $Y = 0 \cdot 0393 + 0 \cdot 9073e^{-0 \cdot 0148X}$, $R^2 = 0 \cdot 96$.

In Fig. 1, the fitted asymptotic barley curve indicates dilution of applied ^{15}N fertilizer by increasing levels of soil mineral N. Chickpea values were low, principally from dilution of ^{15}N assimilated from the soil mineral N pool by ^{14}N in fixed N_2. When the scales of the different ^{15}N units of Figs 1 and 2 are taken into account, the barley ^{15}N natural abundance data reflect a more stable background of ^{15}N in the soil mineral N pool over the range of NO_3-N values compared with the ^{15}N enrichment data. Nevertheless, significant variations are present and are explained by ^{15}N enriching effects resulting from probable denitrification of excess soil NO_3-N and counteracting ^{15}N depletion caused by application of ^{15}N depleted commercial N fertilizer. These effects are detailed more fully in Doughton *et al.* (1991). Where data for chickpea ^{15}N natural abundance were low in comparison to barley, this was again principally due to dilution by ^{14}N in fixed N_2 of ^{15}N assimilated from the soil. The negative $\delta^{15}N$ values for chickpea in Fig. 2 would result from discrimination against ^{15}N in favour of ^{14}N during the process of N_2 fixation (Shearer and Kohl 1986) and/or fractionation of ^{15}N and ^{14}N unequally between different plant parts (Steele *et al.* 1983; Yoneyama *et al.* 1986; Ledgard 1989) leading to ^{15}N depletion of N in tops.

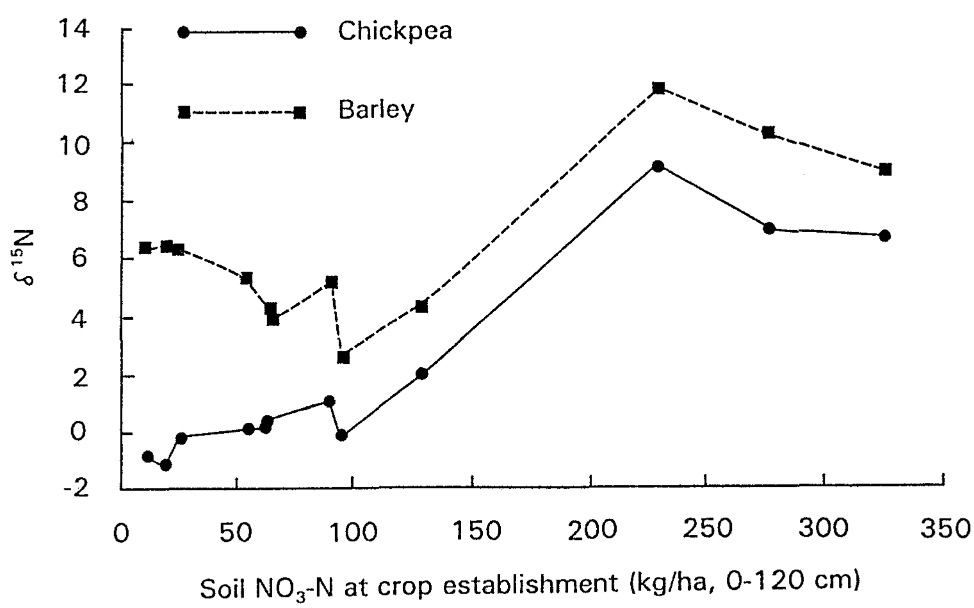

Fig. 2. $\delta^{15}N$ of N in chickpea and barley tops 130 days from planting for various levels of soil NO_3-N at crop establishment (^{15}N natural abundance method).

Fig. 3 compares mean values of % N_2 fixed in chickpea tops estimated by ^{15}N enrichment and ^{15}N natural abundance methods for various soil NO_3-N levels at establishment. The linear relationship for ^{15}N enrichment data in Fig. 3 was fitted by least squares using a weighted regression in which each of the 12 means for % N_2 fixed was weighted in proportion to the reciprocal of its variance ($1/s^2$). The high variances of the three lowest values for % N_2 fixation (see standard errors Fig. 3) resulted in their weightings having an insignificant effect on the fitted regression. The regression line was therefore not extended to include those means.

The ^{15}N natural abundance method provided realistic estimates of % N_2 fixation across all NO_3-N levels at crop establishment with stable standard errors. An asymptotic curve fitted by unweighted regression described a close ($R^2 = 0{\cdot}95$) relationship between these variables.

Both methods for estimating % N_2 fixation gave similar results at high values of % N_2 fixation; however, it was apparent that the ^{15}N enrichment method failed at low values of % N_2 fixation with biologically impossible negative values shown in Fig. 3. Danso *et al.* (1993) and Chalk (1985) in their reviews detail the many problems and potential errors of ^{15}N dilution techniques. Errors that may have influenced our results are discussed below.

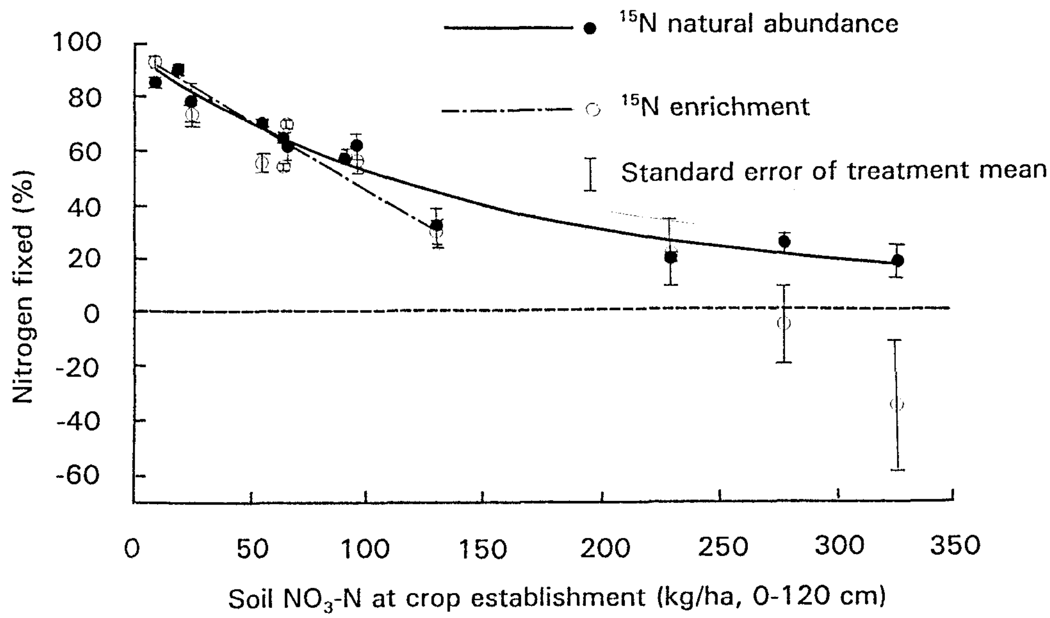

Fig. 3. Comparison of ^{15}N enrichment and ^{15}N natural abundance methods for estimating % N_2 fixation in chickpea tops 130 days from planting for various levels of soil NO_3-N at crop establishment. For fitted curve of ^{15}N natural abundance, $Y = 7{\cdot}05 + 88{\cdot}48e^{-0{\cdot}0070X}$, $R^2 = 0{\cdot}95$. For fitted regression line of ^{15}N enrichment, $Y = 95{\cdot}41 - 0{\cdot}513X$, $R^2 = 0{\cdot}84$.

Methods using ^{15}N dilution to estimate % N_2 fixation in legumes assume that the legume and the non-fixing control plant take up soil mineral N of similar ^{15}N enrichment. Witty (1983) showed that for this assumption to be valid under conditions where ^{15}N enrichment of mineral N varied over time (as it does after application of ^{15}N-enriched fertilizer), then both legume and control plants need to have similar root activity patterns in both time and space. This assists each plant species to synchronize uptake of mineral N of similar ^{15}N enrichment both horizontally and vertically within the soil profile.

Error due to Asynchrony of Mineral N Uptake

Asynchrony of mineral N uptake by legume and control plants due to differing growth patterns can be a major problem where ^{15}N from applied fertilizer declines in the soil mineral N pool over time. Rennie and Rennie (1983) demonstrate this problem clearly with a diagram showing ideal and non-ideal N uptake patterns for legume and control plants.

In Fig. 4 we demonstrate theoretically why asynchrony of N uptake between legume and control plants has more serious consequences at low values of % N_2 fixation than at high values.

The centre line in Fig. 4 is based on synchronous uptake of mineral N resulting in equivalent ^{15}N enrichment of N assimilated from the soil in both legume and control crop. It gives equal values for actual and apparent % N_2 fixation. The upper line is the result of asynchrony of mineral N uptake causing an arbitrary halving of ^{15}N enrichment resulting from soil N uptake in the legume compared with the control crop. The lower line results from a halving of control crop ^{15}N enrichment compared with the legume. The larger potential errors at low % N_2 fixation are clearly demonstrated by the divergence of these lines.

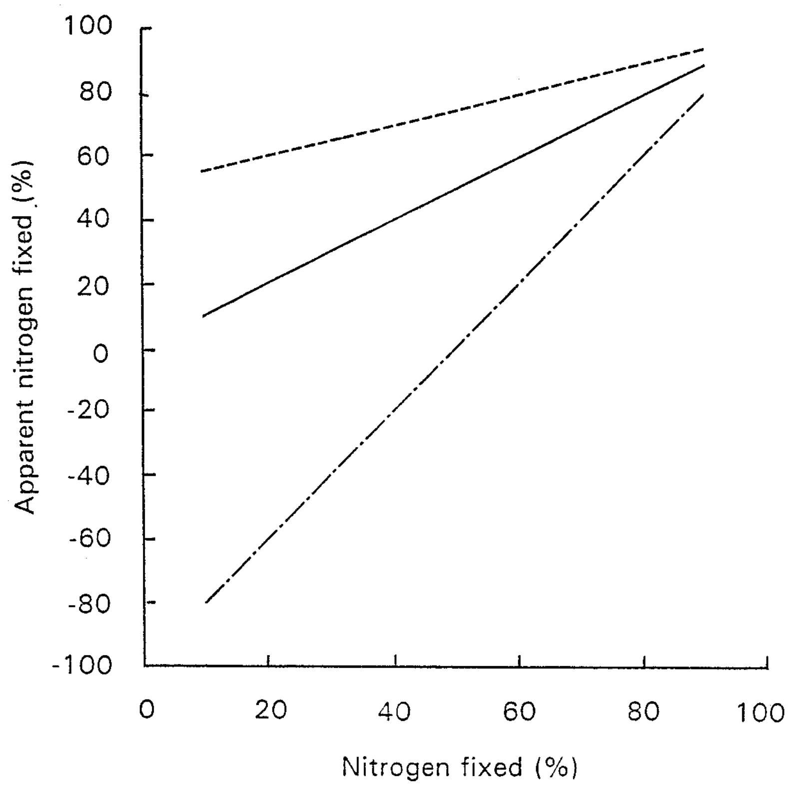

Fig. 4. Theoretical analysis of actual and apparent % N_2 fixation for synchronous uptake of mineral N by legume and control crop and two examples of asynchronous uptake. The three examples are as follows: — indicates ^{15}N enrichment from mineral N uptake similar in legume and control crop (synchronous uptake); ----- indicates ^{15}N enrichment from mineral N uptake in legume halved compared to control crop (asynchronous uptake); —-— indicates ^{15}N enrichment from mineral N uptake in control crop halved compared with legume (asynchronous uptake).

Fig. 4 also shows how apparent negative % N_2 fixation values can occur when the control plant assimilates mineral N of lower ^{15}N enrichment than the legume. This may explain the negative % N_2 fixation results obtained with the ^{15}N enrichment procedure at high soil NO_3-N levels shown in Fig. 3. Danso *et al.* (1993) cite many similar negative results indicating that such unrealistic values only indicate the magnitude of errors associated with these estimations.

Error due to Varying Root Distribution

As alluded to earlier, a similar error to that above occurs when legume and control plant assimilate soil mineral N of differing ^{15}N enrichment due to different root distributions (Peoples and Herridge 1990). This error would similarly be more serious at low values of % N_2 fixation.

Error Associated with Variable ^{15}N Concentration in Soil Mineral N

Hardarson *et al.* (1988) showed theoretically that low values of % N_2 fixation have larger standard deviations than high values. Below we demonstrate how excess soil mineral N contributes to this phenomenon, reducing the accuracy of % N_2 fixation estimated by the ^{15}N enrichment method.

Mineral N dilutes applied ^{15}N in the soil mineral N pool leading to lower mean ^{15}N values in both legume and control plants compared to those grown with less mineral N. This effect can be seen in the control plant ^{15}N values of Fig. 1 and also generally in the legume values if one disregards the ^{15}N diluting effects of N_2 fixation, particularly at low NO_3-N levels. Generally with lower means of atom % excess ^{15}N for both legume and control plants (as a result of high soil mineral N) and assuming that standard errors remain approximately constant, then the respective values of standard error/mean increase. Several of these relationships are demonstrated in Table 1 using hypothetical means and standard errors for legume and control plant ^{15}N enrichment.

In Table 1 a substantial increase in unlabelled soil mineral N (reflected in a 100 fold decrease in atom % ^{15}N excess of the control plant) has reduced % N_2 fixed from 90% to 10% and reduced atom % ^{15}N excess of the legume almost 11 fold. Values for standard errors of legume and control plant ^{15}N enrichment were made equal to fulfil the assumption that they respectively remain approximately constant across a range of means and to simplify comparisons in Table 1. Though arbitrarily selected, mean values approximate actual data. The parameter, 'atom % excess ^{15}N legume/atom % excess ^{15}N control', is a component of Eqn 1 used for estimating % N_2 fixation (^{15}N enrichment method). The standard error of this ratio was calculated using an equation from Kempthorne and Allmaras (1965). In turn the standard error of % N_2 fixed was also derived. Table 1 demonstrates the very large standard errors and standard error/mean ratios at 10% N_2 fixed compared to those at 90% N_2 fixed.

It is apparent that low % N_2 fixation values resulting from excess soil mineral N will be less reliable than those for higher values of % N_2 fixation, and this would have to be taken into account in any statistical comparisons. With our experimental data of percent and total N_2 fixed, transformations were required before means could be compared statistically. These data were scaled to workable units (division by 30 and 60 respectively) after which exponential transformations stabilized variances in the transformed units by giving low weights to large negative estimates associated with low % N_2 fixation levels.

This problem could be reduced by applying larger quantities of ^{15}N-enriched N fertilizer to plots with high mineral N levels so that $^{14}N/^{15}N$ ratios in mineral N remain approximately constant for different soil mineral N levels and therefore different % N_2 fixation measurements. Unfortunately, the cost of ^{15}N fertilizer to do this would often be prohibitive and also the added fertilizer may alter treatment effects.

All of the above indicates that the ^{15}N enrichment technique is suspect at low % N_2 fixation levels. Reichardt *et al.* (1987) showed that a set of field data with high % N_2 fixation values had a lower coefficient of variation than a set of low % N_2 fixed data. Our discussion above and Fig. 5 confirm that the ^{15}N enrichment method becomes progressively unreliable at low % N_2 fixation values.

Table 1. Use of hypothetical means and standard errors for ^{15}N enrichment of legume and control plants in demonstrating calculated differences in standard errors for three values of N_2 fixation (%)

Atom % excess ^{15}N legume			Atom % excess ^{15}N control plant			$\frac{\text{Atom \% excess } ^{15}\text{N legume}}{\text{Atom \% excess } ^{15}\text{N control}}$			N_2 fixation (%)		
Mean	s.e.	s.e./mean	Mean	s.e.	s.e./mean	Mean	s.e.	s.e./mean	Mean	s.e.	s.e./mean
1·000	0·050	0·050	10·000	0·050	0·005	0·100	0·005[A]	0·050	90·000	0·052[A]	0·001
0·500	0·050	0·100	1·000	0·050	0·050	0·500	0·056[A]	0·112	50·000	5·590[A]	0·112
0·090	0·050	0·555	0·100	0·050	0·500	0·900	0·672[A]	0·747	10·000	67·231[A]	6·723

[A] Covariance between atom % excess ^{15}N of legume and control plants has been excluded from these calculations for simplicity.

Data from Fig. 3 based on the ^{15}N enrichment method have standard errors which are dependent on mean values. The variance for these data was stabilized by using an $e^{x/30}$ transformation and the resulting weighted, back-transformed means were compared with untransformed ^{15}N natural abundance data in Fig. 6.

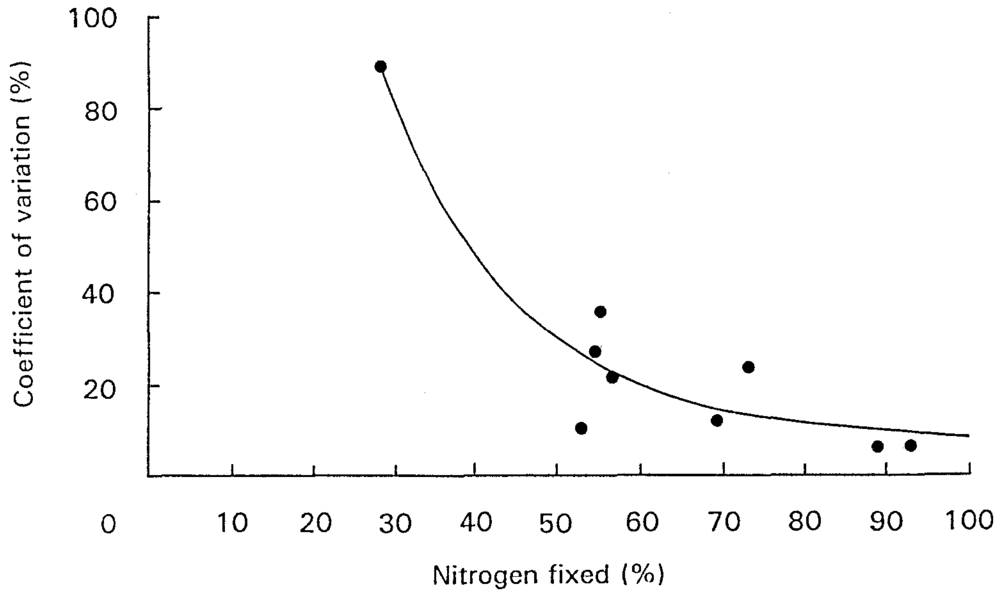

Fig. 5. Coefficients of variation for various values of % N_2 fixed in chickpea tops 130 days from planting estimated using the ^{15}N enrichment method. Data exclude three points of extreme variability associated with low or negative % N_2 fixation. For fitted curve, $Y = 7 \cdot 14 + 445 \cdot 86e^{-0 \cdot 0599X}$, $R^2 = 0 \cdot 90$.

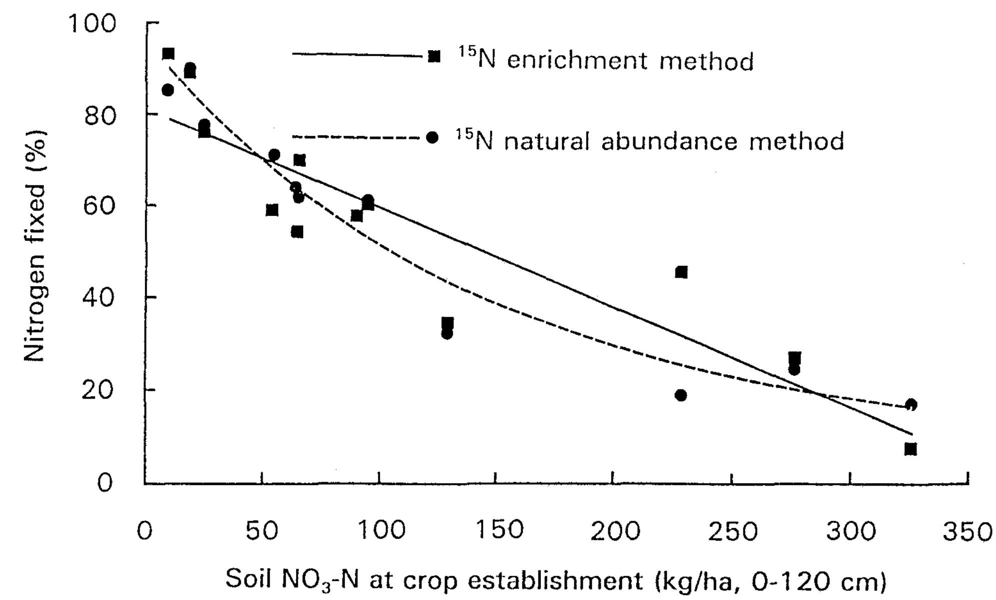

Fig. 6. Percent N_2 fixation in chickpea tops 130 days from planting for various soil NO_3-N levels at crop establishment estimated by ^{15}N natural abundance and ^{15}N enrichment (after an $e^{x/30}$ transformation) methods respectively. For fitted curve of untransformed ^{15}N natural abundance data, $Y = 7 \cdot 05 + 88 \cdot 48e^{-0 \cdot 0070X}$, $R^2 = 0 \cdot 95$. For fitted regression line of back-transformed means of ^{15}N enrichment data, $Y = 80 \cdot 97 - 0 \cdot 215X$, $R^2 = 0 \cdot 82$.

Similarly, the transformed ($e^{x/60}$) means for total N_2 fixed in chickpea (^{15}N enrichment method) were back-transformed and compared with untransformed means from the natural abundance method in Fig. 7.

While transformation of data derived from the ^{15}N enrichment method in this experiment allows generation of stable variances at low N_2 fixation levels, the intrinsic problems with the method remain and similar data sets would probably require transformation. When single low N_2 fixation values are determined by this method, their high variability would give little confidence in the values estimated. Those using the method should be aware of these shortcomings and consider using the ^{15}N natural abundance method in preference when low N_2 fixation is expected.

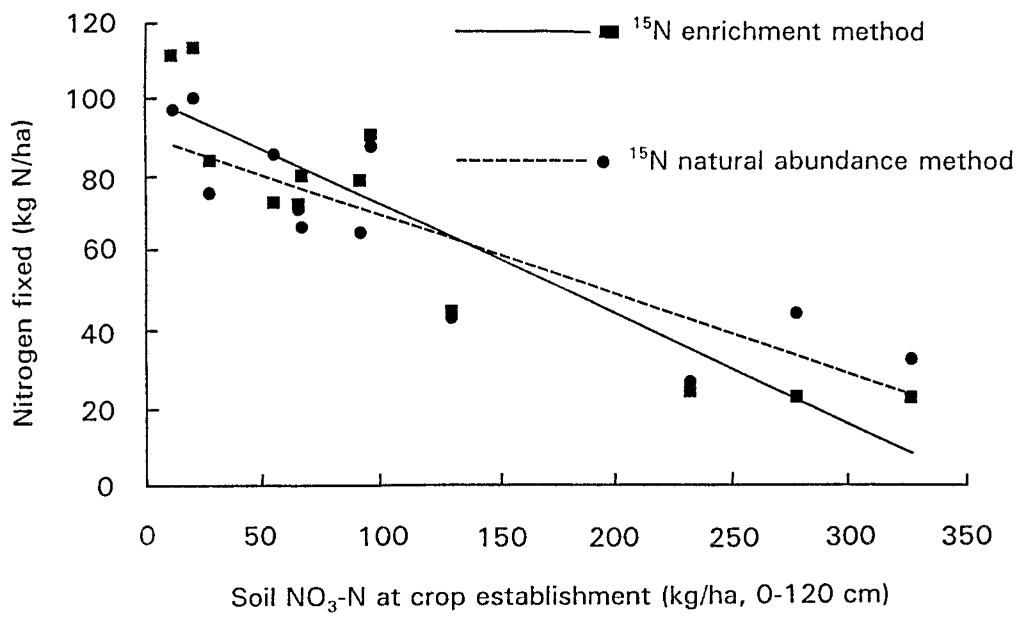

Fig. 7. Total N_2 fixation (kg N/ha) in chickpea tops 130 days from planting for various soil NO_3-N levels at crop establishment estimated by ^{15}N natural abundance and ^{15}N enrichment (after an $e^{x/60}$ transformation) methods respectively. For fitted regression line of untransformed ^{15}N natural abundance data, $Y = 89 \cdot 73 - 0 \cdot 202X$, $R^2 = 0 \cdot 74$. For fitted regression line of back-transformed means of ^{15}N enrichment data, $Y = 100 \cdot 59 - 0 \cdot 280X$, $R^2 = 0 \cdot 84$.

Variability in the accuracy of estimates of % N_2 fixation over the range of possible values is a shortcoming inherent in the ^{15}N enrichment technique that is not widely appreciated. It is fortunate that high values of % N_2 fixation, which are generally of most interest, are likely to be the most accurate.

Many of the problems of the ^{15}N enrichment method result from variable ^{15}N labelling of the soil mineral N pool both spatially and temporally. Fried *et al.* (1983) and Witty (1983) suggested several techniques to stabilize ^{15}N levels in soil mineral N to overcome these problems. These included the direct incorporation of ^{15}N labelled organic matter, the use of sites fertilized with ^{15}N in previous years, the addition of a readily available carbon source along with ^{15}N fertilizer to temporarily immobilize ^{15}N and the use of slow release ^{15}N fertilizers. The use of multiple control plant species was also suggested as a technique for better estimation of mean ^{15}N values in soil mineral N.

The ^{15}N natural abundance method is less subject to these problems as natural ^{15}N labelling of the soil mineral N pool is generally more uniform throughout the soil profile and less variable over time (Ledgard *et al.* 1985). The levels of ^{15}N enrichment are, however, much lower requiring greater precision in sampling and ^{15}N determination than with the ^{15}N enrichment method.

The $\delta^{15}N$ of barley control plants increased with level of accumulated soil NO_3-N in contrast with the large decreases in ^{15}N enrichment of barley control plants with increased levels of soil NO_3-N in the ^{15}N enrichment method. In this experiment this increase potentially improved the accuracy of estimates of low % N_2 fixation values while the relative uniformity of ^{15}N levels in barley control plants avoided many of the problems associated with the ^{15}N enrichment method. Nevertheless, it was apparent that barley comparisons (^{15}N natural abundance) were necessary for each individual plot estimate of % N_2 fixed in chickpea and that a single barley comparison for the total experiment would be inadequate.

This study indicates that the ^{15}N natural abundance method can be a precise and accurate method for estimating % N_2 fixation in chickpea. When estimates of % N_2 fixation are low, it has several advantages over the ^{15}N enrichment method, particularly when N_2 fixation is reduced as a result of excess soil mineral N. Further, the choice of an appropriate control plant is less critical with the ^{15}N natural abundance method (Peoples and Herridge 1990), so the need for multiple control plants is avoided.

Acknowledgments

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