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Chapter 5

THEORETICAL EVALUATION OF ^{15}N ISOTOPIC METHODS FOR MEASURING SYMBIOTIC NITROGEN FIXATION IN THE FIELD

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Abstract

Isotopic methods for the measurement of symbiotic N_2 fixation by leguminous plants in the field rely on the use of differences in ^{15}N enrichment between the N sources potentially available for leguminous crops, soil mineral N and atmospheric N_2. This methodology has been fully documented, especially concerning limitations due to non uniform and non constant distribution of ^{15}N and to the use of a reference plant to measure it. Although all authors recognise the necessity of isotopic methods for giving yield independent and time-integrated estimates of symbiotic fixation, they also agree that these methods intrinsically remain imperfect. Our aim in this chapter is (i) to briefly review the three major isotopic methods and recall the main assumptions they involve, (ii) to evaluate the theoretical precision of those methods by performing sensitivity analysis to all their parameters, in the perspective of precisely delimiting their validity domain and (iii) to quantify the error made when using the method with the largest spectrum of application and to propose solutions to minimise it.

The natural abundance method (NA) is the simplest method as no added fertiliser is required. The method isotopic dilution (ID) requires ^{15}N-labelled fertiliser application to increase ^{15}N soil enrichment. The multi-enrichment technique (MET) relies on the use of several treatments receiving the same amount of fertiliser but labelled at variable ^{15}N enrichments. Using an original mathematical analysis, we show that the precision of NA and MET is likely to be low if the difference in ^{15}N abundance between the soil and atmosphere is low (lower than 8 ‰). Otherwise, the use of NA or MET requires a very precise determination of the isotopic fractionation rate due to symbiotic fixation (ε_{Fix}). The ID method circumvents this problem. It has the largest validity domain as it can be used in soils slightly enriched in ^{15}N

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and does not require such precise determination of ε_{Fix}. However, the main assumption of the ID method is that the relative uptake of soil and fertiliser N is identical for the fixing and the non fixing plants. If this assumption is not valid, large discrepancies can occur between the actual and calculated contributions of symbiotic fixation to the overall N acquisition by the plants (p_A). This error was evaluated as a function of the level of p_A, ^{15}N enrichments of the soil and fertiliser and the proportion of fertiliser N retrieved by the legume to total mineral N. Recommendations for optimal application of ^{15}N labelled fertiliser used to enrich the soil mineral N are given.

Key words: symbiotic fixation, isotopic dilution, natural abundance, ^{15}N, method.

Introduction

Isotopic methods for the measurement of symbiotic N_2 fixation by leguminous plants in the field were first described by McAuliffe et al. (1958). They rely on the use of differences in ^{15}N enrichment between the N sources potentially available for leguminous crops (soil mineral N and atmospheric dinitrogen) that either exist naturally (Natural Abundance method: NA; Amarger et al, 1979) or are artificially induced (Isotope Dilution method: ID; Witty, 1983). Methodology associated to isotopic measurement of symbiotic N fixation in the field has been fully documented (Rennie and Rennie, 1983; Chalk, 1985; Shearer and Kohl, 1986; Hardarson and Danso, 1993; Unkovich et al., 1994; Chalk and Ladha, 1999; Unkovich and Pate, 2000). Although all authors recognise the necessity of isotopic methods for giving yield independent and time-integrated estimates of symbiotic fixation, they also agree that these methods intrinsically remain imperfect.

Limits of isotopic studies mainly reside in the level and measurement of the ^{15}N enrichment of the soil (E_{Soil}) that is commonly performed by the mean of a non fixing reference plant. Hence, to provide realistic estimates of the ^{15}N abundance resulting from mineral N uptake by the fixing plant, it is commonly admitted to use a non-fixing reference plant whose ^{15}N enrichment represents that of the soil mineral N available for the plant, as it is its only N source. Importantly, hypothesis associated with the use of a reference plant differ with the method used and this will be first discussed here for three major isotopic methods: NA, ID and MET. Difficulties then often arise either i) from low level of E_{Soil} that may be insufficiently different from that of the atmospheric dinitrogen (E_{N2}) ii) from precision on measurement of isotopic fractionation at the site of symbiotic fixation within infected cells in nodules ($\varepsilon_{Fix} = E_{N2}$) in case of low level of E_{Soil} iii) from spatial and temporal variability of E_{Soil}, or iv) from inadequacy of the reference plant used to measure E_{Soil}. Thus, the second aim of this chapter was to theoretically and quantitatively analyse the impacts of these factors on the evaluation of the percentage of symbiotic fixation (p_A) by those three isotopic methods. To that purpose, we performed sensitivity analysis on each method to all of its parameters. Since ID is the most widely used method and which presents the largest spectrum of application (Unkovich and Pate, 2000), we calculated the error which may be committed with this method and analyzed its sensitivity to its most important parameters. Finally we tried to give recommendations for the use of the three methods stated above.

1. Description of Three Major Isotopic Methods

Natural Abundance Method (NA)

The natural abundance (NA) method exploits the natural differences in ^{15}N enrichment usually encountered in the soil relative to the atmosphere (Amarger et al., 1979): arable soils are generally enriched in ^{15}N, to usually less than 10 ‰ (Shearer and Kohl, 1986) and with an observed maximum $\delta^{15}N$ of 17 ‰ (Cheng et al., 1965). A mean value of 8.8 ± 1.2 ‰ was observed in Canada (Rennie and Rennie, 1983), a range from 5.1 and 12.3 ‰ was reported from 20 states in the United States (Shearer et al., 1978) and a range from 2.9 to 4 ‰ was reported in a study in south-west Australia (Unkovich et al., 1994). The method is simple since tracer or fertiliser is not required and it has been successfully used in many studies (Unkowich et al., 1994). The percentage of N derived from symbiotic fixation (p_A) can be expressed as the ratio of the difference between the isotopic excess of assimilated soil N (E_S) (as measured by the reference plant (E_{ref})) and the isotopic excess of the legume plant (E) over the differential between the isotopic excess of absorbed soil N and the isotopic fractionation for symbiotic N fixation (ε_{fix}) (Shearer and Kohl, 1986; Mariotti, 1980; 1983) as follows (see appendix A):

$$p_A = \frac{E_S - E}{E_S - \varepsilon_{fix}} = \frac{E_{ref} - E}{E_{ref} - \varepsilon_{fix}} \tag{1}$$

The isotopic excess of soil N (E_S) is given by the isotopic enrichment of a non fixing reference plant (E_{ref}). Thus, ^{15}N enrichment of soil N absorbed by the reference plant is supposed to be similar to that absorbed by the legume, which constitutes the main hypothesis of the NA method. The isotopic fractionation for symbiotic nitrogen fixation (ε_{fix}) is determined by measuring the ^{15}N enrichment of a legume solely dependent on N_2 fixation (Mariotti et al., 1980, 1983), i.e. grown without any combined nitrogen. The main advantage of NA method lies in its simplicity since no specialised experimental set up is needed and calculations are straightforward. The main drawback of NA is that its precision becomes critical when the difference in ^{15}N abundance between the soil and atmosphere is low (Unkovich et al., 1994) or if there a large spatial variability in natural ^{15}N abundances (Holdensen et al., 2007).

Isotope Dilution Method (ID)

The ID method, which consists in applying ^{15}N labelled fertiliser to enhance the difference in ^{15}N enrichment between the soil and atmosphere, solves this problem. However, when labelled fertiliser is applied to the N_2 fixing crop, the soil mineral nitrogen available for the legume plant has two origins: that applied as fertiliser and the one resulting from the soil (i.e. mineral N present in soil + mineralisation of soil organic N). The equations (given in appendix A) are basically similar to those for NA method except that the isotopic excess of the legume plant is compared to that of the soil enriched with ^{15}N-labelled fertiliser (E_{FS}) to calculate the percentage of fixed N by the legume crop $p_{A:}$

$$p_A = \frac{E_{FS} - E}{E_{FS} - \varepsilon_{fix}} = \frac{E_{ref} - E}{E_{ref} - \varepsilon_{fix}} \quad (2)$$

The ^{15}N enrichment of soil N enriched with ^{15}N labelled fertiliser (E_{FS}) is given by ^{15}N enrichment of a non fixing reference plant (E_{ref}). Because extra labelled N has been applied to the soil, the difference between the isotopic composition of soil N and that of the legume crop is high enough to calculate precisely the amount of fixed nitrogen, at least if the isotopic excess of fertiliser has been chosen judiciously. Another advantage of the ID method is that the addition of fertiliser gives access to the study of mixed nitrogen regimes necessary for the understanding of the compensatory response of the two pathways of N acquisition (i.e. absorption of soil mineral nitrogen and symbiotic N_2 fixation, Voisin et al., 2002). Rennie and Rennie (1983) suggested that ID and NA may have a similar precision because the lower spatial variability of the natural ^{15}N abundance of the soil mineral N pool may be compensated by a higher precision of the ID technique.

Multi-Enrichment Technique (MET)

Instead of comparing a legume to a reference plant to measure symbiotic N fixation activity, the idea proposed by Ledgard et al. (1985a) was to use different ^{15}N enrichment treatments for the same legume. This method called here Multi Enrichment Technique (MET) relies on the use of several treatments receiving the same amount of fertilizer but labelled at different ^{15}N enrichments. Varying only the ^{15}N enrichment does not disturb the mass flow of N in between treatments (Ledgard et al., 1985a). For each treatment and during the growth cycle, the legume will accumulate an amount of N coming from three distinct N pools: i) soil, ii) fertiliser and iii) air. At any given time the ^{15}N enrichment of the legume is linearly related to that of the fertiliser. The percentage of N derived from the fertiliser is the slope of the above stated relationship. The relative contribution of the soil mineral N and of the atmospheric N_2 (p_A) to the overall N acquisition by the legume crop can be determined using both the slope (β) and the Y-intercept (α) of the relationship between legume plant and fertiliser ^{15}N enrichments. It also involves ^{15}N enrichment of the soil (E_S) (as measured by the reference plant enrichment: E_{ref}) and isotopic fractionation due to symbiotic N fixation (ε_{Fix}):

$$p_A = \frac{(1-\beta)\cdot E_S - \alpha}{E_S - \varepsilon_{Fix}} = \frac{(1-\beta)\cdot E_{ref} - \alpha}{E_{ref} - \varepsilon_{Fix}} \quad (3)$$

In order to determine α and β, at least two ^{15}N enrichment levels of the fertiliser are necessary. The method is applicable if the ^{15}N composition of the indigenous soil N (estimated by the ^{15}N enrichment of a non-fixing plant: E_{ref}) is significantly different from the ^{15}N composition of the atmosphere (ε_{Fix}, estimated by the ^{15}N enrichment of a plant relying exclusively on symbiotic N fixation).

2. About the Choice of the Reference Plant: What Are the Main Hypotheses?

As it should obtain soil N at the same isotopic composition as the legume plant during its growth cycle, the choice of the non fixing reference plant prerequisites a number of precautions due to spatial and temporal variation of ^{15}N soil enrichment (Danso et al., 1993; Chalk and Ladha, 1999). However, the importance of adequacy of the reference plant with the leguminous plant varies with isotopic methods. For the NA method, the reference plant is used to measure ^{15}N natural abundance of indigenous soil N. Since there is no addition ^{15}N-labelled fertiliser, variations of ^{15}N are usually small with depth (Rennie and Rennie, 1983; Ledgard et al., 1984; Shearer and Kohl, 1986). The NA method is therefore relatively insensitive to the choice of the reference plant, provided it explores a similar soil volume as the legume (Bergersen et al., 1989; Ladha et al., 1993). Moreover, similar rooting pattern is more important that rooting architecture (Shearer and Kohl, 1986) since soil N rapidly decreases with soil depth (Ledgard et al., 1985c; Bergersen et al., 1985). When ID method is used, labelled fertiliser is applied to the N_2 fixing crop and the soil mineral N available for the legume plant has two origins: that applied as fertiliser and that present (and mineralised) in the soil. Therefore, the ID method invokes the hypothesis either that soil has been uniformly labelled or that the fixing and reference plants retrieve mineral N in similar proportions from the indigenous soil N pool and from the added fertiliser N pool. The first hypothesis seems almost impossible to be met because, in addition to the fact that the ^{15}N enrichment of the soil is naturally slightly variable in depth, labelled fertiliser cannot be mixed uniformly within soil layers up to the maximum rooting depth, the latter varying with time (Smith et al., 1996). The second hypothesis is less severe but may be wrong due to the different behaviour (often observed) between the fixing and the non fixing plants relative to mineral N uptake. When the ID method is used, it is therefore essential that the time course and patterns of mineral N uptake are similar for the legume of interest and the reference plant (Chalk and Ladha, 1999). This assumption requires a good knowledge of both soil N dynamics and of its retrieval by both plants species in space and time, which is rarely achieved and difficult to assess before the end of the experiment. In all cases, a special care to the evaluation of ^{15}N enrichment of soil mineral N (E_{Soil}) is recommended to minimise possible error due to sampling error and variations across the site. As such, several authors advocate numerous replicates (5-16) for the measurement of E_{Soil} through the reference plant (Warembourg, 1993; Ledgard et al., 1985 b; Unkovich et al., 1994). To measure the error due to inadequate matching of ^{15}N acquisition of the reference plant and the legume, Shearer and Kohl (1986) suggested growing as many different species as possible. However, it was opposed that in addition to being labour intensive and often unrealistic, this would not help choosing the right reference plant (Chalk and Ladha, 1999). The only adequate method to independently measure N acquisition from soil and fertiliser for the reference plant and the legume species is that proposed by Ledgard et al. (1985a), called here the Multi Enrichment Technique (MET). However, it is also labour intensive and has its own limitations (Shearer and Kohl, 1986). Instead of comparing a legume to a reference plant to measure symbiotic N fixation activity, MET relies on the use of several legume treatments receiving the same amount of fertiliser but labelled at variable ^{15}N enrichments. For each treatment and during the growth cycle, the legume will accumulate an amount of N coming from three distinct N pools: i) soil, ii) fertiliser and iii)

air. In order to determine the proportion of N derived from symbiotic N fixation, at least two ^{15}N enrichment levels of the fertiliser are necessary. The method is applicable if the ^{15}N composition of the indigenous soil N (estimated by the ^{15}N enrichment of a non-fixing plant) is significantly different from the ^{15}N composition of the atmosphere (E_{N2}). It makes the assumption that either the soil is relatively homogenous in enrichment along the profile or that ^{15}N enrichment of the soil mineral N retrieved by the reference plant represents that retrieved by the legume. To these points MET resembles NA but is theoretically more powerful because the applied ^{15}N fertiliser gives supplemental precision. The comparison of legume treatments among them does not imply the assumptions concerning identical N retrieval of different species in indigenous and fertiliser soil N pools like ID.

3. Determination of the Domain of Validity of Three Isotopic Methods from Sensitivity Analysis of Three Isotopic Methods

To our knowledge, our study is the first of its kind that theoretically and quantitatively analyse factors affecting precision of the three isotopic methods described here (NA, MET and ID) in an aim to delimit their domain of validity. To that purpose, sensitivity analysis was performed for each method to all of its parameters (Tab. 1) and recommendations were given for the use of those methods (Tab. 2).

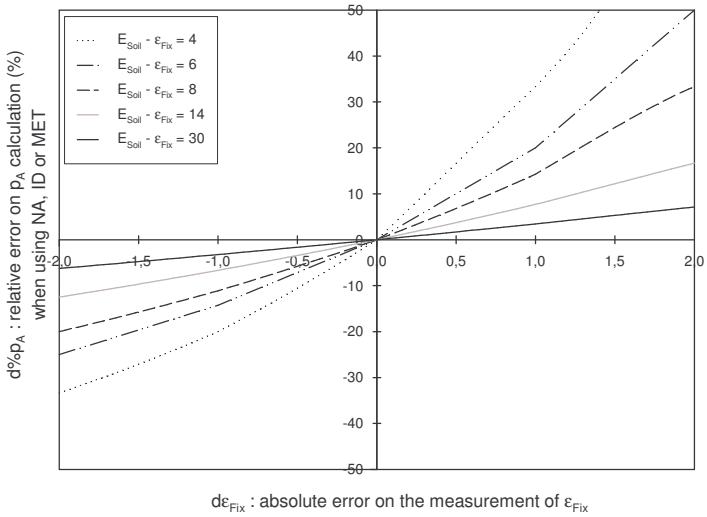

Figure 1: Sensitivity analysis of three isotopic methods used for the measurement of N_2 fixation in the field (NA, ID and MET) to isotopic fractionation due to symbiotic N_2 fixation (ε_{Fix}). Relative error on p_A ($d\%p_A$) was plotted as a function of absolute error on the measurement of ε_{Fix} ($d\varepsilon_{Fix}$), according to eq. 1 and 2 in Tab. 1. Various levels of differential between soil ^{15}N enrichment (E_{Soil}) and ε_{Fix} were represented in different abacus. In NA and MET, E_{Soil} represents ^{15}N enrichment of indigenous soil N, while in ID, it represents ^{15}N enrichment of soil plus fertiliser. Arrows show that when E_{Soil}- ε_{Fix} = 8‰, an absolute error on ε_{Fix} of +1‰ (-1‰ resp.) leads to relative error on p_A of 15% (11% resp.).

The three isotopic methods were shown to be equally sensitive to ε_{Fix} (eq. 1 and 2 (Tab. 1) and Fig. 1), depending on the level of differential ^{15}N enrichment between the soil and atmosphere (E_{Soil}-ε_{Fix}). As such, when ε_{Fix} is known with low precision (±1‰, Unkovich et al., 1994), a minimal level of (E_{Soil}-ε_{Fix}) of 8‰ is required so that the relative error on p_A remains lower than 15%, whatever the level of p_A (Fig. 1). Therefore, when ^{15}N enrichment of indigenous soil N is lower than 8‰, it can be considered that NA and MET methods are applicable only provided ε_{Fix} has been precisely measured (Tab. 2). Therefore, in that situation, the ID method is the most appropriate, as the addition of labelled fertiliser enables to artificially increase the ^{15}N enrichment of the soil plus fertiliser mineral N pool above 8‰. Unkovich and Pate (2000) stated that error on p_A associated to inaccurate measurement of ε_{Fix} is likely to be small for p_A measurements higher than 0.85 only (after Unkovich et al., 1994). Actually, we showed that the effect of inaccurate measurement of ε_{Fix} on error on p_A did not depend on the level of p_A but on the level of (E_{Soil}-ε_{Fix}) (eq. 1 (Tab. 1) and Fig. 1).

Figure 2: Sensitivity analysis of the NA (2A), ID (2B) and MET (2C) methods to ^{15}N enrichment of soil N (E_{Soil}), according to eq. 3 and 4 (Tab. 1). In case of ID, soil N includes the fertiliser N that was added to increase ^{15}N enrichment of mineral N pool, while in NA and MET soil N represents indigenous soil N only. For NA and ID (2A and 2B respectively) the minimal value of differential between ^{15}N soil enrichment (E_{Soil}) and ε_{Fix} so that relative error on p_A remains lower than 15 % was plotted as a function of p_A. Various levels of absolute error on the measurement of E_{Soil} (dE_{Soil}) were represented in different abacus in Fig. 2A and 2B. Despite the same equation applied for both NA and ID methods, the extent of the (E_{Soil}-ε_{Fix}) greatly differs between methods. Therefore, a different graph was shown for each method (Fig. 2A and 2B). The different values of (E_{Soil}-ε_{Fix}) (as Y-axis) and of dE_{Soil} (as abacus) were selected according to our own observations (unpublished data associated to Voisin et al., 2002). For the MET method (2C), the minimal value of differential between ^{15}N soil enrichment (E_{Soil}) and ε_{Fix} so that relative error on p_A remains lower than 15 % was plotted as a function of p_A, with absolute error on the measurement of E_{Soil} (dE_{Soil}) fixed to 2‰. To represent the effect of variations of p_F (eq. 4, Tab. 1), which is the proportion of N in the by legume that was retrieved from fertiliser, we used another variable x_F representing the proportion of fertiliser retrieval relative to total mineral N. Various levels of x_F were represented as abacus of Fig. 2C. x_F was preferred to p_F as it is independent from p_A (contrary to p_F). $x_F = p_F / (p_F + p_S)$ with p_S he proportion of N in the by legume that was retrieved from indigenous soil N. Figure 2 must be interpreted as follows: in the case of the ID method (Fig. 2B), if measurement of (E_{Soil}-ε_{Fix}) is about 60 ± 5 ‰, the minimal p_A value that can be measured with relative error on p_A remaining lower than 15 % is 0.4. In that case, measurement of p_A values lower than 0.4 may be associated to $d\%p_A$ higher than 15 %.

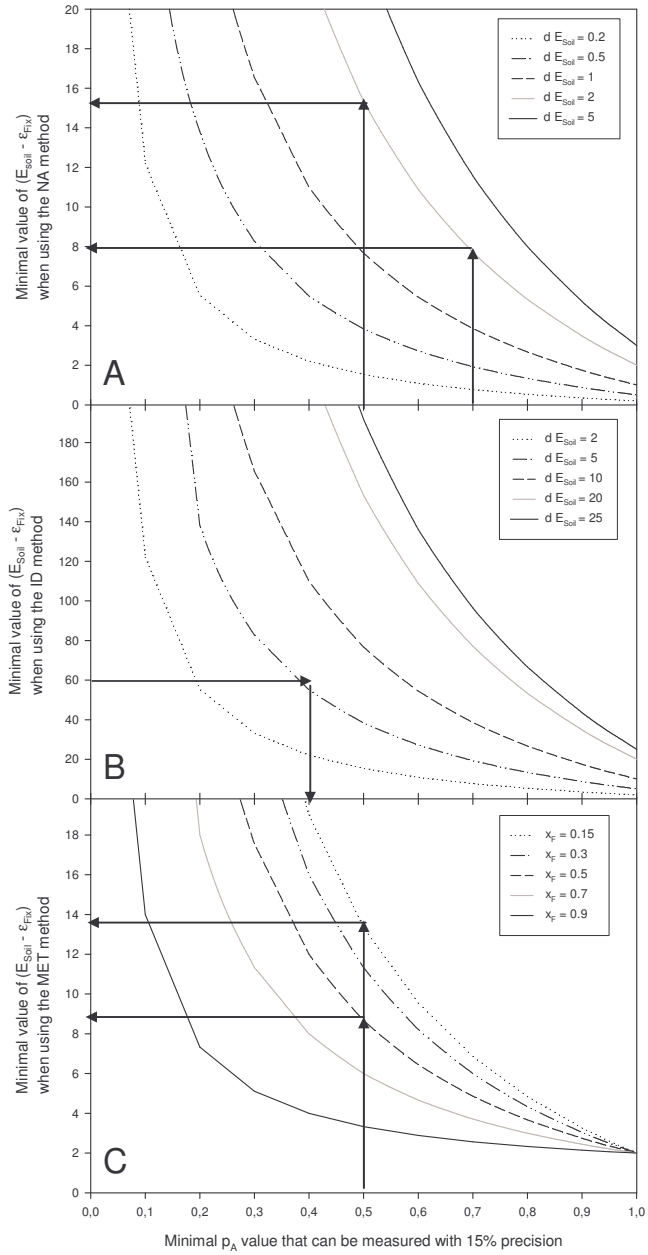

Figure 2: Sensitivity analysis of the NA (2A), ID (2B) and MET (2C) methods to ^{15}N enrichment of soil N (E_{Soil}), according to eq. 3 and 4 (Tab. 1).

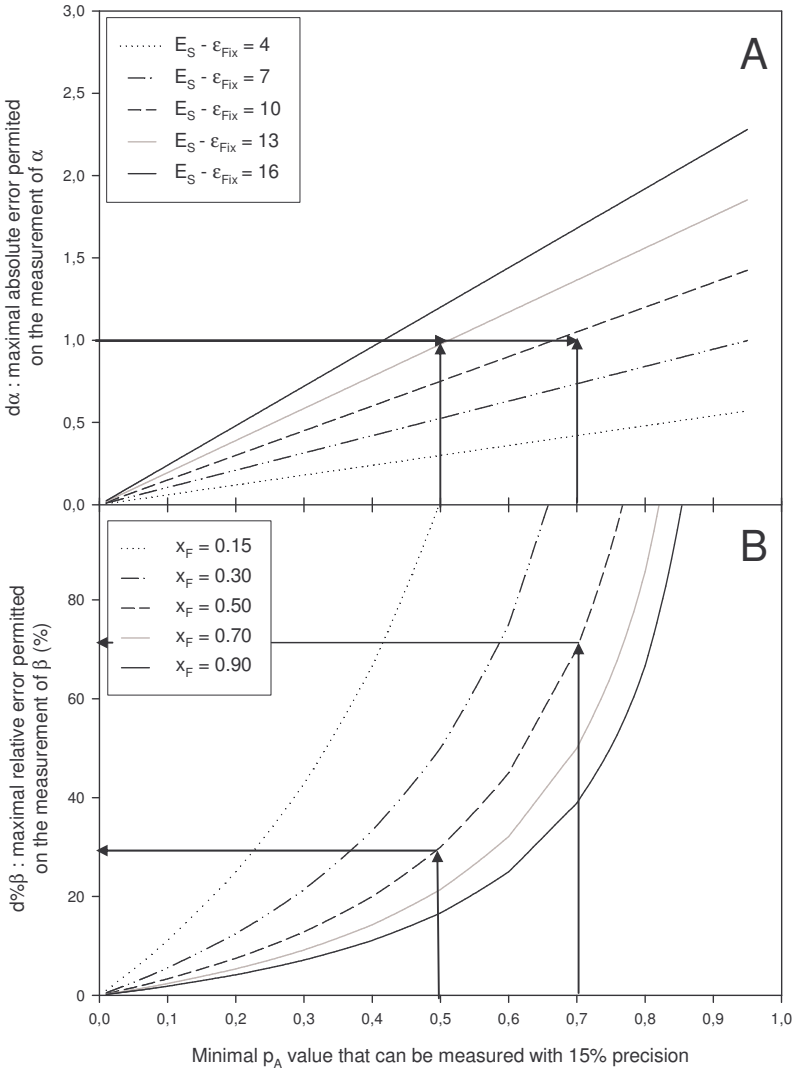

Figure 3: Sensitivity analysis of the MET method to measurement of the Y-intercept α (Fig. 3A) and the slope β (Fig. 3B) of the relationship between legume plant ^{15}N enrichment and fertiliser ^{15}N enrichment, according to eq. 5 and 6 (Tab. 1). 3A: Maximal absolute error of α (dα) permitted (i.e. minimal precision) so that relative error on p_A remains lower than 15 % as a function of p_A. Various levels of differential between ^{15}N enrichment of indigenous soil (E_{Soil}) and ε_{Fix} were represented in different abacus. 3B: Maximal relative error on measurement of β (d%β) permitted (i.e. minimal precision) so that relative error on p_A remains lower than 15% as a function of p_A, with soil ^{15}N enrichment (E_{Soil}) fixed to 8 ‰ and ε_{Fix} fixed to 0 ‰. To represent the effect of variations of p_F, various levels of the proportion of fertiliser retrieval relative to total mineral N (x_F) were represented in different abacus, for the same reasons as in Fig. 2. Arrows in Fig. 3A show that when sdα = ±1 ‰, the minimal value of (E_{Soil}-ε_{Fix}) must be as high as 10 ‰ or 13 ‰ to enable measurement of p_A values as low as 0.7 or 0.5 respectively with 15 % precision.

As calculation of p_A involves (E_{Soil}-ε_{Fix}) as denominator (eq. 7 and 8, Tab. 1), all methods were additionally sensitive to the precision and level of (E_{Soil}-ε_{Fix}), but the extent of the error on p_A also depends on the level of p_A (eq. 3 and 4 (Tab. 1) and Fig. 2). In case of NA and ID, a level of (E_{Soil}-ε_{Fix})±dE_{Soil} as high as 8±2‰ enables measurement of p_A in the range [0.7;1.0] with precision equal or lower than 15% while a level of (E_{Soil}-ε_{Fix})±dE_{Soil} as high as 13±2‰ is required to measure p_A as low as 0.55 with precision equal to 15%. The use of MET with low amount of fertiliser N retrieved from total mineral N allows to enlarge slightly the spectrum of application of NA for example to measurements of p_A as low as 0.50 with 15% precision for (E_{Soil}-ε_{Fix})±dE_{Soil} equal to 13±2‰ (Fig. 2C and Tab. 2). However, MET is additionally very sensitive to measurement of the Y-intercept α of the relationship between legume plant and fertiliser ^{15}N-enrichments (Fig. 3A) while it is much less sensitive to its slope β (Fig. 3B). Thus, for (E_{Soil}-ε_{Fix})<10‰, the range of p_A values to be measured is likely to be slightly higher with MET than with NA only provided α can be measured with high precision (Fig. 3A and Tab. 2). As α represents the ^{15}N-enrichment of legume grown with unlabelled fertiliser, we recommend a direct measurement on such legume plants in the field instead of calculation by linear regression. Still, as α is likely to be low (due to low enrichment of air and plant available soil nitrogen in that case), precision on this value may be critical and spatially variable. As such, for low level of fertiliser N application, the ID method is recommended for measurements of p_A lower than 0.65 when (E_{Soil}-ε_{Fix})=8-10‰ and for p_A lower than 0.55 when (E_{Soil}-ε_{Fix})=10-13‰ (Tab. 2). NA and MET are not applicable when (E_{Soil}-ε_{Fix})<6‰. When (E_{Soil}-ε_{Fix})=6-8‰, they can be used only for p_A measurements higher than around 0.70 and only provided that ε_{Fix} and/or α can be evaluated with high precision (Tab. 2). Our results are in accordance with authors who stated that a level of E_{Soil} as high as 10-15‰ is sufficient to provide good estimates of p_A (Ledgard and Peoples, 1988), but this must be restricted to measurements of p_A values higher then 0.55. Authors who stated that E_{Soil} levels lower than 6‰ (Unkovich et al., 1994) may be sufficient did not take into account possible variability on measurement of E_{Soil} and inaccuracy on measurement of ε_{Fix}.

Precise determination of ε_{Fix} is of paramount importance for NA (Carlsson et al., 2006) and MET, especially when the natural level of E_{Soil} is lower than 8‰, which is often the case. Indeed, arable soils are generally enriched in ^{15}N, to usually less than 10‰ (Shearer et al., 1978; Rennie and Rennie, 1983; Shearer and Kohl, 1986; Unkovich et al., 1994). ε_{Fix} can be measured as the ^{15}N-enrichment of legume plant relying solely on symbiotic N fixation for its N nutrition. However, ε_{Fix} varies with plant age, species (Unkovich et al., 1994, Mariotti et al., 1980, 1983), rhizobial strain and growing conditions (Unkovich et al., 1994; Ledgard, 1989; Carlsson et al., 2006). Values of ε_{Fix} also vary as a function of the organ being considered, being more negative for roots than for shoot (Unkovich et al., 1994). ε_{Fix} usually decreases with the plant age, being positive at the beginning of the growth cycle as it reflects N from the seed and it decreases thereafter sharply below 0 (Mariotti et al., 1980; Unkovich et al., 1994).

Both the MET and ID methods can also be used with higher levels of fertiliser application to study mixed N nutrition regimes, as increasing amounts of plant available soil N gradually lead to reduced proportion of symbiotic fixation (Voisin et al., 2002). In that case, measurement of p_A by MET is less sensitive to the level of (E_{Soil}-ε_{Fix}) than for low level of fertiliser N retrieval to total soil N (Tab. 1 and Fig. 2C). Therefore, for a given level of

(E_{Soil}-ε_{Fix}), the range of p_A to be measured is higher than for low level of fertiliser retrieval, for the same precision on p_A calculation. As for an example, if the amount of fertiliser N retrieved is equal to that of indigenous soil N, levels of p_A as low as 0.50 (x_F=0.50) can be measured when (E_{Soil}-ε_{Fix}) is in the range 8-10‰. However, MET is still as sensitive to ε_{Fix} and α when labelled fertiliser is added.

For all the methods studied, error on p_A calculation increases as p_A decreases, and as stated by Chalk and Ladha (1999), this has to be as an "intrinsic weakness" of these methodologies. Thus when p_A decreases, the required level of (E_{Soil}-ε_{Fix}) has to be higher and precision for α measurement has to be higher for a given level of error on p_A. This comes from the fact that when p_A decreases, the contribution of soil N to total N acquisition by the plant increases and parameters like (E_{Soil}-ε_{Fix}) and α take more importance and require more precision and/or higher level.

4. Evaluation of p_A When Using ID and Recommendations to Minimise the Error

ID has the largest spectrum of application as, unlike NA and MET, it does not require precise evaluation of ε_{Fix} and is valid for low levels of ^{15}N-enrichment of the soil (E_S) and for measurements of low values of p_A. Therefore, in an aim to optimise experimental set up when using ID, we first to tried to objectively characterise the error made on the calculation of p_A when the main assumption is not met i.e. when the reference plant and the legume do not take up mineral N in identical proportions in indigenous soil N and in fertiliser N pools. To that purpose, the deviation to the theoretical value of p_A using the ID method was calculated (see appendix B). It was expressed as a function of ($x'_F - x_F$) which represents the differential between the reference plant and the legume for proportion of fertiliser N retrieval to total mineral N retrieval, as it is the central hypothesis of the ID method. Sensitivity analysis was then performed and shown in Fig. 4. Relationship between $d\%p_A$ and (x'_F-x_F) followed similar trends in all cases and can be first analysed using Fig. 4A as an example. In all cases, the highest was the absolute value of (x'_F-x_F), the highest was the relative error on p_A. Besides, $d\%p_A$ was higher when (x'_F-x_F) took negative values than when it took positive values. $d\%p_A$ additionally increased when the level of p_A decreased (Fig. 4A), when the level of ^{15}N-enrichment of the fertiliser added E_F increased (Fig. 4B), when the level of ^{15}N enrichment of the soil N E_S decreased (Fig. 4C) and when x_F decreased Fig 4D). Finally, the sensitivity analysis shows that the error on p_A was highly sensitive to the level of p_A (Fig. 4A). It was moderately sensitive to ^{15}N enrichments of the fertiliser (E_F) and of the soil (E_S) (Fig. 4B and 4C) when ^{15}N enrichment of total mineral N pool becomes less and less uniform. Nevertheless, the error on p_A reaches a maximum value when E_F is beyond 500 ‰ (Fig. 4B). The level of x_F (the proportion of fertiliser retrieved to total mineral N) is also of a great importance, as it adversely modulated the error on p_A for a given level of (x'_F-x_F) and its maximal value (Fig. 4D).

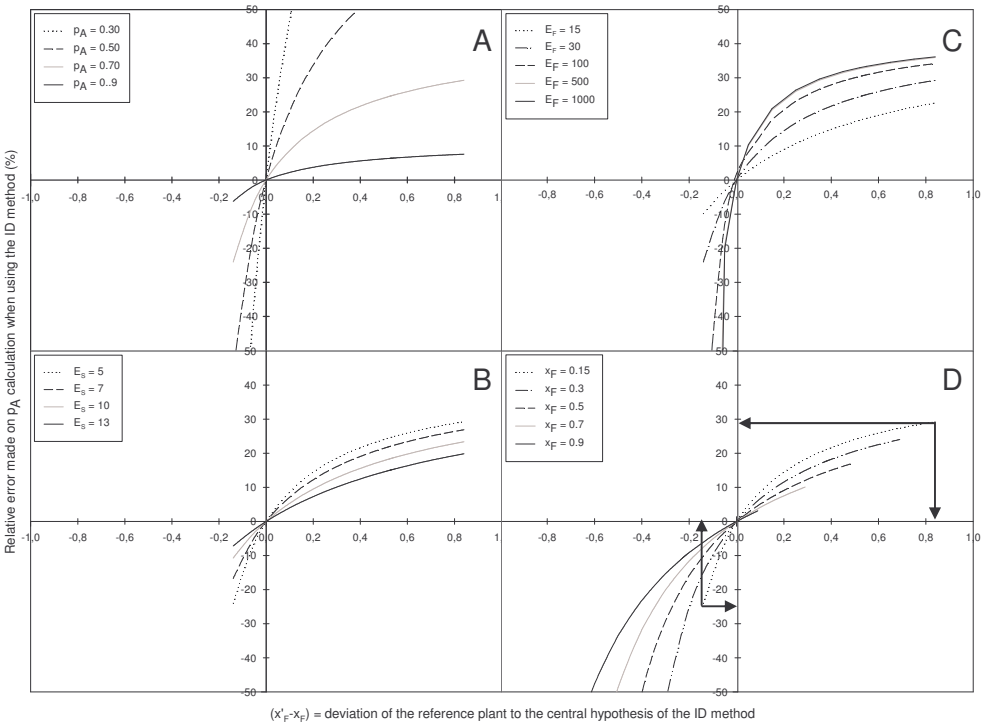

Figure 4: Sensitivity of the error made on p_A when using the ID method (dp_A, see eq. (C13)) to its different parameters: $p_{A:}$ proportion of symbiotic fixation to overall N acquisition (Fig 4A), E_F: ^{15}N enrichment of fertiliser N (Fig. 4B), E_S : ^{15}N enrichment of indigenous soil N (Fig. 4C), $x_{F:}$ proportion of fertiliser taken up by the legume plant relative to total mineral N (Fig. 4D). Relative error made on p_A when using the ID method ($d\%p_A$) as a function of differential proportions of fertiliser N taken up relative to total mineral N between the legume plant (x_F) and the non fixing reference plant (x'_F). Various levels of $p_{A,}$, E_F, E_S and x_F were presented in abacus in Fig. 4A to 4D respectively. It was considered that ε_{Fix} was a constant parameter which was determined by the choice of the legume plant under study (here ε_{fix} = -1, for pea). In each graph, the other parameters were fixed to constant values as follows: E_S = 5 ‰; E_F = 30 ‰; p_A = 0.70; x_F = 0.15; ε_{Fix} = -1 ‰.NB: As x'_F varies between 0 and 1, (x'_F-x_F) only takes values in the range [-x_F ; 1-x_F]. Therefore, $\%dp_A$ tends to finite maximal values when (x'_F-x_F) tends towards -x_F and when it tends towards (1-x_F). As $\%dp_A$ is higher when (x'_F-x_F) takes negative values, when x_F increases, the range of possible (x'_F-x_F) values shifted to lower values and thus yielded maximal $d\%p_A$ globally higher. For example, for x_F = 0.15, (x'_F-x_F) varied in the range [-0.15; 0.85] ‰ and $d\%p_A$ was [-24; +29] % while for x_F = 0.50, (x'_F-x_F) varied in the range [-0.50; 0.50] ‰ and $d\%p_A$ was [-85; +17] (Fig. 4D).

Considering our results (Fig. 2) and others (Ledgard and Peoples, 1988), a level of soil plus fertiliser ^{15}N enrichment E_{FS} of 15-20 ‰ is sufficient to ensure good precision on the measurement of $p_{A.}$ To reach this level, E_{FS} is a combination of the amount n_F (therefore the proportion X_F relative to total mineral N) of the ^{15}N enrichment of the added fertiliser (E_F). Its calculation has to take into account the dilution caused by mineralization of non labelled soil N across the growth cycle. Indeed, ^{15}N enrichment of fertiliser plus soil N at sowing

exponentially decreases across the growing season mainly due to dilution by mineralization of inorganic soil N (Witty, 1983). Simulations were made in Table 3. For a given level of E_{FS}, various combinations of (E_F x X_F) resulted in similar maximal error on p_A (Tab. 3), but this maximal value increased when E_{FS} increased. Indeed, for a given level of $E_{FS,}$ effects of increased levels of x_F (that increased maximal error on $p_{A,}$ Fig. 4D) were compensated by associated decreased levels of E_F (that decreased error on $p_{A,}$ Fig. 4C). However, for a given level of $E_{FS,}$ the error globally decreased when X_F increased. For a given level of E_{FS}, the choice of the most appropriate (E_F x X_F) depends on antagonistic requirements concerning X_F. The amount of fertiliser N added (n_F) increases the amount of total plant available soil N and thus mechanically modify the level of p_A (McNeill et al., 1996; Voisin et al., 2002). Thus on the one hand, n_F and therefore X_F must be low enough so that it does not modify substantially total soil N (thus p_A). On the other hand, increased levels of x_F allow limiting error on p_A. A compromise could be to select an amount of labelled fertiliser in proportion to total N (x_F) between 0.05 and 0.10 (Tab 2). In order to ensure maximal homogeneity, and as the amount of ^{15}N added is low and therefore cost-effective, we also advise to apply it at a large scale, dissolved in water with a field sprayer (with flow of spray proportional to the tractor speed). This is in accordance with the methods proposed by Duc et al. (1988), Reiter et al. (2002) and Voisin et al. (2002).

Conclusion

Danso et al (1993) stated that validity of the ID method was critical for levels of p_A lower than 0.6. However, we showed that judicious application of labelled fertiliser can contribute to reduce potential error on the measurement of p_A. Moreover, in case of low level of ^{15}N enrichment of indigenous soil, ID is the only isotopic method that can be used when ε_{Fix} cannot be measured precisely. It is often the case when numerous measurements are made across the growth cycle and/or when several plant genotypes and/or rhizobial strains are used, as ε_{Fix} should ideally be evaluated for each growth stage x genotype x strain situation. The main problem of the ID method still remains the difficult choice of the reference plant. MET is the only adequate method that allows measuring respective N retrieval by the legume plant in the indigenous soil pool and in the fertiliser pool separately. Thereafter it allows comparing the proportion between these retrievals to that of the reference plant (measured by simple isotopic dilution). However, MET has only been used in the greenhouse (Ledgard et al., 1985b) and remains to be tested in the field. Still, in any case, even if the reference plant does not exactly fit assumptions, ID is probably adequate for measuring symbiotic fixation at the end of the growth cycle when p_A is the highest. It may also be adequate for measuring the amount of N fixed by difference between two dates on a short time step, as the error due to the reference plant, if any, would probably be of similar magnitude on a short period.

Reviewed by Erik Steen Jensen.

Appendix A.

Description of the 3 methods for estimating the proportion of fixed-N
Let be:

N	the amount of N taken up by the legume	(kg ha^{-1})
N_A	the amount of N taken up by the legume derived from atmosphere	(kg ha^{-1})
N_S	the amount of N taken up by the legume derived from soil	(kg ha^{-1})
N_F	the amount of N taken up by the legume plant derived from fertiliser	(kg ha^{-1})
N_{FS}	the amount of N taken up by the legume derived from soil plus fertiliser	(kg ha^{-1})
E	the isotopic excess of the legume	(atom% ou δ‰)
E'	the isotopic excess of the reference plant	(atom% ou δ‰)
E_{N2}	the mean isotopic excess of atmospheric dinitrogen	(atom% ou δ‰)
E_{SOIL}	the mean isotopic excess of the soil	(atom% ou δ‰)
$E_{FERTILISER + SOIL}$	the mean isotopic excess of the soil plus fertiliser N pool	(atom% ou δ‰)
E_A	the mean isotopic excess of the fixed N in the legume	(atom% ou δ‰)
E_S	the mean isotopic excess of the absorbed soil N in the legume	(atom% ou δ‰)
E_F	the mean isotopic excess of the absorbed fertiliser N in the legume	(atom% ou δ‰)
E_{FS}	the mean isotopic excess of the absorbed soil plus fertiliser N pool	(atom% ou δ‰)
$E_{Soil} = E_S$	for NA and MET and $E_{Soil} = E_{FS}$ for ID	(atom% ou δ‰)
p_A	the proportion of fixed N in the legume	($p_A = N_A / N$)
p_S	the proportion of N in the legume plant derived from soil	($p_S = N_S / N$)
p_F	the proportion of N in the legume plant derived from fertiliser	($p_F = N_F / N$)
p_{FS}	the proportion of N in the legume derived from soil plus fertiliser	($p_{FS} = N_{FS} / N$)

1) Natural Abundance Method (NA)

We consider two sources of N for the legume: soil N and atmospheric N.
The N balance and ^{15}N balance can be written:

$$N = N_S + N_A \tag{A1}$$

$$NE = N_S E_S + N_A E_A \tag{A2}$$

The latter equation can be written using p_A, the proportion of fixed N

$$p_A + p_S = 1 \tag{A3}$$

$$E = p_A E_A + (1 - p_A) E_S \tag{A4}$$

The isotopic excess of N assimilated from atmopsheric and soil N pools are respectively:

$$E_A = E_{N2} + \varepsilon_{Fix} \tag{A5}$$

$$E_S = E_{SOIL} + \varepsilon_A \tag{A6}$$

where ε_{Fix} and ε_A are the isotopic fractionation coefficients for symbiotic nitrogen fixation and soil N absorption, respectively.

By definition, $E_{N2} = 0$. Equation (A4) can then be written:

$$p_A = \frac{E - E_{SOIL} - \varepsilon_A}{-E_{SOIL} + \varepsilon_{Fix} - \varepsilon_A} \tag{A7}$$

Hypothesis: the isotopic composition of the reference plant (E') is the same than that of the nitrogen assimilated by the legume (E_A)

$$E' = E_{soil} + \varepsilon_A \tag{A8}$$

Then, p_A can be calculated as follows:

$$\boxed{p_A = \frac{E' - E}{E' - \varepsilon_{Fix}}} \tag{A9}$$

2) Isotope Dilution Method (ID)

The legume receives ^{15}N-labelled fertiliser which is supposed to make a uniformly available pool with indigenous soil N. We then consider two sources of N for the legume: the (soil+fertiliser)-N and the atmospheric N.

The N balance and ^{15}N balance can be written:

$$N = N_{FS} + N_A \tag{D1}$$

$$NE = N_{FS}E_{FS} + N_AE_A \tag{D2}$$

These equations can be written:

$$p_{FS} + p_A = 1 \tag{D3}$$

$$E = p_{FS}E_{FS} + p_AE_A \tag{D4}$$

So that:

$$E = (1 - p_A)E_{FS} + p_AE_A \tag{D5}$$

$$p_A = \frac{E - E_{FS}}{E_A - E_{FS}} \tag{D6}$$

Since

$$E_A = \varepsilon_{Fix} \tag{D7}$$

$$E_{FS} = E_{FERTILISER+SOIL} + \varepsilon_A \tag{D8}$$

where ε_{Fix} and ε_A are the isotopic fractionation coefficients for symbiotic nitrogen fixation and soil N absorption, respectively. ε_A can be neglected compared to $E_{FERTILISER + SOIL}$ which results from addition of ^{15}N enriched fertiliser.

Equation D6 can be written:

$$p_A = \frac{E_{FS} - E}{E_{FS} - \varepsilon_{Fix}} \tag{D9}$$

Hypothesis: the isotopic excess of the reference plant (E') grown with ^{15}N-labelled fertiliser is equal to the isotopic excess of the available N from soil+fertiliser (E_{FS})

$$E' = E_{FS} \tag{D10}$$

$$\boxed{p_A = \frac{E^{'} - E}{E^{'} - \varepsilon_{Fix}}} \tag{D11}$$

3) Multiple Enrichment Technique (MET)

We now consider three sources of N for the legume: soil N, fertiliser N and atmospheric N.

The N balance and ^{15}N balance can be written:

$$N = N_S + N_F + N_A \tag{M1}$$

$$NE = N_S E_S + N_F E_F + N_A E_A \tag{M2}$$

These equations can be written:

$$p_S + p_F + p_A = 1 \tag{M3}$$

$$E = p_S E_S + p_F E_F + p_A E_A \tag{M4}$$

The latter equation shows that the excess of the legume plant is linearly related to the excess of fertiliser. It can be written:

$$E = \alpha + \beta E_F \tag{M5}$$

with

$$\alpha = p_S E_S + p_A E_A \tag{M6}$$

and

$$\beta = p_F \tag{M7}$$

$$E_A = \varepsilon_{Fix}$$

and E_S is given by a reference plant receiving no fertiliser (E').

$$\boxed{p_A = \frac{-\alpha + (1-\beta)E'}{E' - \varepsilon_{Fix}}} \tag{M8}$$

$$p_S = \frac{\alpha - (1-\beta)E'}{E' - \varepsilon_{Fix}} \tag{M9}$$

The three origins of the nitrogen in the legume can be calculated using equations (M7-M9).

Appendix B: Calculations of the Error Committed when Using DI

Let us define:

p_D the estimate of symbiotic nitrogen fixation by the ID method
p_A the (true) value of N symbiotic nitrogen fixation
dp_A the absolute error made on the estimate by the ID method with

$$dp_A = p_D - p_A \tag{C1}$$

p_F and p'_F, the proportion of N derived from fertiliser N to total N uptake in the legume and the reference plant respectively.

p_S and p'_S, the proportion of N derived from soil N to total N uptake in the legume and the reference plant respectively.

k and k', the ratio of fertiliser derived N to soil derived N in the legume and in the reference plant respectively.

$$k = \frac{p_F}{p_S} \tag{C2}$$

and

$$k' = \frac{p'_F}{p'_S} \tag{C3}$$

According to eq. (E1):

$$p_D = \frac{E' - E}{E' - \varepsilon_{Fix}} \tag{C4}$$

$$E = p_A \varepsilon_{Fix} + p_S \left(E_S + kE_F\right) \tag{C5}$$

$$E' = p'_S \left(E_S + k' E_F\right) = \frac{E_S + k' E_F}{1 + k'} \tag{C6}$$

Combining equations C4, C5 and C6 yields:

$$p_D = \frac{E_S + k' E_F - (1 + k')\left[p_A E_A + p_S \left(E_S + kE_F\right)\right]}{E_S + k' E_F - E_A (1 + k')} \tag{C7}$$

The error made with the ID method is

$$dp_A = p_D - p_A = \frac{N}{D} \tag{C8}$$

with $$D = E_S + k' E_F - E_A (1 + k') \tag{C9}$$

and $$N = (E_S + k' E_F)(1 - p_A) - (1 + k') p_S (E_S + kE_F) \tag{C10}$$

Substituting $p_S = \frac{1 - p_A}{k + 1}$ in equation (C10) gives

$$N = \frac{1 - p_A}{k + 1}(k' - k)(E_F - E_S) \tag{C11}$$

Let be x_F and x'_F the ratio of fertiliser derived N to total mineral N accumulated by the legume and the reference plant respectively.

$$x_F = \frac{p_F}{p_F + p_S} \tag{C12}$$

Using equations C8, C9, C10, C12 and C2,

$$\boxed{dp_A = \frac{(1 - p_A) \cdot (1 - x_F) \cdot (k' - k) \cdot (E_F - E_S)}{E_S - \varepsilon_{Fix} + k'(E_F - \varepsilon_{Fix})}} \tag{C13}$$

Equation (C13) shows that the error dp_A is nil if one of the following conditions is met:

1). $1\text{-}p_A = 0$
2). $x_F = 0$
3). $k'\text{-}k = 0$
4). $E_F = E_S$

Conditions (1), (2) and (4) are unlikely to be fulfilled. The use of the isotopic dilution method is not needed when conditions (1) and (2) are true as: $1\text{-}p_A = 0$ (1) means that the legume plant has access to only one source of N (atmospheric N) ; $1\text{-}x_F = 0$ (2) means that N retrieval in indigenous soil N is null i.e. the soil is deprived of mineral N. Fulfilment of condition (4) ($E_F = E_S$) means that the enrichment of the mineral N source including soil plus fertiliser N is uniform, which cannot be achieved considering that fertiliser is added to increase ^{15}N soil enrichment therefore E_F is necessarily higher than E_S. The third condition is fulfilled when the proportions of soil N and fertiliser N derived from total plant available soil N are be identical for the reference plant and for the legume studied (case (3) : k'-k = 0). This is the central assumption of the ID method.

Table 1. **Sensitivity analysis of p_A calculation with three different isotopic method: NA (Natural Abundance), ID (Isotopic Dilution) and MET (Multi-enrichment Technique).**

Sensitivity of p_A to	when using NA or ID	when using MET
ε_{Fix}	$d\%p_A = \frac{d\varepsilon_{Fix}}{(E_{Soil} - \varepsilon_{Fix}) - d\varepsilon_{Fix}}$ (1)	$d\%p_A = \frac{d\varepsilon_{Fix}}{(E_{Soil} - \varepsilon_{Fix}) - d\varepsilon_{Fix}}$ (2)
E_{Soil}	$d\%p_A = \frac{dE_{Soil} \cdot (1 - p_A)}{p_A \cdot (dE_{Soil} + (E_{Soil} - \varepsilon_{Fix}))}$ (3)	$d\%p_A = \frac{dE_{Soil} \cdot (1 - p_A - p_F)}{p_A \cdot (dE_{Soil} + (E_{Soil} - \varepsilon_{Fix}))}$ (4)
α	ND	$d\%p_A = \frac{-d\alpha}{p_A \cdot (E_{Soil} - \varepsilon_{Fix})}$ (5)
β	ND	$d\%p_A = \frac{-d\%\beta \cdot p_F \cdot E_{Soil}}{p_A \cdot (E_{Soil} - \varepsilon_{Fix})}$ (6)

Calculation of relative error made on p_A ($d\%p_A$) when varying each parameter of p_A. For each method, p_A was calculated as follows:

For NA and ID, $$p_A = \frac{E_{Soil} - E}{E_{Soil} - \varepsilon_{Fix}} \quad (7)$$

For MET, $$p_A = \frac{-\alpha + (1-\beta)E_{Soil}}{E_{Soil} - \varepsilon_{Fix}} \quad (8)$$

with E and E_{Soil}: ^{15}N enrichments of the legume and the soil respectively; ε_{Fix}: isotopic fractionation associated to symbiotic fixation; p_F : proportion of N in the by legume that was retrieved from fertiliser. α and β: Y-intercept and slope, respectively, of the relationship between ^{15}N enrichments of the legume and that of fertiliser N added (for MET). E_{Soil} is given by the enrichment of a non fixing reference plant. In NA and MET, E_{Soil} represents ^{15}N enrichment of indigenous soil N. In ID, E_{Soil} represents ^{15}N enrichment of soil plus fertiliser.

Table 2. **Validity domain of three isotopic methods used for the measurement of symbiotic N_2 fixation in the field: NA (Natural Abundance), ID (Isotopic Dilution) and MET (Multi-enrichment Technique).**

Method	x_{F-}	$E_{Soil} - \varepsilon_{Fix} < 6$ ‰	$E_{Soil} - \varepsilon_{Fix} = 6$ -8 ‰	$E_{Soil} - \varepsilon_{Fix} = 8$ -10 ‰	$E_{Soil} - \varepsilon_{Fix} = 10$ -13 ‰
NA	0	not applicable unless for $p_A > 0.75$ with precise ε_{Fix}	$p_{A\ min} = 0.75 - 0.70$ $d\varepsilon_{Fix} = \pm 0.5$ ‰	‰ $p_{A\ min} = 0.70 - 0.65$ $d\varepsilon_{Fix} = \pm 1$ ‰	$p_{A\ min} = 0.65 - 0.55$ $d\varepsilon_{Fix} = \pm 1$‰
MET	0.15	not applicable	$p_{A\ min} = 0.75 - 0.65$ $d\varepsilon_{Fix} = \pm 0.5$ ‰ $d\alpha = \pm 0.6$ ‰	$p_{A\ min} = 0.65 - 0.60$ $d\varepsilon_{Fix} = \pm 1$ ‰ $d\alpha = \pm 0.7$ ‰	$p_{A\ min} = 0.60 - 0.50$ $d\varepsilon_{Fix} = \pm 1$ ‰ $d\alpha = \pm 1$ ‰
ID	0.15	recommended	recommended for $p_A < 0.70$ or when $d\varepsilon_{Fix} = \pm 1$ ‰	recommended for $p_A < 0.65$	recommended for $p_A < 0.55$
MET	0.15	not applicable	$p_{A\ min} = 0.65 - 0.55$ $d\varepsilon_{Fix} = \pm 0.5$ ‰ $d\alpha = \pm 0.6$ ‰	$p_{A\ min} = 0.55 - 0.45$ $d\varepsilon_{Fix} = \pm 1$ ‰ $d\alpha = \pm 0.7$ ‰	$p_{A\ min} = 0.45 - 0.40$ $d\varepsilon_{Fix} = \pm 1$ ‰ $d\alpha = \pm 1$ ‰
ID	0.5	recommended	recommended for $p_A < 0.60$ or when $d\varepsilon_{Fix} = \pm 1$	recommended for $p_A < 0.50$	recommended for $p_A < 0.45$

Recommendations are given for various levels of differential between ^{15}N soil enrichment (E_{Soil}) and isotopic fractionation associated to symbiotic N fixation (ε_{Fix}), and for various levels of proportion of fertiliser N retrieval to total mineral N (x_F). Analysis was not shown for values of ($E_{Soil-}\ \varepsilon_{Fix}$) higher than 13 ‰ as they are unrealistic.

α is the Y-intercept of the linear relationship between ^{15}N enrichment of the plant and ^{15}N enrichment of fertiliser N in the MET method.

Table 3. **Simulations of ^{15}N enrichment level of fertiliser N (E_F) and error made on p_A calculation (for $p_A = 0.7$) considering a targeted level of total mineral soil N (E_{FS}) and a given amount of soil N (n_S), as varied in 3 situations.** The first simulation could correspond for example to a situation where the amount of soil mineral N (n_S) available at sowing was 30 kg N. ha^{-1}. In the second simulation n_S was increased up to 80 kg N. ha^{-1} to simulate a situation where soil mineral N made available during the growing season equal was 50 kg N. ha^{-1}. In these two situations, the targeted level of E_{FS} was 20 ‰. The third situation was identical to situation (1), excepted E_{FS} was 15 ‰.

	n_F	X_F	E_F	$\%dp_A$ with $p_A = 0.7$ for $(x'_F-x_F) = 0.2$	Range of $\%dp_A$ with $p_A = 0.7$ for whole range of (x'_F-x_F)
Situation (1)	1	0.03	255	32 %	[-43%;40%]
$n_S = 30$ kg.ha^{-1}	3	0.09	95	22 %	[-43%;36%]
$E_{FS} = 15$ ‰	5	0.14	63	18 %	[-43%;32%]
	10	0.25	39	12 %	[-43%;26%]
Situation (2)	1	0.01	655	38 %	[-43%;42%]
$n_S = 30 + 50$ kg.ha^{-1}	3	0.04	228	31 %	[-43%;40%]
$E_{FS} = 15$ ‰	5	0.06	143	37 %	[-43%;38%]
	10	0.11	79	10 %	[-43%;34%]
Situation (3)	1	0.03	410	34 %	[-70%;41%]
$n_S = 30$ kg.ha^{-1}	3	0.09	150	25 %	[-70%;37%]
$E_{FS} = 20$ ‰	5	0.14	98	20 %	[-70%;34%]
	10	0.25	59	14 %	[-70%;28%]

n_S: amount of indigenous soil mineral N (kg N.ha^{-1}).; n_F : amount of fertiliser N added

x_F and x'_F : proportion of fertiliser N retrieval to total mineral N for the legume and the reference plant, respectively. (x'_F-x_F) varies in the range $[-x_F; 1-x_F]$.

The ^{15}N enrichment of the fertiliser to add (E_F) were calculated using simple dilution equations as a function of its proportion relative to total mineral N (X_F) and of ^{15}N enrichments of indigenous soil (E_S) and of the soil plus fertiliser pool (E_{FS}). Relative error on p_A ($d\%p_A$) was calculated following eq (C13), with Es = 7 ‰ and ε_{Fix} = -1‰ for various values of (x'_F-x_F). Figure captions:

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