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Assessment of biological nitrogen fixation

S.K.A. Danso
FAO/IAEA Division, Wagramerstrasse 5, P.O. Box 100, A-1400 Vienna, Austria

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Abstract

The four commonly used methods for measuring biological nitrogen fixation (BNF) in plants are: the total nitrogen difference (TND) method, acetylene reduction assay (ARA) technique, xylem-solute (or ureide production) method and the use of ^{15}N labelled compounds.

The TND method relies on a control non-N_2-fixing plant to estimate the amount of N absorbed by the fixing plant from soil. It is one of the simplest and least expensive methods, but works best under low soil N conditions. The ARA technique measures the rate of acetylene conversion to ethylene by the N_2-fixing enzyme, nitrogenase. The ethylene produced can then be converted into N_2 fixed, using a conversion ratio, originally recommended as 3. Although the method is inexpensive and highly sensitive, its major disadvantages are, the short-term nature of the assays, the doubtful validity of always using a conversion ratio of 3 and the auto-inhibition of acetylene conversion to ethylene. The ARA technique is therefore not a method of choice for measuring BNF.

The xylem-solute technique can be used to measure BNF for those species that produce significant quantities of ureide as product of BNF. Although simple and relatively inexpensive, it is an instantaneous assay and also needs to be calibrated against a known method. The most serious limitation is, that only a small proportion of N_2-fixing plants examined are ureide exporters, and the method is therefore not widely applicable.

The ^{15}N methods, classified into the isotope dilution and A-value methods, appear to be the most accurate, but also the most expensive. They involve labelling soil with ^{15}N fertilizer and using a non-N_2-fixing reference plant to measure the $^{15}N/^{14}N$ ratio in the soil. The ^{15}N isotope dilution approach is both operationally and mathematically simpler than the A-value approach. To limit potential errors in the selection of reference crops, it is recommended to use ^{15}N labelled compounds or soil labelling methods that result in the slow release of ^{15}N or the slow decline of $^{15}N/^{14}N$ ratio in the soil. Additionally, the use of several reference plants rather than a single one can improve the accuracy of the results.

Introduction

Nitrogen is a major nutrient element required by crop plants, and its scarcity in soil significantly affects crop yields. On the other hand excessive amounts in soil could result in undesirable environmental effects. Thus, both the gains and losses of N in agricultural soils are important processes which need to be measured.

Depletion of soil N in agricultural soils occurs primarily through plant N uptake. This is often replenished through fertilizer N additions or at least in part through natural processes, the most important of which is biological nitrogen fixation (BNF). It has been estimated that on a global scale, BNF may contribute some 90 million tons ha^{-1} yr^{-1} in agricultural systems of which the *Rhizobium*-legume symbiosis is estimated to contribute about 40 million tons [25]. Accurate measures of BNF are important as a prerequisite in determining how environmental factors can be managed for higher contributions from BNF, and to get a good estimate of N balance in various soil-plant systems.

Several methods have been devised for measuring BNF. However, some of these are only qualitative and not very useful for quantification purposes [8]. The most suitable methods are those that can distinguish between the amounts or proportions of plant N derived from atmospheric N_2 fixation, distinct from the N contribution from soil, and where relevant, from fertilizer applied N. Details of the various methods for mea-

suring BNF have been sufficiently given in several reviews [7-9, 14, 16, 32, 50, 54]. The present paper will therefore not attempt to duplicate these details. Instead, it is intended to critically assess the strengths and weaknesses of those that have been most often used, and to discuss situations under which the methods could most usefully be applied, as well as, efforts to find solutions to some of the problems identified.

Total nitrogen difference (TND) method

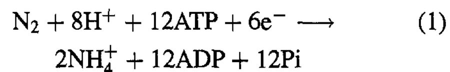
This is one of the oldest and simplest methods, and has provided many valuable estimates of N_2 fixed, upon which several management practices have been based. The TND method measures BNF as the difference between the total N contents of plants that fix N_2 and those that do not derive N from fixation. In essence, the method is based on the assumption that both the N_2 fixing and non-fixing control plants absorb equal amounts of soil N for growth [46]. However, this assumption may not hold under all situations, as it requires that the different plants, in addition to being similar in root morphology and in several physiological attributes, should also absorb their N from similar depths and horizons [11]. The fulfilment of the underlying assumption is therefore the greatest limitation of the TND method [8].

Despite the stated limitations, the TND method has on several occasions provided BNF estimates that are not significantly different from those obtained using more sophisticated and expensive techniques [22, 43]. These similarities therefore justify a close examination of the strengths of the TND method, and when it is most likely to provide satisfactory results. As suggested by Danso [9], and supported by the studies of Paterson and LaRue [40] and Rennie [45], the TND method will often give reliable estimates of N_2 fixed in plants grown in soils or systems in which the initial N content is low. This is because BNF is high under these conditions, in contrast to soil N uptake, and therefore potential errors introduced by the inability of a control plant to correctly assess the amount of soil N absorbed by the fixing plant is of limited effect (compared to where soil N uptake is high and BNF is low). Several such situations exist, e.g., in areas consisting of sand dunes, systems undergoing primary colonization, and in many semi-arid areas. It is therefore not surprising that the values of N_2 fixed assessed by Dommergues [15] for *Casuarina* stands growing on the sand dunes in Cape Verde are still widely quoted, while the BNF studies by Gauthier *et al.* [21] in a sandy soil in Senegal generally showed no significant differences between TND estimates of N_2 fixed and those obtained using the more expensive and more sophisticated ^{15}N methodologies.

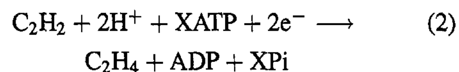
The acetylene reduction assay (ARA) technique

The ARA technique is based on the fact that nitrogenase, the enzyme involved in N_2 fixation is also able to catalyse the reduction of acetylene to ethylene [17]. The ARA technique as used today to measure BNF is based on procedures outlined by Hardy et al. [26]. Simply, the method involves incubating the sample to be assayed, e.g., detached nodules, decapitated plant with roots and nodules attached, whole plants, etc., in a gas-tight chamber containing 0.03 to 0.1% (v/v) acetylene for periods of time ranging from a few minutes to several hours. At the end of the incubation period, a gas sample from the incubation vessel is injected into a gas chromatograph fitted with a Porapak N or P column and assayed for ethylene production [24].

The amount of ethylene produced could itself be used as a measure of nitrogenase or relative N_2-fixing activity. Otherwise, the quantity of ethylene formed can be converted into total amount of nitrogen fixed by multiplying ethylene produced by a conversion factor of 3 [24]. The rationale is, that stoichiometrically, 3 pairs of electrons are used up during the conversion of N_2 to NH_4 compared to the single pair of electrons used in the conversion of acetylene to ethylene, i.e.,



$$N_2 + 8H^+ + 12ATP + 6e^- \longrightarrow 2NH_4^+ + 12ADP + 12Pi \quad (1)$$



$$C_2H_2 + 2H^+ + XATP + 2e^- \longrightarrow C_2H_4 + ADP + XPi \quad (2)$$

The ARA technique therefore indirectly measures BNF by estimating enzyme activity based on electron flux through nitrogenase. The use of the ARA technique has been facilitated by the fact that no end products other than ethylene have been identified in this reduction reaction [24]. Also, the ethylene produced is stable, and can be stored, thus making it possible to analyse the gas samples at a later period after sampling. These advantages, together with simplicity, low cost and high sensitivity made the ARA technique a method of choice, particularly in the 1970s, and together with

the oil crisis, generated great interest in BNF studies.

Although the method is still in use, it is no longer that popular, to the extent that many reviewers and editors of journals often tend to question or reject papers that base their interpretations on ARA measurements [53]. What then could have contributed to this paradox? Detailed accounts of the problems associated with the ARA technique have been provided in several critical reviews [8, 16, 338, 58], and only the major ones will be outlined briefly in this paper.

1. Probably the most apparent problem is that the ARA technique, as frequently used involves short-term assays, in contrast to the process it is intended to measure, BNF that proceeds over long durations in crops. ARA measurements therefore have to be extrapolated to cover several periods over which no measurements were made. The incorrect implication here is, that N_2 fixation rates are constant over long periods. In fact rates of N_2 fixation are known to exhibit very wide diurnal [4, 44] and seasonal variations [61, 62]. To minimise these errors, frequent sampling is required, making the method more tedious than originally intended.
2. Very few ARA measurements have been performed directly on whole plants growing in the field. However, it has been noted that any disturbance in the N_2-fixing system induces an increased resistance to the flow of oxygen into nodules and this adversely affects the rate of acetylene conversion into ethylene [36, 58]; the greater the disturbance, the greater the effect. For different samples, Vessey [53] has thus ranked the inhibition of acetylene conversion as follows: whole nodulated root with shoot intact < detached, whole nodulated roots < partially nodulated roots < nodulated root segments < detached nodules < nodule slices; even the shaking of nodules to remove adhering soil is reported to have adverse effects [58].
3. Most often the sample used for ARA assays consists of uprooted plants. The problem here is, that the amount of ethylene produced will be governed by the proportion of the active nodules that remained intact on the plant in the uprooting process. Utmost care is therefore needed in the uprooting process itself. However, even in normal friable soil or in pot studies, it is difficult to recover 100% of intact nodules, especially where a substantial number of them are located on the lateral roots. Full recovery of nodules becomes an almost impossible task where plants are uprooted from dry and difficult to work soils. Also, as plants grow older, the most active nodules tend to be the newly formed, small nodules located on the distal ends of roots, often the small lateral roots that are at the greatest risk of detaching with the slightest disturbance. This may in part contribute to some of the reported declines in ARA estimates of N_2 fixation, much earlier, e.g. [55] than has been detected when the ^{15}N labelling method was used [32].
4. Serious doubts with the ARA technique as a measure of BNF have been raised; this has been attributed to the validity in using the conversion factor of 3 to derive N_2 fixed. This ratio was actually suggested only on theoretical grounds (see equations 1 and 2). In actual practice, some of the conversion ratios that have been reported for different N_2-fixing systems range from as low as 1.5 to as high as 25 for some non-plant systems [26]. Therefore, there is great uncertainty as to the correct conversion ratio to be used, unless a prior calibration (e.g., by using $^{15}N_2$ gas as a standard) has been done for each system. Even then, for the same *Rhizobium*-plant symbiosis, the conversion ratio does not always remain the same; it has been shown to vary under different environmental conditions [58].
5. The original suggestion to incubate samples in closed containers containing 10% acetylene results in an acetylene-induced inhibition of ethylene production [38]. To reduce such errors, it is recommended to use an open flow-through gas system in which air containing 10% acetylene is passed continuously through the assay vessels and the effluent gas analysed for ethylene [58]. The open flow-through system contrasts with the normal assay procedure in that it measures the rate of ethylene production rather than its accumulated concentration [58]. This again makes the ARA technique more tedious and methodologically more demanding than originally perceived.

Given the above-stated difficulties, several researchers have raised questions as to the best possible use of ARA data. Vessey [53], among many others have suggested the possibility of using ARA measurements for the relative ranking of BNF in different treatments. In doing so, the assumption would have to be, that whatever imperfections there are in the ARA technique, would affect the different treatments equally. This assumption has been refuted by Witty and Minchin [58] and Minchin *et al.* [37]. For example, Minchin *et al.* [37]

called attention to the fact that the acetylene-induced inhibition of ethylene production is inversely related to the stress imposed on the treatment. Thus, it is likely that an incorrect ranking can be made by comparing ARA measurements for different treatments (i.e. under different stresses), one of which is normally an unstressed control.

Analysis of N solutes in xylem exudate and plant parts.

This method is based on the determination of the composition of nitrogen compounds in plant tissues or the N flowing through the xylem sap to the shoot. The solute method was developed as a method for differentiating between fixed N and soil-derived nitrate N in plants [34].

In most agricultural soils, nitrate is the predominant form of N for plant growth [51] and the form of nitrogen most readily assimilated by growing plants. The nitrogen fixed in the nodule is exported via the xylem stream to the shoot either as amides, predominantly asparagine and glutamine or as the ureides, allantoin and allantoic acid [39]. However, substantial ureide production is restricted to some tropical legume species [42] and none of the temperate legumes examined so far has been found to produce ureides. Because ureide-exporting species have low nitrate reductase activity in their roots [1], much of the absorbed nitrate is transported to the shoot unchanged. Therefore the composition of the N compounds in the xylem exudate of nodulated ureide-producing plants change progressively from one dominated by ureides to one dominated by nitrate and amino compounds as the plant's dependence on N_2 fixation decreases in response to increasing contributions from soil N uptake [27, 34]. These changes are so specific that it has been possible to use changes in xylem exudate and in shoot extracts as an indication of the N_2-fixing ability of a legume, and even some quantitative measurements have been made [27, 28, 34]. For measuring the proportion of the plant's N derived from fixation, a calibration relating xylem solute composition in the presence of different levels of nitrate N with measured values of N_2 fixation using e.g., ^{15}N labelling is needed [27].

The sampling and analytical techniques have been described in detail by Peoples et al. [42] and will not be repeated here. The sample collection may be done by root bleeding or tissue extraction, but the original method of decapitating plants and collecting the xylem exudate has proven to be difficult under some circumstances, e.g., when the soil is dry [27, 52]. Pre-watering is therefore sometimes necessary but not always successful. An alternative method developed by Herridge [29] originally using soybean has proved very successful. It involves applying a mild vacuum to the lower end of the detached shoot of the plant and cutting successively from the top end of the shoot to allow the xylem contents to be drawn through. The exudate is trapped in a 5-ml vacutainer connected in series to the source of the vacuum and the shoot.

Compared to the ^{15}N labelling methods, the xylem exudate method is simpler, less expensive, and the method has great potential in many studies, such as in the screening of large germplasm collections for relative N_2 fixation ability. It was initially hoped that it would become a potential tool for measuring N_2 fixation in trees. However, the method is severely hindered by the fact that only a small proportion of known N_2-fixing plants are ureide exporters. For example, of 35 nitrogen fixing trees examined, only two showed a high abundance of ureides in the xylem sap [30], and Sanginga *et al.* [49] could not find any significant correlation between ureide production and BNF in *Leucaena*. Besides, the xylem-exudate method is an instantaneous assay and unlike the ^{15}N soil-enrichment methods, requires the interpolation of several independent estimates of solute composition at different growth stages before a time-integrated estimate of BNF in plants over a growing season may be made.

The ^{15}N labelling methodologies

The ^{15}N methods may be classified into :

- Use of $^{15}N_2$-labelled gas
- The isotope dilution method
- The A-value method

The common principle behind these three methods is that the N_2-fixing plants or systems are grown in soil or an atmosphere containing $^{15}N/^{14}N$ ratio measurably different from the almost constant $^{15}N/^{14}N$ ratio of 0.3663% present in the atmosphere. The incorporation of N_2 from fixation will thus result in a different $^{15}N/^{14}N$ ratio in plant tissue than that of the substrate on which the plant is growing. In the case of fixing plants incubated under $^{15}N_2$ labelled gas, the N in the plant tissues of a N_2-fixing plant will have a significantly increased $^{15}N/^{14}N$ ratio, in contrast to where available soil N rather than the N_2 in air is labelled. These

methodologies have all been extensively reviewed [7, 8, 9, 14, 86, 57]. Details on how to use these methods will not therefore be given in the present paper. Rather, an attempt will be made to give a critical assessment of their limitations and strengths.

The use of $^{15}N_2$ gas is the only direct method for detecting BNF, but has limited practical or field application in terms of quantifying BNF. The limited field applicability stems from the fact that plants can be enclosed for only short durations in sealed containers, and therefore under conditions often completely different from those in the field. Replacing all the air in the field by $^{15}N_2$ labelled gas is an impossible task. The method is therefore, most useful in exploratory studies of plants in ecosystems where not much is known about their N_2 fixing capabilities.

Both the isotope dilution [20, 33] and A-value methods [18] involve growing plants on soil containing higher $^{15}N/^{14}N$ ratio than that of the N_2 fixed from the atmosphere with both methods, the $^{15}N/^{14}N$ ratio of the N_2 that is fixed results in plant tissues accumulating N of a lower $^{15}N/^{14}N$ ratio than that assimilated from the labelled soil. The most important and difficult requirement for these methods is, how to accurately determine the integrated $^{15}N/^{14}N$ ratio of soil -derived N. The difficulty stems from the fact that the $^{15}N/^{14}N$ in a labelled soil does not remain constant with time [19]. In addition, the amounts of this N of changing $^{15}N/^{14}N$ ratio absorbed by a plant can vary drastically with plant age, or time. The estimation of the integrated $^{15}N/^{14}N$ ratio absorbed by a fixing plant from soil cannot therefore be adequately established simply by using instantaneous chemical extraction methods [14]. What has therefore been adopted as an alternative approach is to use the $^{15}N/^{14}N$ ratio in selected non-N_2-fixing plants that accumulated their N from only soil to represent the integrated $^{15}N/^{14}N$ ratio of the soil N absorbed by the fixing plant. The higher the N_2 fixation that occurred, the greater the dilution that will occur in the $^{15}N/^{14}N$ ratio of the tissues of the fixing plant, compared to the N in the reference plant [9].

The A-value method involves applying higher rates of ^{15}N enriched fertilizer to the reference plants (to allow good growth, particularly in N-poor soils) than to the N_2-fixing plant (so as not to severely depress N_2 fixation). The assumption here is, that the available, native (unlabelled) soil N (A_s) is not altered through the addition of different rates of a ^{15}N enriched fertilizer, and thus the A_s assessed by the reference plant is equal to A_s assessed by the fixing crop. Since the A value measured for the fixing crop also includes that for N_2 fixed ($A_s + A_a$), then the A value for fixed N_2, A_a is equal to $A_s + A_a$ (fixing crop) $-$ A_s (reference crop). The proportion of N derived from atmospheric N_2 fixation (% Ndfa) can then be calculated using the following equation [14]:

$$\%\,\mathrm{N}dfa = \frac{\%\,\mathrm{N}dff}{R} \times Aa$$

The A-value method has more underlying assumptions and is more complicated conceptually and mathematically. Therefore, it is not surprising that the A-value method has been criticised more often than the isotope dilution method (14). Consequently, the A-value method has been less often used to measure BNF, compared to the isotope dilution method. The discussion of the ^{15}N soil labelling approach for measuring N_2 fixation will therefore focus largely on the isotope dilution method. However, there are situations where the A-value method gives more reliable estimates than the isotope dilution method [48]. Details of the A-value method for measuring N_2 fixation, including the equations used have been provided elsewhere [9, 12, 18].

The isotope dilution method can be classified into two: (a) one that relies on the inherent higher $^{15}N/^{14}N$ ratios in some soils than that of atmospheric N_2 [50] or (b) where a ^{15}N enriched inorganic or organic N source has been deliberately added to soil to artificially increase any discrepancies between the $^{15}N/^{14}N$ ratio of soil N and that of atmospheric N_2 [20, 33]. In both cases, what is measured is the extent to which the $^{15}N/^{14}N$ ratio of plant tissue N is lowered (relative to soil N) as a result of the assimilation of unlabelled N_2 [14]. A major difference between the two approaches, is that for the ^{15}N natural abundance approach (in contrast to where ^{15}N labelled fertilizer is added), the $^{15}N/^{14}N$ ratios of soil N and atmospheric N_2 are not vastly different. This therefore necessitates far greater precautions and more sophisticated and expensive instrumentation than is necessary when ^{15}N labelled materials are directly applied to soil [14]. These requirements of the ^{15}N natural abundance method tend to limit its use to the more advanced laboratories, mostly in developed countries. For example, emission spectrometers are more rugged and considerably cheaper than mass spectrometers but cannot detect these low levels of differences in $^{15}N/^{14}N$ ratios or $\delta^{15}N$ natural abundance. Besides, as will be discussed later, the application of label to soil is of greater utility in soil N balance studies compared to the ^{15}N natural abundance method which is used mostly

to only measure N_2 fixation. However, a major advantage of the natural abundance method is, that it may be less prone to serious errors from reference plants than where a ^{15}N label is applied to soil, due to the more gradual decline in the natural $^{15}N/^{14}N$ ratios of soils [41].

The accuracy of BNF measurements using the isotope dilution method depends very much on how accurate the soil $^{15}N/^{14}N$ ratio assessed by the reference plant reflects that of soil-derived N in the fixing plant. Consequently, this is the greatest source of error with these ^{15}N methods, especially for the many studies that may have been conducted without prior selection of a suitable reference plant and where the criteria suggested by Fried *et al.* [19] have not been satisfied. In any case prior selection may not work always, as a particularly reference plant may not be satisfactory under all environments [6, 10]. Even some non-nodulating isolines have been found to nodulate in some soils (Danso, unpublished data), and it should therefore not be taken for granted that non-nodulating isolines can be used as non-fixing reference plants without examining their roots for nodules.

There is a need to examine the sources of the problem with obtaining reliable reference plants and to devise remedial measures. The major cause underlying reference plants giving erroneous measures of $^{15}N/^{14}N$ ratio absorbed by the fixing plant has been identified as the rapid decline of the $^{15}N/^{14}N$ ratio with time that occurs in soils into which ^{15}N labelled fertilizers have been added [19, 59]. Further experiments [60] revealed that by using practices that result in fairly stable $^{15}N/^{14}N$ ratios in soil with time, mis-matches between the $^{15}N/^{14}N$ ratio of the N in the reference plant and the true $^{15}N/^{14}N$ ratio of soil sampled by the fixing plant are drastically reduced. Although the ideal situation, a completely stable $^{15}N/^{14}N$ ratio over time is not practically possible in most situations, the aim should be to adopt ^{15}N labelling materials and methods that result in more gradual declines in the $^{15}N/^{14}N$ ratio in soil [14]. These include the use of organic matter-labelled ^{15}N, slow-release inorganic N formulations [19, 60], or the application of the required amount of ^{15}N fertilizer in small splits over time rather than as a single dose [11, 14]. Further improvement in the accuracy of the ^{15}N methods has resulted from using the mean of the $^{15}N/^{14}N$ estimated by several reference plants [3] and possibly in conjunction with practices that result in a slowly declining $^{15}N/^{14}N$ ratio of the soil [5]. However, it is essential to note that reference crop induced errors in BNF measurement are of little importance at high levels of N_2 fixation, in contrast to when N_2 fixation is low [12, 23].

A disadvantage of the ^{15}N methods which has received considerable attention is the high cost in material and equipment. Danso *et al.* [14] have made suggestions on where cuts in the cost of using ^{15}N labelled fertilizers to measure BNF can be made. They pointed out that the use of small isotope sub-plots instead of the traditional yield plots and using fertilizer enriched with 1 to 10 atom % ^{15}N excess rather than highly enriched fertilizers may lower costs without significantly decreasing the accuracy of the determination. Furthermore, emission spectrometers, several times less costly than mass spectrometers are now available, and should in most cases (unless the ^{15}N natural abundance method is used) be capable of providing reliable measures of BNF.

There are several advantages of the ^{15}N methods. These include:

1. Giving truly integrated estimates of BNF for whole growing seasons or up to desired periods.
2. Direct applicability in the field.
3. Ability to distinguish the nitrogen derived from fixation from that derived from soil or fertilizer. This is important for assessing soil N balances or for comparing varieties, species or treatments that accumulate e.g., similar amounts of N but differ in N_2 fixation, or where differences in the genetic potentials for total N accumulation bear no relationship with relative N_2-fixing abilities.
4. Where necessary, treatments can be ranked for BNF ability simply on the basis of differences in their $^{15}N/^{14}N$ ratios. The lower the $^{15}N/^{14}N$ ratio, the higher the N_2 fixing ability.
5. The ^{15}N methods are often more reliable and provide more accurate results.
6. In addition to providing estimates of BNF, the use of ^{15}N labelling methods provides many additional useful agronomic information, such as the fertilizer use efficiency of the legume or reference crop (particularly if it happens to be an important crop). Other information or benefits that can be obtained at no extra cost include the use of such labelled plant material to also label soil N for further BNF studies or to assess ease of decomposition of different organic matter sources and rates of N mineralization. Useful data on N transfer between plants have also been reported from experiments intended to measure BNF. Thus, the often cited high cost of the ^{15}N methodology should always

be viewed in terms of the secondary benefits that can be obtained.

Conclusion

None of the methods for measuring BNF can be singled out as being near perfect. They each have their strengths and weaknesses that need to be considered in deciding when to use what method. The TND method is relatively very simple and inexpensive, and is most reliable in soils with low available N content. Many estimates of BNF have unfortunately been made using more sophisticated and expensive methods in situations where the simpler and cheaper TND method could have been used. Another advantage of the TND method is, that for most experiments total N is determined as one of the routinely examined parameters, in which case TND determinations may not involve extra work other than the growth and total N determination for the control. Experiments are needed to examine whether the reliability of TND estimates may not be improved by using the mean total N from several potential control crops rather than from a single one.

The ARA technique certainly has lost the favour it enjoyed in the early 1970s despite its simplicity and low cost. Although there appears general agreement that the ARA technique may not be valid for quantifying N_2 fixation, ARA could still be a useful tool in some studies. For example, it could be used to provide highly useful indications of potential N_2-fixing species, especially in novel or unexplored environments. It has other qualitative uses, such as for establishing the time of onset of fixation or for screening *Rhizobium* for ineffective strains. One potential area where advantage may be taken of the rapidity, sensitivity and low cost of the ARA technique is, for screening large collections of plants, e.g., in breeding programmes for increasing BNF in crops; the ARA technique should at least be capable of identifying and thus eliminating obvious non- (or very poor) N_2-fixers prior to using more time-consuming and expensive methods on the remaining plants. The ARA technique has also been used as a standard method for screening potential non-N_2-fixing crops to be used as reference plants to estimate BNF by the ^{15}N soil labelling methods.

The xylem-exudate method, like the ARA technique is simple and inexpensive and very useful for comparing the N_2-fixing abilities of different plant genotypes. However, it suffers from being an instantaneous assay, and would need the extrapolation of several independent measurements to obtain integrated values of N_2 fixed. Besides, the method is only limited to N_2-fixing plants that produce ureide.

The ^{15}N methods have great potential, and in general are capable of providing more accurate data on BNF than most other methods. Their major limitation, that of the accuracy of the reference crop in assessing the integrated uptake of $^{15}N/^{14}N$ ratio of soil N, particularly when N_2 fixation is low can be overcome or reduced to tolerable levels. This may be done by decreasing the rapid rate at which the $^{15}N/^{14}N$ ratio of soil N declines, e.g. by incorporating ^{15}N labelled fertilizers that release their ^{15}N slowly in soil, using ^{15}N labelling procedures that result in an operationally steady level of ^{15}N with time, and better still in conjunction with the use of several rather than only one reference crop. The ^{15}N methods give an integrated measure of BNF, can be applied directly in the field, are able to distinguish N_2 fixed from soil-derived N, and are good for many studies on N turnover in soils, in addition to their being used to measure BNF.

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