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An Appraisal of Methods for Measuring Symbiotic Nitrogen Fixation in Legumes

F. Azam and S. Farooq
Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan

Abstract: Symbiotic nitrogen fixation by legumes is the major natural process of adding nitrogen into the biosphere amounting to about 35 million tons annually. The process of nitrogen addition to the ecosystems and its further fate is such as to pose minimum threat to environmental cleanliness relative to N used as chemical fertilizers. Therefore, it has been of great interest not only to understand the basics of nitrogen fixation process but also to quantify the amount of N added to a system under different conditions. This is important in order to quickly screen the available germplasm for its potential of biological N_2 fixation and to devise strategies for further improving the process under different ecological conditions. A critical evaluation of some common methods of studying N_2 fixation in legumes is presented.

Key words: Acetylene reduction assay, Added nitrogen interaction, A-value, BNF, Isotope dilution, Priming effect, Ureides

INTRODUCTION

Symbiotic nitrogen fixation in legumes is a fundamental process for maintaining soil fertility and the continued productivity of natural and agroecosystems. Of a total of 139 million tons biologically fixed N added to the system annually on a global scale, 25% comes from legumes i.e., 35 million tons which is only slightly less than that supplied to agroecosystems through chemical fertilizers. This figure demonstrates the significance of legumes in agricultural and natural N cycles. Most of the legumes meet >70% of their N demands through this process in addition to augmenting N supplying potential of the ecosystem as a whole. Under favourable conditions, legumes such as faba beans, pea and cowpea can derive as much as 80-90% of their N requirement through N_2 fixation (Eaglesham *et al.,* 1977), values for soybeans being reported at 40-60% (Ham and Cladwel, 1978 and Rennie *et al.,* 1982). The amounts of N fixed may vary from 57 to 600 kg N yr^{-1} ha^{-1}, minimum being for soybean (57-94 kg N yr^{-1} ha^{-1}) and maximum (128-600 kg N yr^{-1} ha^{-1}) for alfalfa.

The amounts of N_2 fixed and the N contribution from leguminous crops are influenced by a number of environmental factors, including soil type, nutritional status of soil, species and varieties, water availability and temperature as well as soil and crop management (Ledgard and Steele, 1992). As a result, a wide variation is generally reported for the amount of N_2 fixed and percent of plant N derived from fixation. One of the more important reasons for the variation in N_2 fixation estimates is, however, the method used. Generally, biological N_2 fixation should lead to a detectable/measurable increase in the total N content of the system. However, because of the relatively low additions to an already big pool of soil N, realistic estimates of biological N_2 fixation are hardly possible. Therefore, in order to exploit the N_2 fixing potential of different legumes under divergent agro-climatic and management conditions, it is important to identify/use suitable methodology that can also distinguish between the contribution from this and other sources like soil organic matter and chemical fertilizers. This review presents a critical evaluation of different methods used for the determination of nitrogen fixation in legumes.

Methods of determining BNF

Determination of dry matter: This is the simplest and most easy method of getting a relatively rough estimate of BNF. Since legumes may meet up to 90% of their N requirements through BNF and the fact that biomass yield of crops is dependent on the N content, dry matter accumulation by plants could be used as a measure to compare the efficiency of N_2 fixation of different cultivars. However, reliable quantitative estimates of the fixed N are difficult to obtain because of the inherent differences in the cultivars for exploiting the native soil N. In addition, presence and absence of relevant rhizobia and the extent of effective nodulation will also have a significant bearing on N_2 fixation and consequent dry matter accumulation by different plant types. Nevertheless, the method has been used by several workers (Haydock *et al.,* 1980 and Edwards *et al.,* 1981) and could easily be adopted for large scale screening of germplasm.

Nodule number and mass: This method is dependent on the presence of effective and relevant rhizobia in good

numbers in the plant rhizosphere (Graham, 1981). Since the rhizobia are species and cultivar specific so far as the efficacy of nodulation and efficiency of N_2 fixation is concerned, the comparisons obtained may not be reliable. In addition, the number and weight of nodules may not necessarily give a reliable clue to the amount of N_2 fixed because of the changes in the carbonaceous compounds being made available at the time of sampling. The problems may also arise because of the ineffective nodulation (formation of nodules but no N_2 fixation), failure of rhizobia to enter into the nodules, death of rhizobia within the nodules or absence of plants' support to sustainable rhizobial N_2 fixation (Azam, 2001). In spite of these difficulties or short comings, however, the method can conveniently be used to ascertain the effect of different agroclimatic conditions on nodulation and N_2 fixation of a particular plant type. The method may also serve to reveal the presence of species- and cultivar-specific rhizobia in a particular soil.

The method relies on the assumption that similar amounts of native soil N are made available to the plants irrespective of their genetic differences i.e., whether they are leguminous or non-leguminous. The amount of N fixed can thus be determined by using the expression:

Fixed N= Total N (fixing crop)–total N (non-fixing crop)

This would imply that leguminous (fixing) and non-leguminous (non-fixing, e.g., cereals) plants will have an equal access to the soil N already available or that mineralized under the influence of plants. This method of calculating biologically fixed N does not account for the inherent differences in plant types in affecting the mineralization and availability of soil N. These differences may indeed be significant as cereals are found to obtain a higher amount of soil N as compared to legumes. In addition, no consideration is given to the differences in rooting characteristics and hence the soil volume/depth being explored for N acquisition. These factors are known to significantly influence the uptake of soil N by different plant types and will therefore affect the proportion/amount of plant N attributed to BNF. In spite of these differences, the method has frequently been used by different workers (Broadbent *et al.,* 1982; Talbott *et al.,* 1982; Hardarson *et al.,* 1984; Rennie, 1984 and Vasilas and Ham, 1984). The real advantage of the N difference method is that N fertilizer addition is not required, thus avoiding the potential complication as discussed later.

A supplementary approach to the above method may involve the use of different levels of fertilizer N for the non-leguminous reference crop. The level at which the N yield of fixing crop becomes equal to that of fixing crop is considered equivalent of the amount of N_2 fixed by the legume. The limitation, however, is that this approach does not take into account the amount of fertilizer N lost that may be significant (up to 30% of the applied N) and significantly different with the type of non-legume crop used. It is also known that fertilizer N leads to enhanced uptake of soil N through the so-called priming effect or added nitrogen interaction (Jenkinson *et al.*, 1985; Hart *et al.,* 1986; Woods *et al.,* 1987; Azam, 1990, 2002 and Kuzyakov *et al*., 2000). The amount of extra soil N released may be significant and increases with the amount of applied N (Azam *et al.,* 1993). These shortcomings could probably be adjusted by using non-nodulating isolines. Again the problem may be the differences in rooting characters and acquisition of native soil N by the fixing and non-fixing isolines.

Acetylene reduction assay (ARA): It is inexpensive, rapid, sensitive and apparently accurate method (Hardy *et al.,* 1968; Goh *et al.,* 1978; Hudd *et al.,* 1980; Turner and Gibson, 1980 and Edwards *et al.,* 1981) and has been used extensively for the field measurement of N_2 fixation. The assay gives an estimate of the activity of nitrogenase, an enzyme that is involved in the reduction of several compounds including N_2. Its ability to reduce acetylene (C_2H_2) to ethylene (C_2H_4) has found a good utility in indirectly measuring N_2 fixation at any point of time. Continuous monitoring of enzyme activity has been made possible through frequent sampling of air stream (containing small concentrations of C_2H_2) passing over the nodules followed by measurement of C_2H_4. However, the method needs to be calibrated against one of the most direct methods of measuring N_2 fixation in spite of the fact that a linear relationship between the methods is hard to obtain.

The normal assay (Hardy *et al.,* 1973) involves the incubation of detached nodules, nodulated root pieces, or detopped root system with 10% acetylene in a closed container of known volume. Gas phase samples are analyzed by gas chromatography to measure the concentration of accumulated ethylene. In principle, the method measures the electron flux through nitrogenase in the sample material under the conditions which prevail during the assay period. However, to obtain useful practical information from such data, a number of assumptions have to be made or conversion factors used. The error thus arising may vary between 30 and 60%. The assumptions include i) all nodules are recovered in which case a conversion factor of 1 is used, ii) the rate of activity during the measurement is equal to the pre-assay rate, iii) the electron allocation coefficient to H_2 is 0.25 leading to a conversion factor of 4 i.e., 4 moles of C_2H_2 to 1 mole of N_2 reduced.

The first assumption normally does not hold and estimates of N_2 fixation on per plant basis are limited by the incomplete recovery of nodules especially from deep-rooted plants or under low moisture (dry) situations. In addition, washing and nodule detachment cause a reduction in nitrogenase activity (Mague and Burris, 1972; Hardy *et al.*, 1973, 1977 and Wych and Rains, 1978). The second assumption mentioned above is also faulty because C_2H_2 itself is a strong inhibitor of nitrogenase, while enzyme activity is significantly affected by cutting and handling of root. Using an open flow-through gas system, Minchin *et al.* (1983) reported that the decline begins within minutes of exposure to C_2H_2 and continues for at least 30 min. In a closed system measuring accumulated ethylene, nitrogenase activity may be underestimated by up to 50%. Measurements of nitrogenase activity and respiration at different external O_2 concentrations show that the true cause of the C_2H_2 effect is O_2 limitation of bacteroid respiration because of the resistance induced in nodules to O_2 diffusion (Witty *et al.*, 1984, 1986 and Minchin *et al.*, 1986).

To obtain values for the rate of N_2 fixation from rates of acetylene reduction (with reference to assumption iii above), an appropriate conversion ratio for C_2H_2 reduced to N_2 fixed is needed. In earlier studies, a ratio of 3 was used on the premise that 6 electrons are required for the reduction of N_2 to $2NH_3$ and 2 for C_2H_2 to C_2H_4 (Hardy *et al.*, 1973). Later, it became apparent that with each N_2 reduced at least two protons were also reduced to H_2. However, the proportion of electrons allocated to H_2 is fairly variable, making any generalizations difficult if not impossible and thus rendering the comparative data for different strains invalid. This is because the strains with highest acetylene reduction activity may have a low electron allocation to N_2 and consequently fix less N_2 than those having the reverse of this allocation of electrons. The factors that affect the allocation include temperature, irradiance (Bethlenfavay and Phillips, 1979), or treatments which modify the rate of electron flux through nitrogenase (Hageman and Burris, 1980).

Due to the difficulties outlined above, the data obtained with normal ARA (measuring accumulated ethylene) cannot be reliably extrapolated to estimate seasonal N_2 fixation. However, it could be a good tool for measuring instantaneous rates of nitrogenase activity in studies of crop physiology. The flow through system with open-ended assay chambers could be modified to suit the study conditions. An alternative to measuring maximum rate of activity is to use non-saturating concentrations of acetylene (0.2%) which do not induce decline in nitrogenase (Denison *et al.*, 1983). However, necessary calibrations (another source of error) to determine inhibitory levels of acetylene are required. Once such difficulties are overcome, flow-through systems could be used not only for studying nitrogen fixation but soil respiration as well thus providing the information on allocation to the roots of photosynthates and their use efficiency.

Content of ureides and other metabolites: In legumes, N-containing compounds originating from BNF (ammonia) are incorporated into glutamine and glutamate via glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase pathway, respectively. Tranamination of these compounds leads to the production of aspartate and other amino acids. Some of the amino acids are incorporated into purines which are oxidatively degraded to yield ureides. These compounds are chemically different from those derived from soil N and are transported to the aerial parts in the xylem sap. Samples of the sap can be obtained as bleeding sap produced from the stump of decapitated plants by rot pressure, or as smaller samples obtained from stems by vacuum extraction. Analysis of these samples can yield information on the relative dependence of plants on soil N and BNF (Pate and Atkins, 1983). In general, well-nodulated legumes export (from root to shoot) amides or ureides as products of BNF, while those depending mainly on the soil N have xylem sap rich in NO_3 because of negligible NO_3-reductase activity at the root level. Relative concentration of ureides and NO_3 in the xylem sap has thus been used as a rough measure of N_2 fixing ability of the legumes (Pate *et al.*, 1980; Herridge, 1982, 1984 and Dakora *et al.*, 1992). Under conditions of relatively high NO_3 availability that is inhibitory to nodulation and N_2 fixation, higher concentrations of NO_3 rather than ureides in the xylem sap are bound to be determined. Hence, this approach is not only useful in comparing different plant types for N_2 fixation, but also to study the NO_3 tolerance of N_2 fixing machinery. This is a particularly important requirement for leguminous crops grown in conjunction with non-legumes and the later supplied with chemical fertilizers. However, the premise of the method is that minimum NO_3 reduction should occur at the root level, a condition hard to be met in different genotypes that vary in the level of nitrate reductase.

Methods involving the use of ^{15}N: Most of the techniques discussed above are based on indirect criteria and cannot distinguish between sources of plant N. The advantages and disadvantages of these techniques have been discussed in detail (Hardy *et al.*, 1968; Knowles, 1981; Fried *et al.*, 1983 and Rennie and Rennie, 1983). Alternatively, several approaches have been tested and effectively employed for the measurement of N_2 fixation in legumes using stable isotope of nitrogen i.e., ^{15}N. The so-

called isotopic dilution approach (McAulife *et al.*, 1958) exploits the differences in ^{15}N abundance of different N sources. Development of relatively simpler and less expensive methodology (optical emission spectrophotometry as against more expensive mass spectrometry) for the determination of isotope ratios has made the use of ^{15}N in BNF research fairly convenient and straight forward. Different approaches employing the use of ^{15}N include i) exploiting the difference in δ N^{15} of different N sources i.e., soil, atmosphere and fertilizer, ii) enrichment of soil N, iii) use of A-value modification of ^{15}N enrichment method, and iv) exposure of N_2 fixing sites, e.g. plant roots, to N_2. Some advantages of these methods are that i) they give a truly integrated value for N_2 fixation, ii) can be used directly in the field situations, iii) can differentiate between the sources of plant N, iv) simultaneously provide information on the fertilizer use efficiency of both legume and non-legume reference crop, v) the proportion of Ndfa can be measured even if plants are partially damaged, vi) it is not necessary to grow the reference crop without or with only low N fertilization, vii) useful in ranking the genotypes for N_2 fixing efficiency under different conditions, viii) more sensitive and accurate, ix) the cost of use and analysis has decreased significantly over the years, x) once ^{15}N is applied to the soil as fertilizer or labelled plant residues, the soil (and unused plant material) can be used over extended periods for N_2 fixation studies since highly sensitive instruments are now available to help work even at natural ^{15}N abundance levels, xi) gives best results in studies comparing genotypes or rhizobial strains under any given set of conditions, especially when working with natural abundance levels. In addition, these methods provide yield independent and time-integrated estimates of P_{atm} (proportion of N derived from the atmosphere) differentiating these methods from those which are yield dependent (N difference) or point-in-time acetylene reduction assay (ARA).

In spite of the long list of advantages, the methods are not free of difficulties. The precision of N_2 fixation estimates made with these techniques are strongly influenced by the reference crop used to assess the $^{15}N/^{14}N$ or available N in the soil. The errors are small at high levels of fixation and vice versa. In the past, major disadvantage has been the high costs of ^{15}N-labelled fertilizers and the analytical methodology. This has been overcome by using i) low levels of enrichment, ii) previously ^{15}N-labelled soil, and iii) high precision, low cost mass spectrometers. However, except for method (iv) above i.e., exposure of N_2 fixation sites to N_2, the remaining 3 methods share a major disadvantage that arises from the use of reference crop. A critical and so far insurmountable obstacle to obtaining realistic amounts of N_2 fixed by using isotopic dilution technique (as well as A-value technique described next) is the requirement of a reference crop that may not necessarily behave the way it is assumed to. When using ^{15}N for determining N_2 fixation, it is important to distinguish between the real effects of fertilizer uptake on growth and N_2 fixation of the crop and errors arising from the mismatch of a reference crop (Giller and Witty, 1987). In order for the reference crop to yield reliable results of N_2 fixation, it should be i) non-fixer, ii) obtain N from soil resources in amounts similar to that found in the fixing crop, and iii) similar to fixer in rooting characteristics, N uptake behaviour, and growth period etc. In addition, there should not be any transfer of N between the fixing and non-fixing crops in a mixed cropping system. These conditions are seldom met completely. Of the different reference crops, non-nodulating isolines have been found to be more appropriate as they share many characteristics with the test crop (Vasilas and Ham, 1984). However, differences in the uptake of N from fertilizer and soil have been reported (Harper, 1974; Ruschel *et al.*, 1979; Boddey *et al.*, 1984).

Another possibility is to use un-inoculated controls in situations where artificial inoculation is necessary to achieve nodulation i.e., native rhizobia are ineffective although use of additional reference crops is strongly recommended (Fried *et al.*, 1983). In this particular case, however, care needs to be taken that effects other than N_2 fixation (e.g., release of phytohormones and denitrification) are not involved due to rhizobial inoculation. If that is the case, the control is inoculated with non-fixing bacteria (Fried *et al.*, 1983). Yet another approach is to use high rate of fertilizer N for control (reference crop) to disable it for nodulation and N_2 fixation (Eaglesham *et al.*, 1982), although reservations persist as for the complete inhibition of N_2 fixation (Richards and Soper, 1979; Wagner and Zapata, 1982) and the possible priming effects of the added N. Cereals have also been recommended as non-fixing controls (Broadbent *et al.*, 1982; Fried *et al.*, 1983 and Rennie, 1984) although the difference in growth habit may have serious implications to the validity of the results obtained. This difference also includes that resulting from differences in root-induced N mineralization as well as the extent of soil exploration. Species vary in their root growth and in the rate and timing of N uptake (Boller and Nösberger, 1988). Over the time there will always be a dilution effect caused by the mineralization of non-labelled soil N (Witty, 1983). Some of these difficulties can possibly be overcome by the use of multiple reference plants as suggested by Doughton *et al.*, (1995).

***^{15}N natural abundance or $\delta^{15}N$ technique*:** This technique exploits the difference in natural ^{15}N abundance of soil/fertilizer and atmospheric N (Broadbent *et al.*, 1982;

Rennie and Rennie, 1983; Ruschel *et al.*, 1979; Vasilas and Ham, 1984 and Herridge *et al.*, 1995). It is well established that atmospheric N has lower ^{15}N abundance than the native soil N and there are valid reasons for this isotopic difference to develop. Soil N is continuously being enriched due to a selective loss of ^{14}N through processes such as NH_3 volatilization/denitrification from soil and gaseous emission through plant foliage; selective uptake by plants of ^{14}N compounds notwithstanding (Kohl and Shearer, 1980). Conversely, the heavier isotope ^{15}N from atmosphere is being incorporated into soil organic matter and selectively retained there merely because of its mass; ^{15}N enrichment of soil N is the net result (Turner *et al.*, 1983). Thus the processes of N_2 fixation, N losses and plant uptake will continuously cause ^{15}N enrichment of soil N and depletion of atmospheric N. This difference in the natural abundance of soil and atmosphere has conveniently been exploited for the measurement of biological N_2 fixation by using isotopic dilution equations and/or δ ^{15}N values which can be determined as:

Because of the low values of ^{15}N abundance being dealt with, the expression is changed from atom percent to atom thousand i.e., % The δ ^{15}N of atmospheric N is 0% while that of the fixing or non-fixing system could vary from -1 to 8% (Bergersen and Turner, 1983; Riffkin *et al.*, 1999; Snoeck *et al.*, 2000). It can be assumed that δ ^{15}N of the non-fixing system will be the same as that of readily mineralizable N in soil.

Because of the difference in natural ^{15}N abundance, the N_2 fixing plant that depends on soil N and BNF, will have low ^{15}N abundance than a non-fixing plant that obtains N from the soil alone (Kohl and Shearer, 1980; Danso *et al.*, 1993). However, isotopic fractionation that is reported to occur during biochemical reactions (Yoneyama *et al.*, 1986; Ledgard, 1989 and Doughton *et al.*, 1995) may lead to discrepancies in the estimates of N_2 fixed depending upon the magnitude of fractionation. Host plant, parts of the plant selected for ^{15}N analysis, and rhizobial strain are important factors that influence the isotopic fractionation (Steele *et al.*, 1983; Ledgard, 1989 and Kyei-Boahen *et al.*, 2002). In addition, isotopic discrimination between ^{14}N and ^{15}N is associated with the process of N_2 fixation and needs to be taken into account while calculating Ndfa (Shearer and Kohl, 1986). The degree of discrimination, i.e., the B value, is usually determined as the δ ^{15}N of the N_2 fixing plant grown with atmospheric N_2 as the only source of N (Bergersen and Turner, 1983). However, ^{15}N abundance in soil may change with depth (Steele *et al.*, 1981) and therefore the plant types differing in rooting depth will show difference in ^{15}N abundance because of this variation in addition to that caused by N_2 fixation. The method can conveniently be used to screen/grade leguminous germplasm or breeding material for relative N_2 fixing ability without the involvement of a reference crop. Again the problems may arise from variable amounts of soil N taken up by different plant types mainly because of the differences in rooting characteristics and the consequent effects particularly the volume of soil being explore. The later deficiency can, however, be overcome by using a fixed/limited amount of soil i.e., by screening the germplasm in greenhouse using pots rather than under field conditions with unlimited/variable amount of soil being explored.

The major disadvantage of the δ ^{15}N method is the requirement of a high precision mass spectrometer. This difficulty has been overcome to a sufficient degree by the development/availability of dedicated instruments with high throughput and ease of preparing, shipping and handling of samples.

***Enrichment of soil N*:** This approach is particularly useful for labs that have relatively less sensitive analytical facilities, like optical emission spectrophotometry. The method relies on enriching the soil N by i) labelling native organic matter with ^{15}N, ii) addition of ^{15}N-labelled plant materials, iii) addition of ^{15}N-labelled fertilizer together with some easily oxidizable C source like sucrose, cellulose or glucose etc. Since mineral N (especially NO_3-N) is known to affect nodulation as well as nitrogenase enzyme, immobilized N (using easily available C source to immobilize added ^{15}N; Fried *et al.*, 1983) as well as ^{15}N-labelled plant residues (Broadbent *et al.*, 1982) and ^{15}N from previous soil applications i.e., ^{15}N-enriched soil (Broadbent *et al.*, 1982 and Fried *et al.*, 1983) have been used. This is meant to get slowly mineralizable N in soil to minimize the effect of mineral N on the process of N_2 fixation. Since the isotopic composition of the soil N and its availability to both the fixing and non-fixing crop is assumed to be similar, any dilution in plant N is assumed to result from biological N_2 fixation. Intensity of N_2 fixation is determined by the extent to which plant N is diluted; differences due to rooting characteristics notwithstanding. Using this approach, the proportion (P) of plant N derived from the atmosphere (atm) can be determined by as follows (McAuliffe *et al.*, 1958):

$$P_{atm} = 1 - (\text{atom \% }^{15}\text{N excess legume/atom \% }^{15}\text{N excess reference})$$

Fried and Middelboe (1977) used the following isotope dilution equations to express N_2 fixation in two different ways:

$$\%Ndfa = \left(1 - \frac{\text{Atom \%}^{15}\text{NEx}_{FC}}{\text{Atom \%}^{15}\text{NEx}_{NFC}}\right) \text{ X } \mathbf{0}$$

A derivation of the above equation proposed to obtain the amounts of N_2 fixed Ndfa (N derived from the atmosphere) is:

$$N_2\text{fixed (kg ha}^{-1}) = \frac{\%\text{Ndfa X total Nin fixing crop}}{100}$$

Recently, Reiter *et al.* (2002) have introduced a low-level, large-scale ^{15}N application technique to measure N_2 fixation. According to these authors the technique gives more precise results and can be applied when fixing and reference crops differ in the pattern of N uptake from soil.

However, this approach does not take into consideration the possible "priming" effects and thus may lead to underestimates of N_2 fixation by the legume. In addition, uniform distribution of small quantities of NO_3-N i.e., 26 g ha^{-1} (10 atom % ^{15}N) particularly in vertical direction may become a major concern.

***Use of A-value modification of ^{15}N enrichment method*:** This concept is based on the assumption that the assimilation of N by plants from soil, fertilizer and atmosphere is in direct proportionality to the N available from each source and that the rate of fertilizer N application will have no bearing on the availability of N from other sources, particularly from soil (Fried and Broeshart, 1975; Wagner and Zapata, 1982; Vasilas and Ham, 1984). Initially, the concept was introduced to study the uptake of fertilizer N relative to native soil N and the A-value was determined as:

$$\text{A-value} = \frac{\%\text{N derived from the soil}}{\%\text{N derived from the fertilizer}} \text{ X rate of fertilizer N applied (Kg ha}^{-1})$$

Subsequently, however, it was extended to studies on BNF and its contribution to nitrogen nutrition of plants vis-à-vis fertilizer N and native soil N. For the A-value concept to be applied, a non-fixing reference crop (NFC) has to be included parallel to the fixing crop (FC) and involves the use of ^{15}N-labelled fertilizer. The reference or non-fixing crop is given higher levels of ^{15}N in order to remove N limitation and get good growth. The following formulae can be used to calculate N fixed by the crop:

$$fa = \left(\frac{\text{A-value}_{FC} - \text{A-value}_{NFC}}{100}\right) \text{ X FU}$$

$$\%\text{Ndfa} = 100\left(1 - \frac{\text{Ndff}_{FC}}{\text{Ndff}_{NFC}}\right) + \%\text{Ndff}\left(\frac{1}{n} - 1\right)$$

In N_2-fixing plant, A-value includes available soil N and atmospheric N. Therefore, difference of A-value between fixing and non-fixing reference plant gives the estimate of availability of atmospheric N. However, it is essentially in the perspective of applying higher levels of ^{15}N-labelled fertilizer to the reference crop that makes this approach highly unrealistic. The errors generated by real and apparent added N interactions (Jenkinson *et al*., 1985; Hart *et al*., 1986 and Azam, 2002) are of particular concern where the A-value modification is used. The assumption that fertilizer N will have no bearing on the availability to plants of N from soil has been the subject of serious criticism ever since the concept was introduced (Vasilas and Ham, 1984 and Chalk, 1985). Over the years, it has become increasingly apparent that fertilizer N, as well as any other sources containing easily available N (e.g. green manures), exert a positive influence on the mineralization and plant availability of N from native soil organic matter through the so-called priming effect or added nitrogen interaction (Jenkinson *et al.*, 1985; Hart *et al.*, 1986; Woods *et al.*, 1987 and Azam, 1990, 2002). The ANI (added nitrogen interaction) is found to increase almost linearly with the amount of fertilizer applied and is particularly more with NH_4-N than NO_3-N and is affected by the enhanced C supplies through organic amendment and/or root exudation and will therefore be more in plants showing higher rhizodeposition (Azam *et al*., 1989a, 2002). Fertilizer N not only affects the availability of soil N through enhanced mineralization of the later, but leads to higher proliferation of roots and hence an increase in the soil volume being explored for nutrient (including N) uptake. Thus, if the concept of pool substitution (Jenkinson *et al.*, 1985 and Hart *et al.*, 1986) whereby fertilizer N stands proxy for the soil N is accepted as valid, underestimates of fertilizer N and overestimates of soil N uptake will certainly be obtained affecting the A-value. However, according to Fried *et al.* (1983), it is not necessary for the reference crop and fixing crop to absorb same quantity of total N as long as they absorb soil N and fertilizer N in the same ratio. They asserted that the root systems differing in both size and structure can still give valid quantitative comparisons (Fried *et al*., 1983).

In view of the above considerations, the phenomenon of ANI will have a significant bearing on the uptake of soil N by a non-leguminous (non N_2 fixing) reference crop that must be fertilized for normal growth. Not only this, but the difference in rooting behaviour of fixing and non-fixing crops and thus the exploitation of native soil N (as well as root-induced N mineralization) will affect the uptake of the later and thus the estimates of BNF. Because of such difficulties, the approach of using non-nodulating isolines was introduced for use as a reference to study N_2 fixation in the normal plant types. In this case, however, fertilizer N must be applied to obtain

normal growth of non-fixing isolines; same problems as faced by using non-leguminous reference crop. Fertilizer N seriously hinders the process of N_2 fixation right from the start i.e., nodule formation to the activity of nitrogenase enzyme; the effect is more intense with NO_3 than NH_4 (Steerer, 1988).

Despite its limitations, several studies using this concept have reported satisfactory estimates of N_2 fixed (Wagner and Zapata, 1982). This approach may particularly be applicable under conditions of low N availability when the growth of non-fixing reference crop (control) will be uncharacteristically low thereby making the use of ^{15}N isotopic technique invalid; addition of a higher rate of N to inhibit N_2 fixation in the non-fixing crop will give reasonably reliable estimates of N_2 fixed by the legume. For example, in some studies aimed at estimating N_2 fixation by isotopic method in chickpea, non-leguminous reference crop has also been grown on the same nutrient-deficient and marginal soil (Hafeez, 1998). The results obtained cannot be valid as the growth of reference crop will be poor leading to overestimates of N_2 fixation in the test crop.

***Exposure of plant root system to $^{15}N_2$*:** One of the first applications of mass spectrometry to the use of stable isotopes in biology was the analysis of the incorporation of $^{15}N_2$ gas into culture of *Azotobacter vinelandii* (Burris and Miller, 1941). It has often been used to study asymbiotic N_2 fixation and the fate of fixed N (Witty and Day, 1978 and Azam *et al.*, 1988,1989b). The method remains the most sensitive and accurate for measurement of N_2 fixation in laboratory experiments or in controlled conditions although its field applications are limited due to the requirement of plant enclosure in a chamber and the cost of $^{15}N_2$.

Concluding remarks: Not a single method of determining absolute amounts of fixed N has so far been identified. Each of the methods discussed above have one or the other disadvantage making the results flawed. The problems associated with the use of a reference crop in methods employing ^{15}N isotopic dilution approach could be considered as the most serious, while N difference method is flawed due to the widely divergent N acquisition pattern of the fixing and non-fixing crops. Nevertheless, a choice of methods can be made depending upon the available facilities and the objectives. In view of the facts that legumes may derive up to 90% of their N from rhizobial fixation and existence of a close correlation between plant N and the biomass yield, the later could serve as the simplest and least expensive index of the extent of N_2 fixation. This approach is particularly useful for screening leguminous germplasm used in breeding programmes. It will be desirable, however, to ascertain the presence of effective and pertinent rhizobia in sufficient numbers. This can be achieved by studying the root system for nodulation at appropriate plant growth intervals and devising appropriate inoculation strategies. More sophisticated methods e.g. the ones involving the use of isotopes may be adopted when the objective is to differentiate between the sources of plant N i.e., soil and atmosphere, while soil enriched with ^{15}N can be used for more promising selections. Limiting the amount/volume of soil (i.e. by carrying out studies in pots for a limited time period) could eliminate some of the problems assigned to the variation in the volume of soil being explored by plants roots.

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