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Measurements of nitrogen fixation in fababean at different N fertilizer rates using the ^{15}N isotope dilution and 'A-value' methods

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Abstract

The ^{15}N isotope dilution and A-value methods were used to measure biological nitrogen (N_2) fixation in field grown fababean (*Vicia faba* L.), over a 2-year period. Four N rates, 20, 100, 200 and 400 kg N ha^{-1} were examined. The two isotope methods gave similar values of % N derived from the atmosphere (%Ndfa). With 20 kg N ha^{-1}, %Ndfa in fababean was about 85% in both years. Increasing the N rate to 100 kg N ha^{-1} decreased N_2 fixation slightly to 75%. Further reductions in N_2 fixed to 60 and 43% occurred where 200 and 400 kg N ha^{-1} were applied, respectively. Thus even higher rates of N than normally applied in farming practice could not completely suppress N_2 fixation in fababean.

We also devised one equation for both the isotope dilution and A-value approaches, thereby (i) avoiding the need for different calculations for the ^{15}N isotope methods, and (ii) showing once again that the isotope dilution and A-value methods are mathematically and conceptually identical.

Introduction

The two most common approaches for using ^{15}N enriched fertilizers to measure N_2 fixed are the isotope dilution (ID) and the A-value (AV) methods. Although different equations have been used for each method (Fried and Broeshart, 1975; Fried and Middelboe, 1977), conceptually and mathematically, both are similar (Danso *et al.*, 1986). They only differ in whether the same amount of ^{15}N labelled fertilizer is applied to the fixing (F) and the non-fixing (NF) crops (*i.e.* the ID method) or whether different N rates are applied to the F *versus* NF reference crops (*i.e.* AV method). The more straightforward concept, the single N rate and the less complicated nature of the calculations involved make the ID approach the much preferred of the two methods. The AV method is therefore used in cases where a higher N rate is imperative to support the satisfactory growth of the NF crop (Fried and Broeshart, 1975). Because this N rate might inhibit nodulation and nitrogen fixation, a lower and therefore different rate has to be applied to the F crop.

Hardarson *et al.* (1988) indicated that values of N_2 fixed measured by ^{15}N methodology can have larger errors when the proportional nitrogen fixed is very small. In comparing the ID and AV methods at different N rates therefore, it is necessary to ensure that a legume capable of achieving a satisfactory level of N_2 fixation is used. Nitrogen fixation in crops like soybean and commonbean have been shown to be very sensitive to nitrogen fertilization in several studies (*e.g.* Rennie *et al.*, 1978), while fababean was quite tolerant (Richards and Soper, 1979). Fababean being able to fix N_2 at high N fertilizer rates therefore appears to be a suitable fixing crop for comparing the ID and AV methods at these very different rates.

Fababean was therefore selected to verify the potentials and limits of the ID and AV methods. The N_2 fixation in fababean, as measured by the two approaches at various levels of inorganic fertilizer applications was compared. We also described a new equation for calculating N_2 fixed when either only one or more than one N rates are applied to the F and NF crops. Although mathematically similar to the equation of Fried (1985), it is much simpler, and does not require the calculation of A-values nor is it based on A-values.

Materials and methods

Fababean (*Vicia faba* L. var. *minor*) cv. Wieselburger as fixing (F) and barley (*Hordeum vulgare*), as a non-fixing (NF) reference crop were grown on adjacent plots measuring 2.4 m^2 and 0.9 m^2, respectively. The plant spacing was 40 cm between rows and 8 cm within a row of fababean; barley was planted with 15 cm row spacing and 4 cm between plants in a row. Treatments were arranged in a randomized block design with five replications. The field experiments were conducted from April till July of 1985 and 1987 at the IAEA laboratory at Seibersdorf, Austria. Detailed characteristics of the soil classified as Typic Eutrocrepts have been described by Zapata *et al.* (1987). Ammonium sulphate enriched with 5.0, 1.0, 0.5 and 0.5 atom % ^{15}N excess was applied at the rates of 20, 100, 200 and 400 kg N ha^{-1} respectively, to the whole fababean and barley plots. The ^{15}N labelled fertilizer was dissolved in water and applied at the time of sowing. The 400 kg N ha^{-1} rate was applied only in 1987. The fababean seeds were inoculated at sowing with a liquid suspension containing an effective strain 175 FI of *Rhizobium leguminosarum* biovar *viceae*, obtained from the Nitragin Co.

At physiological maturity of fababean (approximately 95 days after planting in both years) plants in the three central rows of each plot were harvested at about 2 cm above ground. The length of each row harvested was 1.2 m. Plant samples were separated into reproductive (pods or spikes) and vegetative (straw) parts before sub-sampling. After chopping with a forage chopper, sub-sampling and drying (70°C), the plant samples were ground to pass through a 0.5-mm sieve. Total N was determined by Kjeldahl analysis (Eastin, 1978) and the N isotope ratio on a VG 602 mass spectrometer (Fiedler and Proksch, 1975). The dry weights of nodules from five fababean plants/plot were determined in 1985.

The amount or proportion of N derived from atmosphere (%Ndfa) was calculated on weighted averages of atom % ^{15}N excess for above ground plant parts by the ID (McAuliffe *et al.*, 1958; Fried and Middelboe, 1977) and the AV methods (Fried and Broeshart, 1975) using Equations (1) or (6) described in the present study.

Results and discussion

When 20 kg N ha^{-1} of ammonium sulphate was applied as fertilizer, 80 to 90% of the N in fababean, was derived from the atmosphere (%Ndfa) (Table 1). Increasing the N rate to 100 kg N ha^{-1} reduced %Ndfa only slightly to about 70 to 80%. Even with the application of 200 and 400 kg N ha^{-1}, around 60 and 43%, was derived from the atmosphere, respectively. Both years showed similar trends of slightly increased amount of nitrogen fixed at 100 compared to 20 kg N ha^{-1} fertilizer application (Table 2). Nitrogen fixation was significantly reduced ($P < 0.01$) at higher N rates, but in all cases, more than 90 kg N ha^{-1} was fixed. Nodule dry weight was 176 mg $plant^{-1}$ at both 20 and 100 kg N ha^{-1} fertilizer applications in the 1st year's experiment and was significantly ($P < 0.05$) reduced to 128 mg $plant^{-1}$ at the 200 kg N ha^{-1} rate (data not presented). The total N and dry matter yield of fababean were not affected by N fertilizer treatments (Tables 3 and 4). In contrast, the total N yield of barley was significantly ($P < 0.05$) less at 20 kg N ha^{-1} compared to the higher N fertilizer applications.

Adverse effects of large amounts of N fertilizer on symbiotic N_2 fixation by a number of

Table 1. Percentage N derived from atmosphere (%Ndfa) of fababean as calculated by the isotope dilution (ID) and A-value (AV) methods at various rates of N fertilizer applications

Year	N fertilizer application ($kg\,N\,ha^{-1}$)	ID	AV			
			N($kg\,ha^{-1}$) application to NF crop			
			100	200	400	$LSD_{0.05}$
1985	20	85	90	89	ND	NS
	100	79		78	ND	NS
	200	61			ND	
	$LSD_{0.05}$	13		NS		
1987	20	79	85	83	81	NS
	100	74		71	68	NS
	200	64			62	NS
	400	43				
	$LSD_{0.05}$	10		6		

ND: Not determined.
NS: Not significant.

Table 2. Amount of N derived from atmosphere (kg N/ha) of fababean as calculated by the isotope dilution (ID) and A-value (AV) methods at various rates of N fertilizer applications

Year	N fertilizer application ($kg\,N\,ha^{-1}$)	ID	AV			
			N($kg\,ha^{-1}$) application to NF crop			
			100	200	400	$LSD_{0.05}$
1985	20	159	167	166	ND	NS
	100	183		182	ND	NS
	200	122			ND	
	$LSD_{0.05}$	34		NS		
1987	20	159	172	168	164	NS
	100	168		161	154	NS
	200	134			129	NS
	400	91				
	$LSD_{0.05}$	26		21		

ND: Not determined.
NS: Not significant.

Table 3. Dry matter at physiological maturity of fababean at different rates of N fertilizer applications

N fertilizer application, ($kg\,N\,ha^{-1}$)	Dry matter yield ($ton\,ha^{-1}$)			
	1985		1987	
	Pods	Total	Pods	Total
20	1.3	9.0	1.8	7.2
100	2.1	11.0	1.8	7.3
200	1.8	10.3	1.7	7.3
400	ND	ND	1.8	7.5
$LSD_{0.05}$	NS	NS	NS	NS

ND: Not determined.
NS: Not significant.

Table 4. Total N yield of barley and fababean at different rates of N fertilizer application

N fertilizer application ($kg\,N\,ha^{-1}$)	Total N yield ($kg\,N\,ha^{-1}$)			
	1985		1987	
	Barley	Fababean	Barley	Fababean
20	48	186	44	202
100	82	232	131	227
200	89	201	95	208
400	ND	ND	97	211
$LSD_{0.05}$	NS	NS	29	NS

ND: Not determined.
NS: Not significant.

legumes is well documented (Oghoghorie and Pate, 1971; Richards and Soper, 1979). Also, the differential inhibition of fixation by N fertilization has been found between soybean cultivars (Hardarson *et al.*, 1984; Herridge and Betts, 1988) and soybean cultivar-Bradyrhizobium combinations (Senaratne and Hardarson, 1987). Nitrogen fixation in fababean on the other hand has been shown to be more tolerant to elevated N levels, with the present experiment supporting the findings of Roughley *et al.* (1983) who found that *ca* 50 kg N ha^{-1} was fixed at 450 kg N ha^{-1} fertilizer application. This characteristic may be of advantage under conditions of mixed cropping or crop rotation. In these cases it may be necessary to apply high N rates to the non-fixing crop, which could inhibit nitrogen fixation by the companion crop. Fababean would therefore be very suitable in a multiple cropping system with a non-fixing crop demanding high nitrogen application.

Fababean was very suitable for the objective of this study, in that substantial N_2 was fixed in all treatments even when 400 kg N ha^{-1} was applied.

Estimates of %Ndfa did not differ between the ID and AV methods (Tables 1 and 2). Several investigators prefer to use the ID method rather than the AV method because of (i) misconceptions about dissimilarities in the two approaches even though Fried and Middelboe (1977) have shown the similarities of these two methods and Danso *et al.* (1986) further showed that %Ndfa measurements by both ID and AV are yield independent; and (ii) the AV approach requiring a series of equations to arrive at the same %Ndfa provided by the following single and more simple ID equation (Fried and Middelboe, 1977):

$$\%\text{Ndfa} = \left(1 - \frac{\%\text{Ndff}_{\text{F}}}{\%\text{Ndff}_{\text{NF}}}\right) \times 100 \qquad (1)$$

which is derived from equation (2) below, *i.e.*:

$$\text{a)} \quad \frac{\%\text{Ndff}_{\text{NF}}}{\%\text{Ndfs}_{\text{NF}}} = \frac{\%\text{Ndff}_{\text{F}}}{\%\text{Ndfs}_{\text{F}}} \qquad (2)$$

and the two equations describing the N sources of the NF

$$\text{b)} \quad \%\text{Ndff}_{\text{NF}} + \%\text{Ndfs}_{\text{NF}} = 100 \qquad (3)$$

and the F crop

$$\text{c)} \quad \%\text{Ndff}_{\text{F}} + \%\text{Ndfs}_{\text{F}} + \%\text{Ndfa} = 100 \qquad (4)$$

where %Ndff and %Ndfs are % N derived from fertilizer and soil, respectively. Equation (2) presents the only assumption made in the ID method, *i.e.* that the ratio of %N derived from fertilizer over %N derived from soil is the same in the F and NF crops. Different rooting and N uptake patterns by the F and NF crops can affect the validity of this assumption (Witty, 1983). It cannot be tested directly, but it was indirectly examined by determining whether the proportion of fertilizer and soil S taken up by F and NF crops were the same (Wagner and Zapata, 1982). In that study sulphur A-values were similar among fababean, sudangrass and barley, but lower for oil radish, which proved to be an inappropriate reference crop because it took up much more S and by inference, more fertilizer N relative to soil N than the other crops.

The AV method as described by Fried and Broeshart (1975) needs a series of equations to arrive at %Ndfa, making it quite cumbersome. Besides, it tends to give the impression that ID and AV methods are different. This is however not so. Equation (2) can be rewritten for conditions when different N rates are applied to the F and NF crops (*i.e.* AV method), by inserting a factor n (n is the amount of fertilizer applied to the F crop divided by the amount of fertilizer applied to the NF crop):

$$\frac{\text{n} \times \%\text{Ndff}_{\text{NF}}}{\%\text{Ndfs}_{\text{NF}}} = \frac{\%\text{Ndff}_{\text{F}}}{\%\text{Ndfs}_{\text{F}}} \qquad (5)$$

In this equation n × $\%\text{Ndff}_{\text{NF}}$ stands for the calculated %Ndff of the NF crop at the N rate applied to the F crop. The calculated and the measured values should be very similar which only occurs if the $\%\text{Ndff}_{\text{NF}}$ increases proportionally and linearly with increased rate of fertilizer application (an assumption of this method). Erroneous %Ndfa (using the AV method) can be obtained if this does not hold. Although experiments using AV method are done at two different N rates, the N_2 fixation is quantified by calculating the expected %Ndff of the NF crop at the N rate of the F crop.

The following equation:

$$\%\mathrm{Ndfa} = 100\left(1 - \frac{\%\mathrm{Ndff}_F}{n \times \%\mathrm{Ndff}_{NF}}\right) + \%\mathrm{Ndff}_F\left(\frac{1}{n} - 1\right) \quad (6)$$

which is derived from (3), (4) and (5) as shown below can be used to calculate %Ndfa when different rates of N are applied to the F and NF crops.

Equation (4) can be written as follows:

$$\%\mathrm{Ndfa} = 100 - \%\mathrm{Ndff}_F - \%\mathrm{Ndfs}_F \quad (7)$$

From (5)

$$\%\mathrm{Ndfs}_F = \frac{\%\mathrm{Ndff}_F \times \%\mathrm{Ndfs}_{NF}}{n \times \%\mathrm{Ndff}_{NF}}$$

and from (3)

$$\%\mathrm{Ndfs}_F = \frac{\%\mathrm{Ndff}_F}{n \times \%\mathrm{Ndff}_{NF}} \times (100 - \%\mathrm{Ndff}_{NF})$$

or

$$\%\mathrm{Ndfs}_F = \frac{100\%\mathrm{Ndff}_F}{n \times \%\mathrm{Ndff}_{NF}} - \frac{\%\mathrm{Ndff}_F}{n} \quad (8)$$

Introducing (8) into (7)

$$\%\mathrm{Ndfa} = 100 - \%\mathrm{Ndff}_F - \left(\frac{100\%\mathrm{Ndff}_F}{n \times \%\mathrm{Ndff}_{NF}} - \frac{\%\mathrm{Ndff}_F}{n}\right)$$

from which Equation (6) is derived.

Results obtained by applying identical or unidentical N rates to F and NF crops can all be calculated using Equation (6). Where the same rate of fertilizer is applied to the NF and F ($n = 1$), Equation (6) is identical to Equation (1).

Equation (6), which is based only on the original isotope dilution method as presented by McAuliffe *et al.* (1958) and Fried and Middelboe (1977) is mathematically identical to the A-value calculation (Fried and Broeshart, 1975), except that one does not need to first calculate A-values.

In the present study, we tested whether $\%\mathrm{Ndff}_{NF}$ at the higher N fertilizer rate (Table 4) multiplied by n is equal to $\%\mathrm{Ndff}_{NF}$ at the N fertilizer rate applied to the F crop. Figure 1 shows that this is only true when the fertilizer rates to F and NF crops were relatively close, *e.g.* 20 and 100 Kg N ha^{-1}, respectively. Similar linear increase in fertilizer uptake with increased rate has been reported for non-fixing crops (Fried, 1978). The measured and the calculated values deviatiated however significantly when highly divergent fertilizer rates were applied, *e.g.* 20 and 200 or 400 to F and NF crops, respectively. This suggest that in these cases erroneous measurements of nitrogen fixation could be expected. However, in the present study, although there were significant differences between the observed $\%\mathrm{Ndff}_{NF}$ and the calculated $\%\mathrm{Ndff}_{NF}$ differences eventually did not result in significant difference in %Ndfa (Tables 1 and 2). The reason for this is that the method is not affected by these small differences. For example at fertilizer application of 200 kg N ha^{-1} in 1987 the calculated value for $\%\mathrm{Ndff}_{NF}$ is 51.0% and the measured value 40.3 resulting is 83%

Table 5. %N derived from fertilizer (%Ndff) of fababean and barley at various N fertilizer rates

N fertilizer application (kg ha^{-1})	%Ndff					
	1985			1987		
	Fababean	Barley	$LSD_{0.05}$	Fababean	Barley	$LSD_{0.05}$
20	0.9	6.2	1.7	1.1	5.1	0.6
100	6.8	33.0	5.8	7.2	28.6	3.4
200	19.0	48.6	8.6	14.2	40.3	6.5
400	ND	ND		30.8	54.2	5.6
$LSD_{0.05}$	3.6	7.7		4.8	3.0	

ND: Not determined.

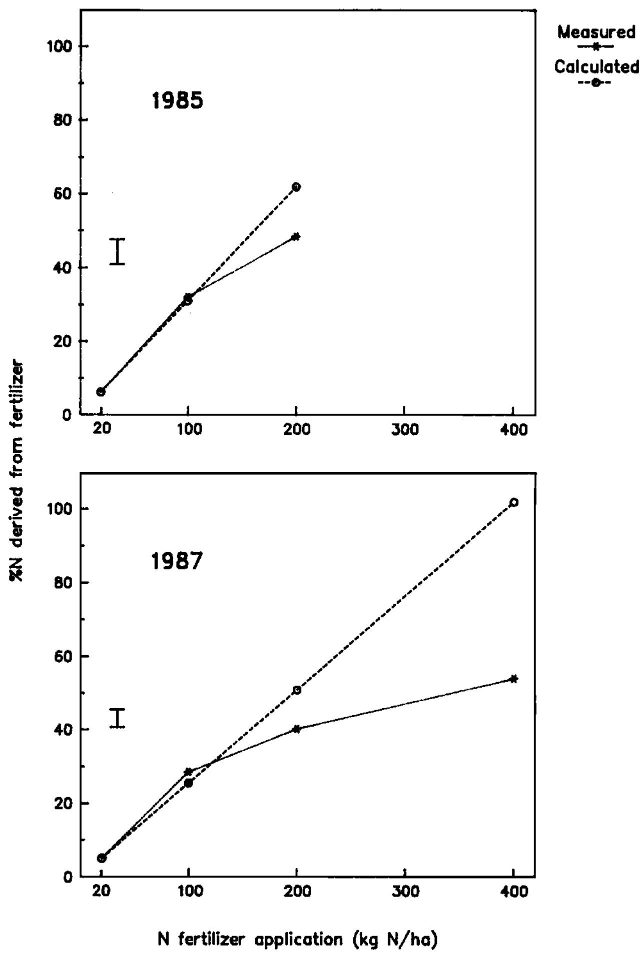

Fig. 1. Measured (%Ndff) and calculated (n × %Ndff) % N derived from fertilizer in barley at various N fertilizer levels. Vertical bars represent the pooled LSD values at $P < 0.05$.

Ndfa. Had the $\%Ndff_{NF}$ been much lower *i.e.* 30 or 20% would the respective values for fixation only have been reduced 73 and 55%.

Examples to calculate %Ndfa using the ID, Equation (6) or the AV method of Fried and Broeshart (1975) are presented in Table 6.

In this study the ID and AV methods gave similar results. Considering that the ID method is conceptually simpler and involves less assumptions, not to mention the fact that only a single N rate is applied, it seems more appropriate to use the ID method where the NF crop can grow well with low fertilizer applications. When the use of the AV method is unavoidable, *e.g.* under conditions of low soil fertility, N rates as close as possible should be preferred. Under these circumstances, the chances that the calculated n $\%Ndff_{NF}$ and measured $\%Ndff_{NF}$ at the N rate of the F crop would be similar are very high. Thus there is no need to use 100 kg N ha^{-1} on a reference crop which would grow satisfactory on 50 kg N ha^{-1} or less, when the F crop is grown at 20 kg N ha^{-1}. Potential errors introduced by the use of different N rates may thus be minimized.

It is very clear that the ID and AV methods are based on the same principles. Equations (1) and (6) are closely related and using them makes it much easier to calculate %Ndfa from ^{15}N data than was previously possible, especially since calculations based on A-values (Fried and Broeshart, 1975) are more complicated than the ID and undoubtedly can lead to misinterpretation of data.

It is important to note that the above

Table 6. Example to calculate %Ndfa using the ID, Equation (6) or the AV method of Fried and Broeshart (1975). The data are from the 1985 experiment

	Fababean	Barley	Barley
Fertilizer application	20 kg N ha^{-1}	20 kg N ha^{-1}	100 kg N ha^{-1}
% ^{15}N a.e.$_{fertilizer}$	5.108	5.108	0.921
% ^{15}N a.e.$_{plant}$	0.047	0.317	0.304
%Ndff[a]	0.920	6.208	33.01
%Ndfa ID[b]	85.18		
AV[c]	89.74		

[a] $\%\text{Ndff} = \frac{\%\ ^{15}\text{N a.e.}_{plant}}{\%\ ^{15}\text{N a.e.}_{fert.}} \times 100$

[b] By Equation (1):

$$\%\text{Ndfa} = \left(1 - \frac{0.920}{6.208}\right) \times 100 = 85.18$$

[c] a) by Equation (6):

$$\%\text{Ndfa} = 100\left(1 - \frac{0.920}{0.2 \times 33.01}\right) + 0.920\left(\frac{1}{0.2} - 1\right) = 89.74$$

b) by the AV method of Fried and Broeshart (1975):

$$A_{soil} = \frac{100 - \%\text{Ndff}_{NF}}{\%\text{Ndff}_{NF}} \times A_{fert} = \frac{100 - 33.01}{33.01} \times 100 = 202.9$$

$$A_{soil+air} = \frac{100 - \%\text{Ndff}_F}{\%\text{Ndff}_F} \times A_{fert} = \frac{100 - 0.920}{0.920} \times 20 = 2153.9$$

$$A_{air} = 2153.9 - 202.9 = 1951$$

$$\%\text{Ndfa} = A_{air} \times \frac{\%\text{Ndff}_F}{A_{fert}} = 1951 \times \frac{0.920}{20} = 89.74$$

methodologies give correct estimates of %Ndfa only when the assumptions on which the methods are based are met. Therefore, for successful use of ^{15}N to quantify symbiotic nitrogen fixation more research is needed to determine how well the above assumptions hold under various experimental conditions.

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