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Full Length Research Paper

Appraisal of the ^{15}N-isotope dilution and ^{15}N natural abundance methods for quantifying nitrogen fixation by flood-tolerant green manure legumes

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Quantification of biological nitrogen fixation (BNF) is fundamental to identifying potential green manure legumes for their enhanced nitrogen contribution to sustained crop production. A screenhouse experiment compared BNF by *Aeschynomene afraspera* L. as affected by phosphorus, using the ^{15}N-isotope dilution and the ^{15}N methods. Rice (*Oryza sativa* L.) was grown as a non -fixing plant. Phosphorus significantly increased the percent N derived from the atmosphere (% N_{dfa}), whereby the ^{15}N method tended to overestimate BNF contribution on average by 20% compared to the ^{15}N dilution. Data confirmed rice as a non-fixing plant for estimation of BNF by flood-tolerant legumes.

Key words: *Aeschynomene afraspera*, Biological nitrogen fixation, ^{15}N-isotope dilution, ^{15}N natural abundance.

INTRODUCTION

Nitrogen fixation by legumes (BNF) is an affordable alternative to N fertilizer for small- scale farmers (Ladha et al., 1996). Phosphorus applied to green manure legumes (GMs) has been shown to enhance their contribution to nitrogen (N) accumulation through BNF (Somado et al., 2003). Quantification of the percent of N derived from fixation is essential to identifying GMs for enhanced BNF.

The ^{15}N enriched isotope dilution technique (^{15}NE) and the δ ^{15}N natural abundance (^{15}NA) method have been used extensively for the estimation of BNF. Using the ^{15}NE approach, enriched ^{15}N-fertilizer is applied to both legume and reference crop and the differences in ^{15}N dilution between the two crops are used to calculate N_2 fixation. In contrast, the ^{15}NA approach uses the naturally occurring differences in atom% ^{15}N of soil available N and atmospheric N_2 to estimate BNF. Again, differences in dilution of ^{15}N between the legume and the reference crop are used to calculate BNF. Previous studies reported that both methods provided similar estimates of N_2 fixation (Peoples and Herridge, 1990; Doughton et al., 1995). Subsequently, the validity of the ^{15}NA approach to estimate BNF under field conditions has been questioned (Handley and Scrimgeour, 1997). These authors have argued that the ^{15}NA and NE approaches essentially reflect different processes.

The ^{15}NE is believed to be the most accurate technique to measure the N fixation by plants (Danso, 1988). On the other hand, the ^{15}NA method does not require the use of costly ^{15}N fertilizers, and if proved as accurate as the former, may be promptly adopted by resource-limited scientists in Africa.

There is genetic variability for N fixation among and within legume species (Sanginga et al., 1990). Screening of GMs that have high N fixation potential is needed for enhanced N contribution to sustain food crop yields. The rationale for using GMs in crop production systems is

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Abbreviations: BNF, Biological N fixation; **%Ndfa**, percent of N derived from the atmosphere; **^{15}NE**, ^{15}N enriched isotope dilution technique; and **^{15}NA**, ^{15}N natural abundance.

their potential to meet food crops' N requirement, and reduce farmers' dependency on external mineral inputs for sustainable food production (Ladha et al., 1992).

The main objective of this study was to (i) determine the effect of P application on the percent of N derived from fixation (%Ndfa) of a short-duration flood-tolerant legume and (ii) appraise BNF estimates, using the ^{15}N - isotope dilution and the ^{15}N natural abundance methods.

MATERIALS AND METHODS

A screenhouse experiment was carried out at the main research station of the Africa Rice Center (WARDA) at Mbé (derived savannah zone, 7.5^{o}N, 5.1 ^{o}W, 280 m altitude) in Cote d'Ivoire. The experimental soil (Ultisols) was pH 5.2 with 4 mg/kg Bray-1 total P, 1.1% total carbon and 0.05% total nitrogen (Okalebo et al., 1993). The soil was sieved (< 2 mm), homogenized and 6 kg (dry weight basis) was placed into eight liter glazed pots of 40 cm diameter each. The seeds of the flood-tolerant N-fixing *Aeschynomene afraspera* were scarified for 30 min in concentrated sulphuric acid (commercial grade) to break dormancy and achieve a high and even germination rate. The seeds were then rinsed with tap water and air-dried. To inoculate the legume plant, fresh nodules of *A. afraspera* were collected in a nearby field, and squashed in distilled water. The scarified seeds were soaked overnight in the rhizobial suspension before planting. Pre -treated seeds were dibble-seeded at a density of 100 seeds m^{-2} (0.10 x 0.10m) in the pot under flooded conditions. Scarified and inoculated seeds of *A. afraspera* were then thinned to ten seedlings pot^{-1} after emergence. Rice (*Oryza sativa* L., cv. WITA 1) was used as a non N_2-fixing reference plant for BNF estimation (Pareek et al., 1990). Triple super phosphate (TSP, 19.6% soluble P) was applied at 60 kg P ha^{-1} to study the effect of P on %Ndfa. Potassium (K) was also applied to all pots at 60 kg K ha^{-1} as potassium chloride (KCl). The soil was saturated with distilled water and puddled manually after application of P and K. A solution containing ^{15}N-labeled ammonium sulphate fertilizer (10 atom % excess) was added to all pots to supply 0.1g ^{15}N g^{-1} soil (Hardarson and Danso, 1990) to estimate the proportion of legume-N accumulated that was derived via BNF. In addition to ^{15}N-labeled fertilizer material, BNF-N was measured, using the ^{15}N natural abundance method. For BNF estimation by the ^{15}N natural abundance method, additional pots were included.

The pots were arranged in a randomized complete block design with three replications (No-P applied and TSP). The soil was submerged under 3-5 cm water layer and this was maintained throughout the growing period. The legume and rice plants were harvested eight weeks after sowing (at the onset of flowering, in the case of *A. afraspera*). At harvest, plant shoots were cut at the soil level from the pot, and weighed to determine the fresh matter yield of the legume plants. Dry weight was then determined after oven drying at 70^{o}C for three days. Above and belowground plant part were oven-dried. The oven-dried material was ground further into a fine powder using a ball-mill. 5 mg sub-samples were weighed into small tin capsules, which were then closed and rolled into a ball. These samples were then analyzed for total N and ^{15}N using an Elemental N analyzer connected to a mass -spectrometer (Reineking et al., 1993). The proportion of plant N derived from atmosphere ($\%N_{dfa}$), was calculated using the ^{15}N dilution and the^{15}N natural abundance methods, as appropriate.

^{15}N-isotope dilution technique: The following equation (1) of Hardarson and Danso (1990) was used to compute $\%N_{dfa}$:

$$\% N_{dfa} = [1 - (N_{fix}/N_{ref}) \times 100] \quad (1)$$

Where N_{fix} is the ^{15}N atom% excess of the N- fixing plant (*A. afraspera*), and N_{ref} the ^{15}N atom% excess of the non-fixing reference plant (rice).

Natural abundance method: The following equation (2) of Shearer and Kohl (1986) was used to calculate $\%N_{dfa}$:

$$\%N_{dfa} = [1 - (^{15}N_{ref} - ^{15}N_{fix}) / (^{15}N_{ref} - B)] \times 100 \quad (2)$$

Where $^{15}N_{ref}$ is the ^{15}N of the non-fixing reference plant, $^{15}N_{fix}$ is the ^{15}N of the fixing legume plant and B the ^{15}N of the fixing plant grown hydroponically on N-free media. B value for *Aeschynomene* spp. was: - 0.7 $^{o}/_{oo}$ (Yoneyama et al., 1991).

$^{15}N = 1000 (R_{sample} - R_{air\ N_2}) / R_{air\ N_2}$

R = mass 29/mass 28 = $^{15}N^{14}N/^{14}N_2$

The proportion of N derived from the fertilizer (N_{dff}) was estimated using the following equation:

$$\%N_{dff} = (N_{plant} / N_{fert}) * 100 \quad (3)$$

Where N_{plant} is the ^{15}N atom% excess of the plant (rice or *A. afraspera*), and the ^{15}N atom% excess of the labeled fertilizer.

The proportion of N derived from the unlabeled soil ($\%N_{dfS}$) was calculated with the assumption that both non-fixing (rice) and fixing legume took up N from unlabeled soil and labeled fertilizer in the same ratio, i.e.

In the fixing crop, $\%N_{dff} + N_{dfS} + N_{dfa} = 100$ (4)

In the non-fixing crop, $\%N_{dff} + \% N_{dfS} = 100$ (5)

Data were analyzed using an analysis of variance (ANOVA) procedure of the SAS program (SAS, 2001). Unless otherwise indicated the probability level of 5% was considered statistically significant.

RESULTS AND DISCUSSION

Accuracy of measurements of nitrogen fixation

Results of the present study support observations made by Pareek et al. (1990) that rice (*O. sativa*) is an appropriate reference crop for estimation of N_2 fixation by semi-aquatic legume plants. Moreover, this finding satisfies the basic requirements (Danso et al., 1993) for measuring N_2 fixation using the ^{15}N-isotope dilution technique in that both the N_2 fixing legume and the reference non-fixing plant used soil N of identical ^{15}N enrichment (Table 1). Table 1 summarizes mean values of the proportion of ^{15}N derived from ^{15}N-labeled ammonium sulphate fertilizer (N_{dff}) and from soil (N_{dfS}) and the $^{15}N/^{14}N$ ratio (R) of the N_2 fixing *A. afraspera* and the non-fixing reference rice plant. Data in Table 1 show that both the fixing legume and the non-fixing reference plants assimilated similar proportion (R) of total N from ^{15}N ammonium sulphate fertilizer and from soil N.

Table 1. Mean values of the proportion of nitrogen derived from air (%Ndfa), the proportion of ^{15}N derived from ^{15}N-labeled ammonium sulphate fertilizer (%Ndff) and from soil (%NdfS) and the $^{15}N/^{14}N$ ratio (R) of the N2 fixing *A. afraspera* and non-fixing reference rice (*O. sativa*, cv. WITA1) crop grown in pot under flooded conditions[§].

Plant species		**P fertilizer**	**%Ndff**	**%NdfS**
		0 P	9	35
A. afraspera				
	$LSD_{0.05}$	TSP	5 2	24 7
		0 P	20	80
Rice (cv. WITA 1)				
	$LSD_{0.05}$ $R=^{15}N/^{14}N$ ratio R= Ndff/NdfS	TSP R=0.234 (*Legume*) R=0.230 (*rice*)	17 3	83 3

[§] Means of three replicates
0 P, Unfertilized control; TSP, triple super phosphate

Table 2. Percent N derived from atmosphere (Ndfa) and % ^{15}N atom excess (^{15}NAE) in above and underground biomass of *A. afraspera*, using rice (*O. sativa*, cv. WITA1) as a reference non-fixing plant[§].

BNF Estimates Method	**P fertilizer**		**^{15}N atom excess (%)**					**%Ndfa**
			A. afraspera		**Rice**			
			Above	Under	Above	Under		
	0 P		0.198	0.315	0.760	0.845		56
^{15}N dilution								
	TSP		0.047	0.068	0.666	0.614		71
		$LSD_{0.05}$	0.077	0.124	0.195	0.143	Mean = (%Ndfa)	63* ($LSD_{0.05}$=8.51)
			Delta ^{15}N ‰					
			A. afraspera		Rice			%Ndfa
			Above	Under	Above	Under		
	0 P		3.534	5.431	26.392	25.816		81
Delta ^{15}N								
	TSP		0.728	2.452	16.350	12.737		87
		$LSD_{0.05}$	0.742	1.389	4.148	6.183	Mean = (%Ndfa)	84* ($LSD_{0.05}$=6.04)

[§] Means of three replicates
*, Pair-wise *t*-test significant at p <0.001 (means of three replicates)
0 P, Unfertilized control; TSP, triple super phosphate

Effect of P application on percent nitrogen derived from fixation

Irrespective of the method of BNF estimates used, P fertilization caused a significant increase in the percentage of legume N-derived from fixation ($\%N_{dfa}$).

Using the ^{15}N isotope dilution technique, $\%N_{dfa}$ was 56% (unfertilized legume) and 71% (TSP). These values were higher (80 and 87% for non-P and TSP, respectively) when estimated by the $\delta^{15}N$ natural abundance method (Table 2). The specific role of P in BNF has been controversial. For example, Sanginga et al. (1996) observed that the effect of P on N fixation was mainly in the total amount of N fixed rather than on the $\%N_{dfa}$. Their conclusions were based on root-nodulating legume species. In contrast, Becker et al. (1991) and Engels et

al. (1995), growing a stem-nodulating flood-tolerant legume (e.g. *A. afraspera*) reported increase in N_{dfa} as a result of P fertilization. Israel (1987) noted that P requirement for nodulation, nodule activities and N fixation are higher than that for host plant growth.

Therefore, it can be reasoned that the impact of P supply on BNF is likely to be more pronounced in stem-nodulating legume species such as *A. afraspera*, because of higher number of nodules (root + stem) than in root-nodulating legumes.

Screening of GMs that have high N fixation potential is needed for enhanced N contribution to sustain food crop yields. The present study was undertaken with the objective to appraise BNF by the flood-tolerant N fixing *A. afraspera* as affected by the application of P fertilizer, using the ^{15}N-isotope dilution and the ^{15}N natural abundance methods.

Quantifying nitrogen derived from fixation, labeled fertilizer, and unlabeled soil using the δ^{15}N natural abundance and ^{15}N-isotope dilution methods

The pair-wise comparison *t* test indicated that the mean BNF-N estimates by the dilution technique significantly ($p<0.001$) was on average 20% lower than the mean BNF-N determined using the δ^{15}N method. The results suggest that the two methods do not provide similar results on BNF by *A. afraspera*. Spatial variability in N accumulation by legumes across landscapes and agroecosystems has been reported (People and Herridge, 1990). The two methods of measuring BNF by field-grown legumes have been reported to provide similar estimates of N_2 fixation (People and Herridge, 1990; Doughton et al., 1995). Subsequently, Handley and Scrimgeour (1997) disagreed, arguing the two methods reflect fundamentally different processes. According to Boddey et al. (2000), the observed lack of a significant correlation between the ^{15}N-isotope dilution technique and the δ^{15}N natural abundance method is likely caused by high spatial variability of the controlling biotic and abiotic factors for BNF. In the present study under pot conditions, perhaps the BNF spatial variability was minimized, as the relative δ^{15}N uptake of the plants were restricted to the soil in the containers. Our data support the observation made by Handley and Scrimgeour (1997) and suggest that the two methods differ in providing BNF estimates by the legume evaluated.

Furthermore, the % ^{15}N atom excess and the delta ^{15}N in the above and the underground part of the rice plant were higher than that in the legume species, indicating the occurrence of N fixation in *A. afraspera* (Table 2). Sanginga et al. (1992) and Ndoye et al. (1995) have reported that, failing to take into account roots in BNF estimates may result in significant underestimation of nitrogen fixed in some N-fixing trees. In the present study, and irrespective of the P fertilization regime used, both the % ^{15}N atom excess and the delta ^{15}N measured in the underground biomass of *A. afraspera* were higher than that determined in the aboveground biomass (leaves and stems). Thus, in the flood-tolerant legume under study, ignoring roots may have resulted in a bias in the BNF estimates. The proportion of nitrogen derived from the soil ($\%N_{dfS}$) is higher in *A. afraspera* than that derived from the fertilizer ($\%N_{dff}$) (Table 1) . The rationale for using GMs in crop production is the legume's potential to improve the quality of soil. Screening of legume plants that have a high potential for N fixation and at the same time have a low proportion of N derived from the soil should be a priority.

CONCLUSION

The ^{15}N- isotope dilution and δ^{15}N natural abundance methods for estimating BNF might not be interchangeable in measuring the contribution of BNF to the legume's N accumulation under the flooded conditions of the present study. However, if the precise quantification of N_2 fixation is not needed, then the use of the δ^{15}N natural abundance method may be adequate for screening of legumes for BNF. The δ^{15}N natural abundance method does not require the use of costly ^{15}N fertilizers, and may then be affordable to resource-limited scientists in the region. This is of particular importance in view of the urgent need to identify green manure legume for their potential of enhanced BNF contribution for sustained crop production.

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