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Quantitative Methods/ Original Article

Comparison of ^{15}N isotope methods to determine the recovery efficiency of nitrogen from green manure

Abstract – The objective of this work was to compare three methods of ^{15}N isotope application to the soil in order to determine the recovery efficiency of nitrogen derived from green manure by corn (*Zea mays*). The used experimental design was a randomized complete block with six replicates. The treatments consisted of three ^{15}N isotope methods: indirect method I, isotope dilution with ^{15}N-labelled synthetic fertilizer applied through a small hole next to each corn plant; indirect method II, isotope dilution with ^{15}N-labelled synthetic fertilizer spread in the furrow next to the corn planting row; and direct method, application of a legume straw as green manure, treated with 2.23% excess ^{15}N. The green manure used was jack bean (*Canavalia ensiformis*). Applying synthetic ^{15}N to the soil (indirect methods I and II) did not interfere in the estimation of the recovery efficiency of N derived from green manure. The recovery efficiency of N from green manure was 17% for the indirect methods, overestimated when compared with that of 7% for the direct method. The direct method is the most adequate to determine the recovery efficiency of N from green manure.

Index terms: *Zea mays*, biological nitrogen fixation, legume.

Comparação de métodos isotópicos de ^{15}N na determinação da eficiência de recuperação do nitrogênio da adubação verde

Resumo – O objetivo deste trabalho foi avaliar três métodos isotópicos de aplicação de ^{15}N ao solo para determinar a eficiência de recuperação do nitrogênio derivado da adubação verde pelo milho (*Zea mays*). Utilizou-se o delineamento experimental de blocos ao acaso, com seis repetições. Os tratamentos consistiram de três métodos isotópicos de ^{15}N: método indireto I, diluição isotópica com ^{15}N sintético aplicado por um pequeno orifício ao lado de cada planta de milho; método indireto II, diluição isotópica com ^{15}N sintético espalhado em sulco ao lado da linha de plantio do milho; e método direto, aplicação de palhada de leguminosa como adubo verde, marcada com 2,32% de ^{15}N em excesso. O adubo verde utilizado foi o feijão-de-porco (*Canavalia ensiformis*). A aplicação de ^{15}N sintético no solo (métodos indiretos I e II) não interferiu na estimativa da eficiência de recuperação do N da adubação verde. A eficiência de recuperação do N proveniente do adubo verde foi de 17% nos métodos indiretos, superestimada em comparação à de 7% no método direto. O método direto é o mais adequado para determinação da eficiência de recuperação do N derivado da adubação verde.

Termos para indexação: *Zea mays*, fixação biológica de nitrogênio, leguminosa.

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Introduction

The use of green manure composed of legume straw is a practice aimed at improving the productive potential of the soil by increasing its water retention capacity, controlling nematodes, protecting it against erosion, improving its structure, adding carbon to it, and promoting nutrient cycling, mainly of nitrogen derived from the biological fixation of N from the atmosphere (Carvalho et al., 2011; Chieza et al., 2013).

Green manure, therefore, plays a major role in organic production systems, where the use of N derived from synthetic sources is prohibited. However, for proper green manure management in commercial crops, it is necessary to understand N recovery efficiency by the crops. The uptake of this nutrient by the crop depends on the timing between N mineralization of the legume (green manure) and crop demand. It should be noted that this timing is significantly affected by N mineralization half-life ($t^{1/2}$), which varies among legumes. According to Acosta et al. (2014), N mineralization $t^{1/2}$ for vetch is 30 days, while that for forage turnip is 106 days. The same authors observed an increase in $t^{1/2}$ during a year with reduced precipitation, which shows that climatic conditions are another important factor. Ambrosano et al. (2005) and Scivittaro et al. (2003) found that the recovery efficiency of N from green manure varies, on average, from 5 to 24%, respectively. Therefore, information on the recovery efficiency of N from green manure by commercial crops is essential to ensure a proper planning and adjustment of legume biomass management practices in different edaphoclimatic conditions.

According to Araújo et al. (2011), N recovery efficiency can be evaluated either directly or indirectly using the ^{15}N isotope. The direct method, as described in Cueto-Wong et al. (2001), consists of two steps: in the first, a legume is cultivated in substrate containing excess ^{15}N; in the second, this ^{15}N-treated green manure is applied to the commercial crop. Therefore, any excess ^{15}N found in the crop would have been provided by the legume, indicating the actual amount of N derived from green manure.

Although the direct method enables an assessment of the real N recovery efficiency, it requires a large amount of ^{15}N for the production of the treated biomass, which limits its usefulness in research due to high costs. For this reason, Urquiaga & Zapata (2000) proposed the use of an indirect method for the estimation of the recovery efficiency of N from green manure.

The indirect method involves the comparison of samples from two treatments. In the first, two different N sources are applied to the same plant, one in the form of green manure and the other as ^{15}N-labelled synthetic fertilizer. In the second, only the ^{15}N-labelled synthetic fertilizer is applied. Based on the difference in labelled ^{15}N between plants grown with and without green manure, it is possible to estimate the percentage of N transferred from the green manure to the test crop.

The indirect method has the advantage of being practical, quick, and cost-effective. However, the influence of synthetic N on the mineralization of N from the soil and green manure (priming effect), can affect the estimates of the recovery efficiency of N from green manure, since the N fertilizer has a synergistic effect on the absorption of this nutrient in the soil (Silva et al., 2009; Kuzyakov, 2010). According to Alfaia (1997), the priming effect consists of an increase in organic N mineralization caused by the addition of organic substrates and N fertilizers. In order to reduce this effect, the application of the ^{15}N-labelled fertilizer through a small hole in the soil next to the plant has been recommended empirically, since no known study has confirmed the effectiveness of this method. It has been shown that the indirect method can lead to an overestimation of the recovery efficiency of N from green manure, and the manner in which the ^{15}N-labelled fertilizer is applied to the soil may also interfere in the estimation of N recovery efficiency.

The objective of this work was to compare three methods of ^{15}N isotope application to the soil in order to determine the recovery efficiency of N derived from green manure by corn.

Materials and Methods

The study was carried out in the experimental area of Embrapa Agrobiologia, located in the municipality of Seropédica, in the state of Rio de Janeiro, Brazil (22º45'28"S, 43º41'05"W, at an average altitude of 33 m), during the 2013 crop season. The climate of the region is Aw, according to Köppen's classification, with hot and rainy summers and dry winters. The area had been cultivated with sugarcane (*Saccharum* *officinarum* L.) for approximately ten years and was left fallow for the two years preceding the study.

The experimental design was a randomized complete block with six replicates, totalizing 18 experimental units. Each unit consisted of 5.0x2.5-m plots, containing three corn rows, with the central row left free for circulation. In each plot, subplots of one linear meter were established, where green manure or urea was applied according to each treatment.

The study was conducted in two phases: in the first, jack bean [*Canavalia ensiformis* (L.) DC.], treated with ^{15}N, was used as green manure; in the second, the recovery efficiency of N from green manure by corn was evaluated under field conditions.

In the first phase, the jack bean seeds were previously disinfected in 70% v/v alcohol for 5 min and a 1% v^{-1} sodium hypochlorite solution for 3 min, then washed ten times with distilled water (Lima et al., 2005). Six seeds were sown per pot, each containing about 10 kg substrate (50% washed sand and 50% vermiculite, v/v). The substrate per pot consisted of: 37.87 g single superphosphate (6.8 g P_2O_5), 1.15 g potassium sulfate (0.57 g K_2O), 3.80 g magnesium sulfate heptahydrate (375 mg Mg), 0.9 g dolomitic limestone, and 1 g of the FTE BR 12 micronutrient mixture with 3.9% S; 1.8% B; 0.85% Cu; 2.0% Mn; and 9.0% Zn, besides 1 mL kg^{-1} of the micronutrient substrate solution described by Franco & Döbereiner (1967).

At ten days after sowing (DAS), thinning was conducted, leaving only four plants per pot. For the production of biomass marked with ^{15}N, 30 pots with green manure were used. ^{15}N green manure was labeled according to Araújo et al. (2011).

For the enrichment with ^{15}N, 360 mg N per pot were applied using a solution of urea with 3% excess ^{15}N atoms, divided into five doses throughout the vegetative cycle of the jack bean crop.

Fertilization per pot was performed with Mg and K, using 3.8 g magnesium sulfate, divided into two doses applied at 21 and 47 DAS, and 1.15 g potassium sulfate at 21 DAS, respectively.

At 58 DAS, the jack bean plants produced in the greenhouse were collected and weighed to obtain total fresh weight. To determine humidity, subsamples were taken from the aerial part of the plant and dried in an oven at 65ºC with forced-air circulation until reaching a constant weight. The subsamples were then ground in a Wiley mill using a 2-mm sieve. The subsamples were subsequently taken to the laboratory to determine N content using the Kjeldahl method (Alves et al., 1994).

In order to determine excess ^{15}N, subsamples were powdered in a ball mill, and aliquots containing approximately 40 μg N were weighed for ^{15}N analysis in an automated C and N analyzer coupled to a mass spectrometer (Delta V Advantage, Thermo Fisher Scientific, Bremen, Germany).

In the second phase of the experiment, the corn hybrid AG1051 was planted in an area of 350 m^2 (10x35 m), spaced at 1 m between rows, with five plants per linear meter. According to the Brazilian soil classification system (Santos et al., 2018), the soil of the experimental site is classified as a Planossolo Háplico distrófico, i.e., an Albaqualf, with a low natural fertility at a depth of 0–20 cm and the following chemical characteristics: pH (H_2O) 5.42, 4.12 mg dm^{-3} P, 58.00 mg dm^{-3} K^+, 1.19 $cmol_c$ dm^{-3} Ca^{2+}, 0.69 $cmol_c$ dm^{-3} Mg^{2+}, 0.05 $cmol_c$ dm^{-3} Al^{3+}, base saturation of 47.19%, and 7.4 g kg^{-1} organic matter.

The fertilization of the corn crop consisted of the application of 80 kg ha^{-1} P_2O_5 as thermophosphate, 40 kg ha^{-1} K_2O as potassium sulfate, and 2,000 kg ha^{-1} dolomitic limestone. The untreated green manure biomass, which is necessary to evaluate N recovery efficiency using the indirect method, was produced under field conditions in a soil with the same chemical and physical characteristics of the one of the experimental area cultivated with corn, but without soil fertility correction.

When corn reached the V3 phenological stage, three different isotope treatment methods were applied: indirect method I, isotope dilution with ^{15}N-labelled synthetic fertilizer applied in a small hole next to each corn plant; indirect method II, isotope dilution with ^{15}N-labelled synthetic fertilizer spread in the furrow next to the corn planting row; and direct method, application of a legume as green manure, treated with 2.23% excess ^{15}N.

For the direct treatment method, chopped green manure biomass marked with 2.32% ^{15}N was distributed on the ground next to the corn row. The amount of biomass was 2.5 kg m^{-1} fresh matter, which corresponds to 7.42 Mg ha^{-1} dry biomass of the aerial part of the jack bean plant and 127.66 kg ha^{-1} N.

In the indirect treatment methods, two subplots per experimental unit were used. Both subplots received a small dose of urea with 10% atom ^{15}N in excess

(15 kg ha^{-1} N) to enrich the available soil N, but only one received the unlabeled green manure; the subplot without green manure was used for the calculations of N recovery efficiency. The green manure used was jack bean biomass at a rate of 2.5 kg fresh matter per linear meter, which corresponds to a dose of 6.39 Mg ha^{-1} dry matter and 186.19 kg ha^{-1} N.

The corn plants were harvested when the cobs showed dry stigmas and the tips of the bracts were flexible to the touch, coinciding with the dough stage of the grains. The green cobs were collected and weighed to determine productivity. Sampling consisted of the two central plants in each subplot in order to include the plants that received the labelled ^{15}N. The aerial part of the plant was placed in an oven at 65ºC with forced-air circulation until reaching a constant weight. Both N and ^{15}N were analyzed according to Ramos et al. (2001).

For the indirect methods, the recovery efficiency of N from green manure was estimated according to Urquiaga & Zapata (2000). The amount of N derived from green manure was calculated using the following equation: $NdGM = ((^{15}NsGM - ^{15}NcGM)/^{15}NsGM) \times 100$, where NdGM is the percentage of N derived from green manure in corn; ^{15}Ns is the percentage of ^{15}N in the corn plant without green manure; and $^{15}NcGM$ is the percentage of ^{15}N in the corn plant with green manure.

The recovery efficiency of N from green manure by the corn crop (NrecEff) was calculated using the equation: $NrecEff\ (\%) = (NdGM/NGM) \times 100$, where NrecEff (%) is the percentage of the recovery efficiency of N from green manure by corn, and NGM is the quantity of N applied to the corn crop in the form of green manure biomass (kg ha^{-1}).

The data were subjected to the analysis of variance, significance was assessed, and the averages were compared using Tukey's test through the Sisvar, version 5.1, statistical package (Ferreira, 2011).

Results and Discussion

The average production of the corn crop was of 10 Mg ha^{-1}, with no difference between treatments (Table 1), since all of them received green manure: biomass marked with ^{15}N in the direct method, and untreated biomass in both indirect methods. This productivity is an indicative that the corn crop was able to recover N derived from green manure. In addition, the obtained result is consistent with that expected for a hybrid cultivar under appropriate management conditions, considering that the productivity of green corn in Brazil varies from 9 to 15 Mg ha^{-1} depending on the region (Paiva et al., 2012), and the state of Rio de Janeiro has a low average corn productivity.

The treatments also did not differ regarding the amount of dry biomass of the aerial part of the corn plants (stalks, leaves, and cob) nor regarding the total amount of N accumulated in the dry biomass. These results are indicative that all treatments enabled the corn crop to develop adequately.

The labelling of corn plants was not influenced by the method used for the application of urea-^{15}N to the soil, both in the presence and absence of green manure. Therefore, the percentages of N from green manure in the corn plant were the same for both indirect methods. This result is an indicative that the application of the ^{15}N-labelled fertilizer in a small hole (indirect method I) causes the same effects as the application of fertilizer in the furrows between crop rows (indirect method II). This way, in future studies, urea-^{15}N can be applied as most convenient, since both spreading it in the furrow or applying it beside each plant individually results in the same marking of the test crop.

Corn plants that received the ^{15}N-treated green manure biomass showed significantly lower levels of isotope marking than those that were treated with

Table 1. Green corn (*Zea mays*) productivity, dry biomass (DB), and nitrogen accumulated in the aerial part of the plant[(1)].

Method[(2)]	Green corn	DB of the aerial part		N in DB
	Corncob	Stalk and leaves	Corncob	Aerial part + corncob
	-------------------(Mg ha^{-1})-------------------			(kg ha^{-1})
Indirect I	12.38a	5.43a	4.46a	80.10a
Indirect II	8.82a	5.19a	3.18a	84.50a
Direct	8.83a	5.42a	3.63a	87.61a
CV (%)	24.28	20.07	26.71	30.44

[(1)]Averages followed by equal letters, in the columns, do not differ by Tukey's test, at 5% probability. [(2)]Indirect I, ^{15}N isotope dilution method with the application of ^{15}N-labelled synthetic fertilizer in a small hole next to each corn plant; Indirect II, ^{15}N isotope dilution method with the spreading of ^{15}N-labelled synthetic fertilizer in the furrow next to the corn planting row; and Direct, direct method with ^{15}N-treated jack bean used as green manure, with 2.32% excess of ^{15}N atoms.

^{15}N-labelled urea (Table 2). The percentage of N from green manure in corn was about 10%, which increased to 32.4 and 45.9% for indirect methods I and II, respectively, in the plants that received urea-^{15}N, probably because synthetic N was more readily available to the corn crop than the N derived from green manure. However, it is important to remember that part of the N derived from green manure can remain in the soil as residual N and can contribute nutrients for subsequent cultivation cycles.

The recovery efficiency of N from green manure was significantly higher in the indirect methods (Table 3), compared with the direct one: 17.39 vs. 7.0%. This shows that the indirect method caused an overestimation of the recovery efficiency of N from green manure. Based on these results, the use of indirect methods can result in misleading information, not being recommended for scientific experiments until calibration curves are developed.

The indirect method has been used to evaluate the biological N fixation rate in legumes and the transfer of N to crops (Sarr et al., 2016). However, the isotope dilution technique should be used with caution as it overestimates the N transfer rate from green manure to the commercial crop.

In the direct method, the recovery efficiency of N derived from green manure treated with excess ^{15}N was lower than that of 9 and 16%, respectively, obtained by Araújo et al. (2011) when using velvet bean (*Mucuna cinereum* Piper & Tracy) and jack bean as green manure for a cabbage (*Brassica oleracea* var. *capitata*) crop. Ambrosano et al. (2011) found that the recovery efficiency of N from sunn hemp (*Crotalaria juncea* L.) by sugarcane varied from 8.8 to 9.98%, based on a single harvest, and from 19 to 21% based on two consecutive sugarcane harvests, which shows that, despite the low recovery levels of N from green manure in one harvest cycle, there is a significant residual effect in subsequent harvests.

According to Acosta et al. (2011), N transfer from vetch (*Vicia villosa* Roth) to a corn crop was about 12% of the total applied. It is important to note that the management of green manure influences N recovery efficiency by crops. Ambrosano et al. (2009) showed that the incorporation of the legume velvet bean to the soil provided greater N accumulation in the soil, recovery efficiency by corn plants, and accumulation in the aerial part of the plants when compared with the straw cover management.

In general, the recovery efficiency of N from green manure is low, rarely exceeding 20% in the first crop after application (Scivittaro et al., 2003; Ambrosano et al., 2005; Araújo et al., 2011), as also observed in the present study.

Table 2. Marking of corn (*Zea mays*) plants in the subplots without and with jack bean (*Canavalia ensiformis*) used as green manure (GM), as well as percentage of nitrogen derived from jack bean (NdGM) in corn(1).

Method(2)	Marking of corn plants (% atoms, excess ^{15}N)		NdGM (%)
	Without GM	With GM	
Indirect I	0.76a	0.50a	32.40a
Indirect II	0.75a	0.40a	45.94a
Direct	-	0.23b	10.20b
CV (%)	21.43	18.27	33.46

(1)Averages followed by equal letters, in the columns, do not differ by Tukey's test, at 5% probability. (2)Indirect I, ^{15}N isotope dilution method with the application of ^{15}N-labelled synthetic fertilizer in a small hole next to each corn plant; Indirect II, ^{15}N isotope dilution method with the spreading of ^{15}N-labelled synthetic fertilizer in the furrow next to the corn planting row; and Direct, direct method with ^{15}N-treated jack bean used as green manure, with 2.32% excess of ^{15}N atoms. - , no result since this subplot was not included in the direct method.

Table 3. Applied amount of nitrogen derived from jack bean (*Canavalia ensiformis*) used as green manure, total N in corn (*Zea mays*) stalks and leaves (aerial part), total N in corncobs, amount of N derived from jack bean (NdGM) in the corn crop, and recovery efficiency of N from jack bean (NrecEff)(1).

Method(2)	Applied N	Aerial N	Corncob N	NdGM	NrecEff
	----------------(kg ha^{-1})-----------------			--------(%)--------	
Indirect I	186.19	48.53a	31.58a	25.96a	13.93a
Indirect II	186.19	50.34a	34.16a	38.83a	20.84a
Direct	127.66	49.02a	38.59a	8.94a	7.00b
CV (%)	-	47.23	44.63	46.37	43.94

(1)Averages followed by equal letters, in the columns, do not differ by Tukey's test, at 5% probability. (2)Indirect I, ^{15}N isotope dilution method with the application of ^{15}N-labelled synthetic fertilizer in a small hole next to each corn plant; Indirect II, ^{15}N isotope dilution method with the spreading of ^{15}N-labelled synthetic fertilizer in the furrow next to the corn planting row; and Direct, direct method with ^{15}N-treated jack bean used as green manure, with 2.32% excess of ^{15}N atoms.

Conclusions

1. The method of applying synthetic ^{15}N to the soil – in a small hole next to the corn (*Zea mays*) plant or spread in the furrow between planting rows – does not interfere in the indirect estimation of the recovery efficiency of nitrogen derived from jack bean (*Canavalia ensiformis*) green manure.
2. The indirect method results in an overestimation of the recovery efficiency of N from green manure.
3. The recovery efficiency of N from green manure by the corn crop is 7% of the applied N.
4. The direct method is the most adequate to determine the recovery efficiency of N derived from green manure.

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