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RESEARCH ARTICLE

BIOLOGICAL NITROGEN FIXATION BY ^{15}N ISOTOPE TECHNIQUES IN THESENEGALESE COWPEA: METHOD OF A VALUE

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ABSTRACT

Niébé or *cowpea* [*Vignaunguiculata*(L.)Walp.] is a grain legume which occupies an important place in african agriculture. The ripe seeds contain high levels of protein, starch, B vitamins (folic acid), and are also rich in essential trace elements (iron, calcium and zinc). Protein-rich tops are also a preferred feed the livestock. In combination or in rotation with other crops, cowpea may yield 60-70 kg / ha of fixed nitrogen for the next crop (Rachie, 1985). The objective of our study is to estimate the biological fixation of the nitrogen of various varieties of cowpea from the national germplasm and to estimate their genetic variability to target interesting varieties. Isotope techniques used for the assessment of biological nitrogen fixation of 16 cowpea varieties have allowed us to classify the different varieties according to their potential for nitrogen fixation (NdFix) and their ability to take nitrogen from the soil. The results obtained allowed to target cowpea varieties that can be introduced into soil fertility improvement programs and increasing crop yields.

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INTRODUCTION

Cowpea is native to Africa, where he was domesticated from the Neolithic (Rawal, 1976; Vanderborght and Baldwin, 2001). It is a plant capable of establishing atmospheric nitrogen - fixing symbiosis with bacterial strains Bradyrhizobium (Sun and Simbi, 1983). The symbiosis is manifested by the formation of a body called lump or nodule on the roots. The nitrogen fixation is the conversion of atmospheric nitrogen N_2 combined to nitrogen usable by plants and animals. This is especially cyanobacteria and some bacteria living in symbiosis with legumes such as cowpeas.It is done by certain bacteria such as rhizobia that live in soil and successfully assimilate diatomic nitrogen N_2. Several research studies have been carried out on the molecular biology of cowpea (Badiane 2011; Ndiaye and al.; 2004) The method of the A value is a variant of the method of isotope dilution (Chalk 1996). It involves applying different doses of labeled nitrogen fertilizer for nitrogen fixing plants (NFP) and for the reference plants (PREF). The concept of the A value (Chalk, 1996; Fried and Dean , 1952) is based on the following hypothesis: when a plant is in the presence of two or more sources of a nutrient , it will absorb this element in proportion to the quantity available in each source called A value.

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MATERIALS AND METHODS

Plant material

The plant material used in our study described in Table I includes 16 varieties of cowpea as nitrogen fixing plant. A variety of souna millet (*Pennisetumgambiense*Stapf and Hubb) provided by the National Agricultural Research Center (CNRA) in Bambey in Senegal and a variety m129 of non nodulating soybean (*Glycine max* L.) provided by the International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeriaare used as no-fixing reference plants.

Table I. Plant material used for the estimate of the amount of nitrogen fixed by cowpea

Vegetables Species	Varieties
Cowpea (*Vignaunguiculata*)	Bambey 21, BayeNgagne, CB 5, Diongoma, Melakh, Mougne, Mouride, Ndiaga Aw, Ndiambour, Ndoute, Variety black (X), Variety white (Y), 58-57, 58-74, 59-9 et 66-35
Soybean (*Glycine max*)	m 129
Millet (*Pennisetum gambiense*)	Souna 3

Methods

Plant grown in soil amended with fertilizer, the plant will absorb the nutrient in question from two sources: the pool of the soil and the amount known in the fertilizer. In this case, it must only determine the respective removals from each source to determine soil A value for this nutrient. A value is independent of the dose of fertilizer made and can be used to estimate the amount of a nutrient soil available for the plant. It is expressed as fertilizer equivalent (IAEA, 1990) and is calculated as follow:

nutrient in the plant from the fertilizer / nutrient element in the fertilizer = nutrient in the plant from the soil / nutrient element in soil (A Value of soil)

Is :

A value of soil = (nutrient in the plant from the soil / nutrient in the plant from the fertilizer) x nutritive element in the fertilizer

If nitrogen (N) is the nutrient:

$$AN = \frac{NdfSol}{NdfFertilizer} \times \text{application rate of fertilizer}$$

For value method A, the basic hypothesis is that the reference plant must absorb the nitrogen from the soil and fertilizer in the same ratio as the fixing plant. The non- fixing plant must have a similarity with the fixing plant studied on:

- The length of the growing season;
- The rate of absorption of the current round of nitrogen;
- Rooting depth.

The basic equation is as follows:

$$\frac{\%NdfFix}{A\ val\ (soil + Fix)} = \frac{\%\ NdfS}{A\ val\ soil} = \frac{\%\ NdfFertilizer}{NfFertilizer} = \frac{\%\ N\ total\ plant}{N\ total\ available}$$

Estimated amount of nitrogen fixed by Cowpea

The experiment was conducted at the IRD-ISRA research center based in Bel Air Dakar with 16 varieties of cowpea leguminous and two no-fixing reference plants. The soil containing approximately 103 g^{-1} bradyrhizobia native (Brockwell, 1982) was taken at Bambey (120 kilometers east of Dakar). This soil was sieved (1 mm) and homogenized. It consists of 3.9 g C kg^{-1}, 0.25 g N $kg^{-1}$and 84 % sand. Soil pH is 7.36 (Soil Survey Staff, 1987). Amount of 30 kg was weighed and placed in cylindrical tubes PVC (30 cm in diameter and 50 cm high) that are buried in the earth and the edge of which emerges at about 5 cm from the ground. Cowpea seeds were sanitized by dipping into $NaOCl_3$ for 3 min and then swollen in tap water for 30 min before being sown in the cylindrical tubes at the rate of one seed per tube. All cowpea seeds were treated with a liquid inoculum of *Bradyrhizobium* strain ISRA 313 from the culture collection of ISRA / MIRCEN. Inoculation was performed at the rate of 1 ml per plant with a concentration of 10^8 cells per ml. There were a total of 18 plants including non nodulating soybean (*Glycine max*), millet Souna 3 (*Pennisetum gambiense*) and 16 varieties of cowpea. ^{15}N ammonium sulphate fertilizer containing 10 atom % ^{15}N excess was applied to all plants. The completely randomized experimental design includes two treatment and 7 repetitions was set up. For the first treatment: the fixing plants receive 20 kg of ammonium sulfate nitrogen per hectare with 5% isotopicexcess and the second treatment: non-fixing plants receive much nitrogen fertilizer with ammonium sulfate 100 kg of nitrogen per hectare with 1% isotopic excess.

Application of nitrogen fertilizer marked

It is necessary to describe the method of calculation used for the preparation of the labeled nitrogen solution. (Nitrogen fertilizer used is in the form of ammonium sulfate $(^{15}NH_4)_2SO_4$ having an isotopic excess nitrogen-15 (^{15}N %) of 10 %.) With this method, the nitrogen rate of 20 kg N/ha was applied to fixing plants and the rate of 100 kg N / ha for non -fixing plants. For a theoretical field density of 160,000 plants per hectare, the amount of nitrogen required by plants has been 0.125 g N (for nitrogen fixing plants) and 0.625 g N (for reference plants).

For fixing plants

Considering the content of nitrogen in ammonium sulfate (21.2%), the need for ammonium sulfate in 112 (16 X 7) plants is 66.04 g. In anticipation of a spray of 100 ml per plant, or 11200 ml total, this amount was reduced to 67.22 g (66.04 x 11400/11200) and dissolved in 11400 ml of water. At the end of the experiment, the remainder (200 ml) was sent to the IAEA in conjunction with the plant samples to determine their isotopic excess. In the equation of isotope dilution that we recall below, the sum of (m1 + m2) represents the quantity of labeled sulfate (m1) and normal sulfate m2) is equal to 67.22 g of sulfate. The equation of isotope dilution is given by the formula of Fried and Middelboe (1977):

$$m1 = \frac{(m1 + m2) \times M1a'}{a'1\ M2 + (M1 - M2)\ a'}$$

This formula calculates the amount of each of these compounds.

M1 = 132.33 g per mol. of ammonium sulfate to 10% marked
M2 = 132.13 g per mol. of normal ammonium sulfate
a '= 5% isotopic excess of the final dilution of ammonium sulfate
a'1 = 10% isotopic excess ammonium sulfate to dilute
m1 = 33.63 g of ammonium sulfate to 10% marked
m2 = 33.59 grams of ammonium sulfate standard

For reference plants

The variety of non-nodulated m29 soybean and millet variety Souna 3 were chosen as reference plants, with seven replicates per variety, which was 7 x 2 = 14 plants. Seedlings were made to one plant per nozzle, with an application rate of fertilizer of 100 kg N / ha. For the theoretical seeding rate of 160,000 plants per hectare, the amount of nitrogen required by plants was 0.625 g N, totaling 8.75 g N for 14 plants corresponding to 41.27 g sulfate ammonium. Here too, in anticipation of a spray of 100 ml per plant, or 1400 ml in total, this amount was reduced to 44.21 g (41.27 x 1500/1400) and dissolved in 1500 ml of water. Thus, the sum (m1 + m2) is equal to 44.21 g of sulfate and a '= 1%. With the isotopic dilution formula we obtained:

m1 = 4,42g of ammonium sulfate marked 10%
m2 = 39.79 grams of ammonium sulfate standard

After 50 days of culture in the nozzles plants are sacrificed, the aerial parts are isolated and then dried in an oven at a temperature of 70 ° C for 3 days. After grinding and weighing 50 mg of each plant were sent to the IAEA laboratory in Seibersdorf for determining nitrogen content (% N) and their isotopic excess (% ^{15}Nei) at the same time as the remaining the solution [15]

N applied to plants.

$$\frac{\%NdfF}{NF1} = \frac{100 - \%NdfF}{A\ (soil + Fix)}$$

$$1\text{-}A\ (soil + Fix) = \frac{100 - \%NdfF}{\%NdfF} \times NF1$$

The control plant is used to calculate:

$$\frac{\%NdfF}{A\ soil} = \frac{100 - \%NdfF}{NF2}$$

$$2 - Asoil = \frac{100 - \%NdfF}{NdfF\ \%} \times NF2$$

(NF1 and NF2 are nitrogen rates applied)

3 - From 1 and 2 we can write that: AFix = A (soil + Fix) – A soil

$$\frac{\%NdfF}{NF1} = \frac{NdfFix}{A\ Fix} = \frac{\%NdfS}{A\ soil}$$

$$4\text{-}\ \%Ndffix = \frac{\%NdfF \times A\ Fix}{NF1} \quad \text{or} \quad \%Ndffix = \frac{\%NdfS \times A\ fix}{A\ soil}$$

RESULTS AND DISCUSSION

Quantity of nitrogen fixed by Cowpea

To calculate the N fixed by the different cowpea varieties only one of the no-fixing plants is used as reference. So the non nodulating soybean m129 which exhibits the highest atom %^{15}N excess than the millet was selected. Soil A value of 1.181 g N sulfate per PVC tube is the average of the A value of tubes receiving 20 kg N (0,125N g/tube) and the A values of tube receiving 100 kg N (0.625 g N/tube). For all cowpea varieties, nodulation and N fixation occur when plants are inoculated with bradyrhizibium strain. Pule-Meulenberg et al. (2010) reported that cowpea growth depends on N_2-fixation. Our results confirme that, in area where soil fertility are continunously declining, the nutrient requirement of crops can be afford by leguminous crop such as cowpea through the process of biological nitrogen fixation. However, a selection of plant varieties with high nitrogen fixing potential would comfort this situation. Table II show that all cowpea varieties fixed nitrogen from atmosphere. Most varieties fixe a significant amount of nitrogen with a highest N fix by the Ndoute variety. In consequence, this variety shows the lowest percentage of NdfS (20.22%). In addition, the percentage of the N-fertilizer (% of NdfF) in Ndoute variety is among the lowest 2.14%. On the other hand, four varieties CB5, Melakh, Bambey 21 and VLY present the lowest N fixed. Bambey 21 nitrogen nutrition is mainly provided by soil and fertilizer. According to previous studies (Ndiaye 2015, Ndiaye et al., 2000), Ndoute should be used as the highest potential fixer variety. However, for the N2 fixation improvement program, it is essential to examine the high potential N2 fixation varieties. In addition, it is necessary to consider those with low capacity to take nitrogen from the soil. This capacity is reflected by their percentage of N derived from the soil (% NdfS).

Table II. Percentage of N derived from fertilizer ((% the NdfF), the A value (A soil + Fix) and (AFix), percentage (% NdfFix) and amount (NdfFix) of N derived from fixation and percentage of N derived from soil (% NdfS) in plant shoots of the different varieties

Varieties	% NdfF	A(soil+Fix)gN/pl	A FixgN/pl	% NdfFix	NdfFix mg/pl	% NdfS
58-74	3.604 bcd	3.377 bcd	2.196 bcd	62.345 bcd	589.121 abcde	34.051 bc
Ndiaga Aw	2.603 cde	5.238 ab	4.057 ab	72.806 abc	843.471 ab	24.591 cde
Diongama	2.903 cde	4.391 abcd	3.210 abcd	69.14abc	738.573 abcd	27.958 cde
59-9	2.326 de	5.298 ab	4.117 ab	75.698 ab	815.329abcd	21.976 de
CB5	3.773 bc	3.185 bcd	2.004 bcd	60.579 cd	484.061 bcde	36.019 bc
Baye Ngagne	2.821 cde	4.377 abcd	3.196 abcd	70.411 abc	769.336 abcd	26.757cde
VLX	3.058 cde	4.160 abcd	2.988 abcd	68.050 abc	841.598 abc	28.892 cde
Melakh	3.593 bcd	3.382 bcd	2.201 bcd	62.456 bcd	458.887 cde	35.166 bc
Bambey 21	5.32 a	2.199 d	1.018 d	41.909 e	268.296 de	52.531a
66-35	3.382 bcd	3.922 abcd	2.741 abcd	64.665 abcd	598.434 abcde	31.953 bcd
VLY	4.468 b	2.725 cd	1.544 cd	53.316 d	445.183 cde	42.215 b
58-57	2.704 cde	4.705 abc	3.524 abc	71.749 abc	925.777 ab	25.547 cde
Mougne	2.822 cde	4.433 abcd	3.252 abcd	70.944 abc	677.433 abcd	26.234 cde
Ndiambour	2.680 cde	4.651 abc	3.470 abc	71.999 abc	841.272 abc	23.620 cde
Mouride	3.012 cde	4.051 abcd	2.871 abcd	68.531 abc	802.105 abcd	28.457 cde
Ndoute	2.140 e	6.164 a	4.983 a	77.641 a	995.758 a	20.219 e
Average	3.193	4.141	2.961	66.390	693.415	27.135
Stand. Deviat.	0.817	1.011	0.979	8.679	201.255	8.023
Coef. Var.%	25.60	24.40	31	13.10	29	29.60

Values for each soil type followed by the same letter are not significantly different at $P < 0.05$.

Conclusion

In the context of low soil fertility and the low utilization of nitrogen fertilizer in Senegal according to high cost and low availability, breeders and agronomists should use cowpea varieties with a high potential of nitrogen fixation and low percentage of N derive from soil. According to its low NdfS (20.2%) and high N drive from atmosphere, Ndoute variety is advised in soil with low rate of fertility.

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