RESEARCH ARTICLE

BIOLOGICAL NITROGEN FIXATION BY 15N ISOTOPE TECHNIQUES IN THE SENEGALESE COWPEA: METHOD OF A VALUE

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ABSTRACT

Niébé or cowpea [Vigna unguiculata (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 for 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.

INTRODUCTION

Cowpea is native to Africa, where it 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 N2 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 N2. 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.

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 sona millet (Pennisetum glaucum) provided by the National Agricultural Research Center (CNRA) in Bamby in Senegal and a variety m129 of non-nodulating soybean (Glycine max L.) provided by the International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria are used as non-fixing reference plants.

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

<table>
<thead>
<tr>
<th>Vegetables Species</th>
<th>Varieties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cowpea (Vigna unguiculata)</td>
<td>Bamby 21, BayeNgagne, CB 5, Diongoma, Melakh, Mougne, Mouride, Ndiaga Aw, Ndambour, Ndoute, Variety black (X), Variety white (Y), 58-57, 58-74, 59-9 et 66-35</td>
</tr>
<tr>
<td>Soybean (Glycine max)</td>
<td>m 129</td>
</tr>
<tr>
<td>Millet (Pennisetum glaucum)</td>
<td>Sona 3</td>
</tr>
</tbody>
</table>

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:

\[ \text{AN} = \frac{\text{A value soil}}{\text{A value fertilizer}} \times \text{application rate of fertilizer} \]

\[ \text{NdfFertilizer} \]

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:

\[ \frac{\%\text{NdfFix}}{\%\text{NdfS}} = \frac{\%\text{NdfFertilizer}}{\%\text{N total plant}} \]

\[ \frac{\%\text{N} \text{ total plant}}{\%\text{N} \text{ available}} \]

Estimated amount of nitrogen fixed by Cowpea

The experiment was conducted at the IRD-ISRA research center in Bel Air, Dakar with 16 varieties of cowpea leguminous and two non-fixing reference plants. The soil containing approximately 103 g m⁻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 NaOCl₃ 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 10⁹ cells per ml. There were a total of 18 plants including non-nodulating soybean (Glycine max), millet Souna 3 (Pennisetum gambiae) and 16 varieties of cowpea, ¹⁵N ammonium sulphate fertilizer containing 10 atom % ¹⁵N excess was applied to all plants. The completely randomized experimental design includes two treatments and 7 repetitions was set up. For the first treatment: the fixing plants receive 20 kg of ammonium sulphate nitrogen per hectare with 5% isotopic excess 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 (¹⁵NH₄)₂SO₄ having an isotopic excess nitrogen-15 (¹⁵N %) 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):

\[ \text{m1} = \frac{(\text{m1} + \text{m2}) \times \text{M1}a'}{ \text{a'(M2 - M1)} + \text{M2} } \]

This formula calculates the amount of each of these compounds.

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

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:

\[
m1 = 4.42 \text{g of ammonium sulfate marked} 10\% \\
m2 = 39.79 \text{grams of ammonium sulfate standard}
\]

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 (% 15N) at the same time as the remaining the solution 

\[
% N_{dfF} = \frac{m1 - m2}{m1} \times 100
\]

\[
% N_{dfS} = \frac{A_{(soil + Fix)}}{A_{(soil + Fix)}}
\]

\[
% N_{dfF} x A_{Fix} = \frac{A_{soil + Fix}}{A_{soil}}
\]

\[
% N_{dfS} x A_{soil} = \frac{A_{soil}}{A_{soil}}
\]

### 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 %15N 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

\[
% N_{dfF} = \frac{m1 - m2}{m1} \times 100
\]

\[
% N_{dfS} = \frac{A_{(soil + Fix)}}{A_{(soil + Fix)}}
\]

\[
% N_{dfF} x A_{Fix} = \frac{A_{soil + Fix}}{A_{soil}}
\]

\[
% N_{dfS} x A_{soil} = \frac{A_{soil}}{A_{soil}}
\]

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

<table>
<thead>
<tr>
<th>Varieties</th>
<th>NdfF</th>
<th>A (soil + Fix)</th>
<th>A Fix</th>
<th>Ndfx</th>
<th>NdfS</th>
</tr>
</thead>
<tbody>
<tr>
<td>58-74</td>
<td>3.604 bcd</td>
<td>3.377 bcd</td>
<td>2.196 bcd</td>
<td>62.345 bcd</td>
<td>589.121 abde</td>
</tr>
<tr>
<td>Ndja Aw</td>
<td>2.603 cde</td>
<td>5.238 ab</td>
<td>4.057 ab</td>
<td>72.806 abc</td>
<td>843.471 ab</td>
</tr>
<tr>
<td>Dongama</td>
<td>2.903 cde</td>
<td>1.931 abcd</td>
<td>3.210 abcd</td>
<td>69.14c bcd</td>
<td>738.573 abcd</td>
</tr>
<tr>
<td>59-9</td>
<td>3.236 de</td>
<td>5.298 ab</td>
<td>4.117 ab</td>
<td>75.698 ab</td>
<td>815.329abecd</td>
</tr>
<tr>
<td>CB5</td>
<td>3.773 bc</td>
<td>3.165 bcd</td>
<td>2.804 bcd</td>
<td>60.579 cld</td>
<td>484.061 bcde</td>
</tr>
<tr>
<td>Baye Ngagne</td>
<td>2.821 cde</td>
<td>6.777 abcd</td>
<td>3.246 abcd</td>
<td>70.411 abc</td>
<td>769.336 abde</td>
</tr>
<tr>
<td>VLX</td>
<td>3.056 cde</td>
<td>1.160 ab</td>
<td>2.488 ab</td>
<td>68.050 abc</td>
<td>841.598 abc</td>
</tr>
<tr>
<td>Melahk</td>
<td>3.593 bcd</td>
<td>3.382 bcd</td>
<td>2.201 bcd</td>
<td>62.456 bcd</td>
<td>458.887 cde</td>
</tr>
<tr>
<td>Bambey 21</td>
<td>5.32 a</td>
<td>2.199 d</td>
<td>1.018 d</td>
<td>41.909 e</td>
<td>268.296 de</td>
</tr>
<tr>
<td>66-35</td>
<td>3.382 bcd</td>
<td>3.922 abcd</td>
<td>2.741 bcd</td>
<td>64.665 abcd</td>
<td>598.434 abde</td>
</tr>
<tr>
<td>VLY</td>
<td>4.468 b</td>
<td>2.725 cd</td>
<td>1.544 cd</td>
<td>53.316 d</td>
<td>445.183 cde</td>
</tr>
<tr>
<td>58-57</td>
<td>2.704 cde</td>
<td>4.705 abc</td>
<td>3.524 abc</td>
<td>71.749 abc</td>
<td>925.777 ab</td>
</tr>
<tr>
<td>Mougue</td>
<td>2.822 cde</td>
<td>4.433 abcd</td>
<td>3.252 abcd</td>
<td>70.944 abc</td>
<td>677.433 abcd</td>
</tr>
<tr>
<td>Ndiambour</td>
<td>2.680 cde</td>
<td>4.651 abc</td>
<td>3.470 abc</td>
<td>71.999 abc</td>
<td>841.272 abc</td>
</tr>
<tr>
<td>Mourioudy</td>
<td>3.012 cde</td>
<td>4.051 abcd</td>
<td>3.271 abcd</td>
<td>68.531 abc</td>
<td>802.105 abcd</td>
</tr>
<tr>
<td>Ndotue</td>
<td>2.140 e</td>
<td>6.164 a</td>
<td>4.983 a</td>
<td>77.641 a</td>
<td>995.758 a</td>
</tr>
<tr>
<td>Average</td>
<td>3.193</td>
<td>4.141</td>
<td>2.961</td>
<td>66.390</td>
<td>693.415</td>
</tr>
<tr>
<td>Stand. Deviat.</td>
<td>0.817</td>
<td>1.011</td>
<td>0.979</td>
<td>8.679</td>
<td>201.255</td>
</tr>
<tr>
<td>Coef. Var.%</td>
<td>25.60</td>
<td>24.40</td>
<td>31</td>
<td>13.10</td>
<td>29</td>
</tr>
</tbody>
</table>

Table shows that all cowpea varieties fixed nitrogen from atmosphere. Most varieties fixe a significant amount of nitrogen with a highest N fix by the Ndoutef variety. In consequence, this variety shows the lowest percentage of NdfS (20.22%). In addition, the percentage of the N-fertilizer (% of NdfF) in Ndoutef variety is among the lowest 2.14%. On the other hand, four varieties CB5, Melahk, Bambey 21 and VLY present the lowest N fixed. Bambey 21 nitrogen nutrition is mainly provided by soil and fertilizer. According to previous studies (Ndaiyee 2015, Ndaiyee et al., 2000), Ndoutef 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).

### 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.

REFERENCES


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