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Biological nitrogen fixation in *Crotalaria* species estimated using the ^{15}N isotope dilution method

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A greenhouse experiment was conducted to measure nitrogen fixation in three *Crotalaria* species : *C. ochroleuca*, *C. perrottetii* and *C. retusa* growing in Senegal by using ^{15}N direct isotope dilution technique. Two non-fixing plants, *Senna obtusifolia* and *Senna occidentalis* served as reference plants. The amount of nitrogen fixed two months after planting was obtained using the average of the two reference plants. The atom % ^{15}N excess in the *Crotalaria* species was significantly lower than that of the reference plants, indicating that significant nitrogen fixation occurred in the three plants. Significant differences were observed between the *Crotalaria* species; *C. ochroleuca* yielded more dry matter weight and total nitrogen than did *C. perrottetti* and *C. retusa*. The % nitrogen derived from atmosphere (%Ndfa) in leaves and stems was also higher in *C. ochroleuca*. There was no significant difference in %Ndfa in the whole plant between the three *Crotalaria* species (47% to 53%). In contrast, interspecific variability was observed based on the %Ndfa. *C. ochroleuca* significantly exhibited the higher amount of total nitrogen fixed, equivalent to 83 kg of nitrogen fixed per hectare. Based on these data, it was concluded that *C. ochroleuca* could be used in multiple cropping systems in Senegal for making more nitrogen available to other plants.

Key words: *Crotalaria* spp, isotope dilution, ^{15}N, nitrogen fixation, reference plant.

INTRODUCTION

In the tropics, soil erosion and depletion are becoming a problem of global proportions and few farming systems are totally immune to it. Legumes, nitrogen fixing trees with high fixing potential, can be used in agricultural systems for replenishing nitrogen, the most limiting growth factor in the soil. Based on the current knowledge (Giller and Wilson, 1991), the challenge is to choose the appropriate legume in a given niche. In tropical areas, spontaneous legumes play an important role in the maintenance and improvement of soil fertility, but they remain largely unexploited. Among them, the legumes belonging to the genus *Crotalaria* are active in fixing nitrogen through the nodules they form on their roots in association with rhizobia. *Crotalaria* is widespread in tropical regions and includes about 550 species in Africa and Madagascar (Polhill, 1982), of which about 33 species are found in Senegal. *Crotalaria* plants have a high dry matter production potential and are able to grow on poor soil with low nitrogen content (Daimon et al., 1995). They have also been reported as good intensive fallowing cover crops to regenerate the soil (Müller-Sämann and Kotschi, 1994). These annual or perennial plants can be used as green manure or in intercropping farming systems. Although several *Crotalaria* nodulated species have been found (Allen and Allen, 1981), no

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information is available on how much nitrogen can be fixed by these plants. The present experiment was designed to estimate the nitrogen fixing potential of three *Crotalaria* species native to Senegal using the ^{15}N isotope dilution method in view to use an elite one in Senegal cropping systems.

MATERIALS AND METHODS

A greenhouse experiment was carried out at the IRD Bel-Air experimental station at Dakar, using a local sterilized sandy soil (Psamment; vernacular name: Dior), with 93% of sand. This soil contains approximately 10^2 native *Bradyrhizobium* per gram counted by infection test method (Brockwell, 1982; Vincent, 1970) using *Crotalaria* seedlings. The soil was pH 7.0 with 1.9% total carbon and 0.025% total nitrogen (Bremner, 1965). The soil was sieved (<1 mm), homogenized and 10 kg was placed into pots of 30 cm diameter.

The seeds of the three fixing species of *Crotalaria* and that of the non-nodulating *Senna occidentalis* and *Senna obtusifolia* (reference plants) were used in these experiments. They were surface-sterilized with sulfuric acid and germinated in petri dishes containing soft agar (0.8%). Seedlings were transplanted one per container after two days, followed by inoculation (5 ml/pot) with a liquid rhizobial inoculum strain containing 10^9 cells/ml. All *Crotalaria* seedlings were then inoculated with appropriate liquid inoculum: *C. perrottetii* was inoculated with ORS 1924 rhizobial strain isolated from *C. perrottetii*, while *C. ochroleuca* and *C. retusa* were inoculated with ORS 1929 rhizobial strain isolated from *C. comosa* (Samba et al., 1999). A solution of $(^{15}NH_4)_2SO_4$ containing 10.9 ^{15}N atom % excess was applied to all pots to supply 0.2 g nitrogen/pot. A basal fertilizer was then applied to each pot equivalent to 0.14 g of K_2HPO_4. The pots were arranged randomly and watered such that soil moisture was kept close to field capacity (14%, v/w). Five replicates of these experiments were made.

The plants were harvested after two months after transplanting into pots. Dry weights of the different plant parts (leaves, stems, roots and nodules) were recorded. Nitrogen content (%N) and atom $\%^{15}N$ excess ($\%^{15}NAE$) were determined for each plant part by the Central service of analysis of CNRS at the University of Lyon.

Nitrogen fixation (%Ndfa) was estimated using the isotope dilution equation (Fried and Middelboe, 1977):

$$\%Ndfa = 1 - \frac{\%^{15}NAE \text{ in fixing crop}}{\%^{15}NAE \text{ in non fixing crop}}$$

A weighted $\%^{15}N$ atom excess (WAE) for the whole plant was estimated as follows:

$$WAE = \frac{AE(sh) \times TN(sh) + AE(st) \times TN(st) + AE(r) \times TN(r) + AE(p) \times TN(p)}{TN(sh) + TN(st) + TN(r) + TN(p)}$$

Where AE(sh), AE(st), AE(r) and AE(p) refer to atom excess in shoots, stems, roots, and pods, respectively. While TN(sh), TN(st), TN(r) and TN(p) designate total nitrogen in shoots, stems, roots and pods, respectively.

Then the %Ndfa for the whole plant was:

$$\%Ndfa = 1 - \frac{\text{WAE in fixing crop}}{\text{WAE in non-fixing crop}}$$

Data were statistically analyzed using Newman and Keuls test (See Dagnélie, 1969).

RESULTS

Dry matter weight and total nitrogen accumulation

There were significant variations in dry matter weight and total nitrogen uptake between the *Crotalaria* species (Table 1). *C. ochroleuca* accumulated five and two times more dry matter than *C. perrottetii* and *C. retusa*, respectively. *C. ochroleuca* also accumulated more nitrogen than *C. perrottetii* and *C. retusa* when either the whole or part of the plant is considered.

Nodulation and nitrogen fixation

Nodules were found in all three *Crotalaria* plants studied. *C. ochroleuca* exhibited significantly higher nodule dry weight (1.54 g/plant) than the two others plants (0.63 and 0.21 g/plant for *C. retusa* and *C. perrottetii* respectively) (Table 1). The % ^{15}N atom excess in each plant part and in the whole plant was higher in the two reference *Senna* plants than in *Crotalaria* species indicating the occurrence of nitrogen fixation in *Crotalaria*. No statistically significant differences were observed in the % ^{15}N atom excess between the *Crotalaria* species for each plant part and the whole plant, although the lowest atom % ^{15}N excess was recorded in *C. ochroleuca*.

Based on the whole plant, no significant difference in % Ndfa (Table 2) was found between the three *Crotalaria* plants (47-53%). However, the % Ndfa of

Table 1. Dry matter weight, % ^{15}N AE, proportions and amounts of total nitrogen in plant parts of three species of *Crotalaria* cultivated in 10 kg pots using *Senna* as reference plants

Plant parts	Species	Dry matter weight (g plant^{-1})	% ^{15}N AE	% N	Total N (g plant^{-1})
Leaves	*Crotalaria ochroleuca*	15.30 a	0.46 b	4.43 a	0.68 a
	Crotalaria perrottetii	4.84 d	0.52 b	3.53 b	0.17 c
	Crotalaria retusa	10.95 b	0.50 b	3.22 c	0.35 b
	Senna obtusifolia	7.80 c	1.09 a	1.35 e	0.11 d
	Senna occidentalis	2.62 e	1.06 a	2.5 d	0.07 d
	CV (%)	16.7	7.9	5.4	16.7
Stems	*Crotalaria ochroleuca*	20.79 a	0.45 b	0.91 b	0.19 a
	Crotalaria perrottetii	2.86 d	0.53 b	1.25 a	0.03 c
	Crotalaria retusa	10.95 b	0.50 b	1.00 ab	0.11 b
	Senna obtusifolia	9.55 b	1.08 a	0.51 c	0.05 c
	Senna occidentalis	6.44 c	1.06 a	0.47 c	0.03 c
	CV (%)	15.6	9.0	23.5	22.7
Roots	*Crotalaria ochroleuca*	21.37 a	0.47 b	0.97 b	0.18 a
	Crotalaria perrottetii	2.66 c	0.50 b	1.20 a	0.03 c
	Crotalaria retusa	6.67 b	0.51 b	1.25 a	0.08 b
	Senna obtusifolia	5.15 b	0.82 a	0.62 c	0.03 c
	Senna occidentalis	8.40 b	0.84 a	0.72 c	0.06 bc
	CV (%)	19.7	8.7	17.9	28.9
Total	*Crotalaria ochroleuca*	57.47 a	0.46 b	2.0 a	1.05 a
	Crotalaria perrottetii	10.36 d	0.52 b	1.99 a	0.24 c
	Crotalaria retusa	28.57 b	0.50 b	1.82 b	0.54 b
	Senna obtusifolia	22.51 c	1.00 a	0.83 d	0.18 c
	Senna occidentalis	17.46 cd	0.99 a	1.23 c	0.16 c
	CV (%)	20.1	8.0	7.0	14.9
Nodules	*Crotalaria ochroleuca*	1.54 a			
	Crotalaria perrottetii	0.21 c			
	Crotalaria retusa	0.63 b			
	Senna obtusifolia	-			
	Senna occidentalis	-			
	CV (%)	35.4			

Values in the same column and for each plant part, values followed by the same letter do not differ significantly at *P*= 0.05. CV = coefficient of variation

leaves and stems was significantly higher in *C. ochroleuca* than in *C. retusa* and *C. perrottetii*. Due to high nitrogen content, *C. ochroleuca* accumulated more fixed nitrogen than the other *Crotalaria* species. As reported in Tables 1 and 3, there were significant differences between the three *Crotalaria* species in nitrogen content and the amount of nitrogen derived from atmosphere. In *C. ochroleuca*, the total amount of nitrogen fixed is equivalent to 83 kg/ha versus 45 kg/ha and 19 kg/ha for *C. retusa* and *C. perrottetii*, respectively. The values of total nitrogen and % Ndfa for the leaves and the whole plant of *C. ochroleuca* were more than 150% greater than those of *C. retusa* and *C. perrottetii*. The proportion of nitrogen derived from soil (% Ndfs) is higher in *C. perrottetii* and *C. retusa* than that from fertilizer (% Ndff) in both species (Table 2).

DISCUSSION

The ^{15}N isotope dilution technique is known to be the most accurate technique to measure the nitrogen fixation by plants (Danso, 1988). The requirement for the choice of suitable reference plants for measuring nitrogen fixation by the ^{15}N isotope dilution method has been discussed by Danso et al. (1992). Following the recommendations of Boddey et al. (1990) and Awonaike et al. (1994), the average of the % ^{15}N atom excess of two reference *Senna* species (*S. obtusifolia* and *S. occcidentalis)* was used in our experiments, since non-nodulating *Crotalaria* isolines have not been identified. No nodules were observed on the roots of both reference plants, indicating the absence of symbiotic nitrogen fixation. It is possible that some nitrogen may have been fixed by free-living root associates, however, this amount would probably be negligible. Although the reference plants do not have equal rooting depth as the *Crotalaria* plants, they were cultivated in identical containers and the relative nitrogen uptake patterns were probably similar. Under these circumstances, errors in estimations of the nitrogen fixation were minimized.

Interspecific differences between *Crotalaria* species has been related by Daimon et al. (1995) for *C. juncea*,

Table 2. Proportions of nitrogen from fixation, fertilizer and soil of three species of *Crotalaria* cultivated in 10 kg pots of soil using two species of *Senna* (*S. occidentalis* et *S. obtusifolia*) as reference plants

Plant parts	Species	%NDFA	%NDFF	%NDFS
Leaves	*Crotalaria ochroleuca*	57.64 a	9.11 b	33.25 c
	Crotalaria perrottetii	52.70 b	10.43 b	38.07 b
	Crotalaria retusa	53.66 ab	9.96 b	36.38 b
	Senna obtusifolia	-	21.76 a	78.46 a
	Senna occidentalis	-	21.24 a	78.77 a
	CV(%)	5.8	7.9	4.2
Stems	*Crotalaria ochroleuca*	57.98 a	10.00 b	33.03 c
	Crotalaria perrottetii	50.57 b	10.57 b	38.86 b
	Crotalaria retusa	53.22 b	8.98 b	36.78 b
	Senna obtusifolia	-	21.54 a	83.59 a
	Senna occidentalis	-	21.23 a	83.30 a
	CV(%)	5.9	9.0	3.9
Roots	*Crotalaria ochroleuca*	42.61 a	9.49 b	47.89 b
	Crotalaria perrottetii	39.13 a	10.07 b	50.80 b
	Crotalaria retusa	38.36 a	10.20 b	51.45 b
	Senna obtusifolia	-	16.41 a	80.10 a
	Senna occidentalis	-	16.70 a	76.32 a
	CV(%)	11.7	8.7	7.8
Total	*Crotalaria ochroleuca*	52.60 a	9.20 b	38.00 c
	Crotalaria perrottetii	47.00 a	10.40 b	42.60 b
	Crotalaria retusa	48.40 a	10.00 b	41.40 b
	Senna obtusifolia	-	19.90 a	80.10 a
	Senna occidentalis	-	19.72 a	80.28 a
	CV (%)	7.2	8.2	4.3

For each plant part, values in the same column followed by the same letter do not differ significantly at *P*= 0.05. CV = coefficient of variation

C. pallida and *C. spectabilis* in terms of germination, root nodules formation, dry matter production, and nitrogen uptake. Similar species variability in nitrogen fixation has been discussed for other *Crotalaria* species (Polhill, 1982). Results from this study show that *C. ochroleuca* has higher dry matter production and greater nodulation.

Our data does not indicate any significant difference in % Ndfa in the roots and the whole plant between the three *Crotalaria* species. These proportions of Ndfa (47-53 %) are similar to those obtained in clover (50 %) under Mediterranean conditions (Carranca et al., 1999), and fallow legumes (30-80%) in savannah and other *Crotalaria* plants (41-77%) grown as fallow crops (Becker and Johnson, 1998). Coefficients of variation were generally low, indicating a good homogeneity of sampling.

Due to the genetic variability for nitrogen fixation observed between and within legume species it is essential to screen and use the species with higher nitrogen fixing potential (Sanginga et al., 1990). The highest % Ndfa values in the whole plant, or in the leaves and stems were recorded in *C. ochroleuca* making it the most suitable species tested to ensure an optimal nitrogen input in cropping systems characterized by similar soil conditions.

The proportion and the amount of nitrogen derived from the soil in the three *Crotalaria* species were higher than those derived from the fertilizer. Nevertheless, further screening of these plants for high nitrogen fixing potential and low proportion of nitrogen derived from the soil is needed. Whether the *Crotalaria* plants in these experiments are suitable as soil-improving legumes remains unknown. The three species of the present study were selected because of their biomass production. *C. ochroleuca* produced more lateral roots and would be likely able to absorb more soil nitrogen, but this does not limit its ability to fix also nitrogen as observed in this study.

Roots have not often been harvested in similar studies, especially in grain legumes, on the assumption that it will not make much difference in the nitrogen fixation values for the whole plant (Danso et al., 1992). In this study, the roots contained on average 8-15% of the amount of nitrogen fixed by the whole plant. This is small and similar to the 12% reported by Carranca et al. (1999) in Pea roots. Danso and Kumarasinghe (1990) suggested that it is essential to distinguish the effect of roots on % Ndfa, distinct from total nitrogen fixed. In *Crotalaria*, ignoring roots may not result in significant errors in % Ndfa estimates and amount of nitrogen fixed as shown in the present study. In contrast, ignoring roots may result in significant underestimation of nitrogen fixed in many nitrogen-fixing trees as have been reported in *Leucaena leucocephala* and *Gliricidia sepium* (Sanginga

Table 3. Amounts of nitrogen from fixation, fertilizer and soil of three species of *Crotalaria* cultivated in 10 kg pots using two species of *Senna* (*S. occidentalis* et *S. obtusifolia*) as reference plants

Plant parts	Species	NDFA (g plant^{-1})	NDFF (g plant^{-1})	NDFS (g plant^{-1})
Leaves	*Crotalaria ochroleuca*	0.39 a	0.06 a	0.23 b
	Crotalaria perrottetii	0.09 c	0.02 c	0.06 a
	Crotalaria retusa	0.19 b	0.03 b	0.13 a
	Senna obtusifolia	-	0,02 c	0.08 a
	Senna occidentalis	-	0,01 d	0.15 a
	CV(%)	16.0	14.6	7.2
Stems	*Crotalaria ochroleuca*	0.08 a	0.02 a	0.06 a
	Crotalaria perrottetii	0.05 a	0.00 d	0.01 d
	Crotalaria retusa	0.05 a	0.01 b	0.04 b
	Senna obtusifolia	-	0,01 c	0.03 c
	Senna occidentalis	-	0,01 c	0.02 c
	CV(%)	12.8	16.3	15.1
Roots	*Crotalaria ochroleuca*	0.08 a	0.02 a	0.09 a
	Crotalaria perrottetii	0.01 b	0.00 c	0.02 c
	Crotalaria retusa	0.03 b	0.01 b	0.04 b
	Senna obtusifolia	-	0.01 c	0.03 c
	Senna occidentalis	-	0.01 b	0.05 b
	CV(%)	37.1	23.0	21.4
Total	*Crotalaria ochroleuca*	0.52 a	0.10 a	0.35 a
	Crotalaria perrottetii	0.12 b	0.02 b	0.09 c
	Crotalaria retusa	0.28 b	0.07 a	0.22 b
	Senna obtusifolia	-	0.04 b	0.14 c
	Senna occidentalis	-	0.03 b	0.12 c
	CV(%)	37.0	36.8	16.4

For each plant part, values in the same column followed by the same letter do not differ significantly at *P*= 0.05. CV = coefficient of variation

et al., 1992), *Acacia* spp (Ndoye et al., 1995), *Faidherbia albida* (Gueye et al., 1997) and *Pterocarpus* spp (Sylla et al., 1999).

These experiments were carried out in greenhouse conditions, but further studies are planned to estimate the actual nitrogen fixation of the *Crotalaria* species in the field.

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