Plant and Soil 106, 269–276 (1988)
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PLSO 6927

Measurements of genetic variability for symbiotic dinitrogen fixation in field-grown fababean (*Vicia faba* L.) using a low level ^{15}N-tracer technique

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Received 3 July 1986. Revised October 1987

Key words: ^{15}N, nitrogen fixation, plant breeding, *Vicia faba*

Abstract

Before starting a breeding program aimed at improving the nitrogen nutrition of *Vicia faba*, the authors tried an alternative technique to the acetylene reduction assay, to measure some genetic variability in the plant material. The quantity of dinitrogen fixed by several cultivars of *Vicia faba* was estimated using a low enrichment ^{15}N tracer method and high precision ^{15}N mass spectrometry. The fababeans were cultivated for two years in two different soils.

The percentage of fixed dinitrogen in the seed varied between genotypes from 40 to 83% of the total nitrogen and was positively correlated with the total seed nitrogen (r = 0.64 to 0.86). A highly significant positive correlation was also found between the total seed nitrogen and the quantity of fixed dinitrogen in the seed (r = 0.95 to 0.99).

The technique used to measure dinitrogen fixation proved to be useful and reliable enough to discriminate between various genotypes, grown over a period of two years in two different soils. However, several non-fixing control plants showed significant differences in their ^{15}N enrichment and the problem of choosing a good reference plant was raised and discussed.

Introduction

To enhance the amount of biologically fixed dinitrogen in cropping systems, increasing attention is being paid to the role of the host plant in symbiotic dinitrogen fixation of Rhizobium-legume associations. Differences in the dinitrogen fixation potential of cultivars of the same legume species occur for both grain (Amarger *et al.*, 1979; Coale *et al.*, 1983; Graham *et al.*, 1984; Hardarson *et al.*, 1984; Hobbs and Mahon, 1982; Ronis *et al.*, 1985) and forage legumes (Barnes *et al.*, 1981; 1984; Seetin and Barnes, 1977; Smith *et al.*, 1982; Teuber *et al.*, 1984) Programs for the improvement of N^2 fixation in soybean and Phaseolus have been proposed (Graham and Temple, 1984) and significant progress has already been achieved in breeding alfalfa for improved dinitrogen fixation (Barnes *et al.*, 1981; 1984; Teuber *et al.*, 1984). However, mutant lines of *Vicia faba minor* attained similar percentages of plant nitrogen derived from fixation (Brunner and Zapata, 1979). Our research objective was to determine the various abilities of *Vicia faba* genotypes from different origins, to fix dinitrogen under field conditions. From among several approaches for determining the quantities of dinitrogen fixed, we chose a modification of the usual ^{15}N fertilizer enrichment method.

When compared to the acetylene reduction assay, the ^{15}N technique offers the following benefits: undisturbed field grown plants are utilised, analysis can be performed after the growing season

and the measurement is an integrated value of the dinitrogen fixing activity for the whole season.

The isotope dilution technique is based on a difference in ^{15}N abundance between the atmospheric N_2 reservoir and the soil N reservoir. The natural difference between the ^{15}N content of the air and soil and the ^{15}N content of the legume has been used to estimate the percentage of plant N derived from fixation (Amarger *et al.*, 1977; 1979; Bardin *et al.*, 1977; Delwiche and Steyn, 1970; Kohl *et al.*, 1980; Mariotti, 1982; 1983; Rennie *et al.*, 1976). Often, differences in natural ^{15}N abundance are too small and enrichment of the soil with ^{15}N labelled fertilizer improves the method (Fried *et al.*, 1983; Heichel *et al.*, 1984; Rennie, 1982). The quantities of ^{15}N added to soil have ranged from 1 to 4 kg of ^{15}N per hectare but only small areas were treated to keep the cost of the ^{15}N at a reasonable level. In order to work with large field areas in our investigation and provide better precision than is possible using natural abundance, we applied small amounts of ^{15}N (close to $50\,g \cdot ha^{-1}$) and used a highly sensitive mass spectrometer.

Material and methods

Agronomic techniques

Two field locations near Dijon, France, were used over a period of two years. The soil at location 1, Epoisses was a clay-loam and at location 2, la Bruyère, it was a sand. The clay contents were 40% and 5%, the pH 7.1 and 6.9, the organic nitrogen content 2‰ and 1‰, for each soil respectively. At both locations, the water table depth varied, during the growing season, from 0.7 to 2 meters. Irrigation water (60 mm) was applied in mid-June at both locations and again in mid-July at location 2, but irrigation at location 2 in 1981 was not sufficient to prevent the crop from suffering from drought stress. *Rhizobium leguminosarum* populations were present in the soil at both locations, at a level of 10^3 to 10^4 bacteria per g of soil. The time of planting was the beginning of March and mid-March for locations 1 and 2, respectively. Soil was fertilized with P ($70\,kg\,ha^{-1}$) and K ($100\,kg\,ha^{-1}$). Simazine (chloro-2, bis(ethylamine)-4,6 triazine-1,3,5) was used to control weeds and protection from aphids was accomplished with pyrimicarb (N,N-dimethyl-carbamate). Harvesting took place during early August.

A randomized complete block design with four replications was used for each trial. The size of plots and the density of sowing for each year was respectively $4.5\,m^2$, 20 plants m^{-2} in 1981 and $15\,m^2$, 50 plants m^{-2} in 1982. Seed samples from small areas at the center of the plots (1.5 and $10.5\,m^2$ for 1981 and 1982 respectively) were used to measure yield, protein content and ^{15}N enrichment.

Plant material

Several genotypes of *Vicia faba* were evaluated for seed yield, protein content and dinitrogen fixation. Some of the genotypes were inbred lines from different geographic origins and were selected at Dijon (33, 106, 127, 196, 240, 249, 252, 316, 319, 370, 437, G 77, PoAd 74), others were commercial or experimental strains (Séville, Blankfila, Hertzfreya, Deiniol, Ascott, Wierboon, PK), and one was an F1 hybrid (Ad23 × HG 115).

To estimate the quantity of fixed dinitrogen, the ^{15}N technique required the use of a non-fixing control plant to indicate the magnitude of the ^{15}N enrichment supplied by the soil mineral nitrogen pool and which was available to the legume plant (Kohl *et al.*, 1980; Mariotti *et al.*, 1983; Rennie, 1982; Wagner and Zapata, 1982). For this purpose, several species were used:

- in each experiment: + oat (*Avena sativa*, cv Selma)
- only in 1982, location 1: + a non-fixing mutant of *Vicia faba* selected at Dijon out of an Indian accession; this character is controlled by the recessive sym-1 gene; + chickpea (*Cicer arietinum*, genotype ILC 482 from ICARDA, Syria) which did not nodulate at our field location without inoculation; + wheat (*Triticum aestivum*, cv Bastion).

Analytical techniques

The soil was enriched with ^{15}N just after sowing, using $5\,kg\,ha^{-1}$ of N (KNO_3 with 1% ^{15}N abundance) dissolved in water and sprayed at a rate of $600\,l\,ha^{-1}$ over the entire experimental area. The flow of spray was proportional to the tractor speed, which provided a uniform enrichment of ^{15}N.

Table 1. Characteristics of fababean seed from two field locations, that relate to yield, quantity of biologially fixed dinitrogen and soil derived nitrogen (1981)

Name of genotypes	Location 1						Location 2					
	Protein		$\delta^{15}N$ ‰[a]	N source			Protein		$\delta^{15}N$ ‰[a]	N source		
	%	t ha^{-1}		Fixation		Soil	%	t ha^{-1}		Fixation		Soil
				%	kg ha^{-1}	kg ha^{-1}				%	kg ha^{-1}	kg ha^{-1}
1. 370	26.3	1.19	15.8cde	70.3	134	56	22.2	0.99	20.2bcd	67.7	107	51
2. G77	29.1	1.53	9.8ab	81.1	198	46	27.1	1.34	16.0ab	74.1	158	56
3. PK	36.0	0.65	20.1c	62.5	66	38	33.7	1.38	15.2ab	75.4	166	54
4. 127	32.4	1.11	10.5ab	79.9	142	35	29.1	1.62	12.7a	79.3	205	54
5. Ascott	31.2	1.23	11.5abc	78.1	154	43	30.2	1.27	16.8ab	72.9	107	51
6. 437	29.9	1.76	9.4ab	81.9	231	51	29.6	1.51	19.5bcd	68.8	166	75
7. Deiniol	31.4	1.23	11.3abc	78.5	155	42	29.2	0.98	14.2ab	76.9	120	37
8. 33	34.4	0.43	27.5f	49.2	34	35	27.7	0.58	29.4bc	53.4	50	43
9. Séville	30.3	1.06	16.7de	68.7	117	53	27.0	0.68	23.6cde	62.4	67	42
10. Blankfila	36.4	1.85	9.6ab	81.5	242	54	31.4	1.15	18.3abc	70.6	130	54
11. Hertzfreya	29.7	1.50	11.8abc	77.5	186	54	27.7	1.35	17.5abc	71.9	155	61
12. 252	27.8	1.25	14.4bcd	72.9	146	54	22.9	0.77	25.2de	59.9	74	50
13. 106	34.4	1.11	11.4abc	78.3	139	38	35.8	1.59	18.2abc	70.8	181	74
14. Wierboon	28.6	1.84	8.7a	83.1	245	50	27.6	1.50	16.8ab	72.9	174	66
15. 249	28.8	1.20	12.1abcd	77.0	147	45	30.1	1.34	16.6ab	73.2	157	58
16. PoAd 74	30.7	1.91	9.0a	82.6	253	53	30.0	1.04	15.2ab	75.4	125	42
Mean	31.1	1.30	13.1	75.2	162	47	28.8	1.19	18.5	70.4	137	54
LSD 0.05	1.3	0.24	4.2	7.8	40	14	1.3	0.28	3.7	5.8	34	13

[a]Duncan test at 5% level.

The nitrogen content of the seed was measured by the near infrared technique (Williams, 1975) with a Neotec G.Q.A. 21, and the Kjeldahl technique (Bremner, 1965) was used in the preparation of references for calibration purposes. For ^{15}N analysis, a sample of 200 mg of seed flour was treated by the Kjeldahl technique with selenium mixture as a catalyst (Mariotti and Létolle, 1978). After distillation of the NH_3, it was fixed in the form of ammonium sulfate which was later treated with lithium hypobromite to release N_2 and the ^{15}N enrichment was enrichment was measured after purification (Mariotti and Létolle, 1978; Mariotti, 1982). Isotope analysis was performed with a Finnigan Delta E mass spectrometer fitted for multiple ion collection and with a dual inlet system. Results are expressed in $\delta^{15}N$ units employed for natural ^{15}N abundance variations, using atmospheric nitrogen as the standard (Mariotti, 1983; 1984), using the formula below

$$\delta^{15}N\ (‰) = \left[\frac{R_{sample} - R_{standard}}{R_{standard}}\right] \times 1000,$$

where R is the nitrogen isotope ratio.

Calculation of the percentage of fixed nitrogen was made using the following formula (Amarger *et al.*, 1977; 1979; Mariotti *et al.*, 1983):

$$x = 100.\frac{\delta^{15}N_{legume} - \delta^{15}N_{control\ plant}}{\varepsilon_{fix} - \delta^{15}N_{control\ plant}}$$

where:

x = percentage of dinitrogen fixed by the legume plant.

$\delta^{15}N_{legume}$: isotopic composition of the legume plant (‰)

ε_{fix} = isotopic enrichment factor associated with N_2 fixation process. For *Vicia faba*, the value is −0.6‰ (Mariotti *et al.*, 1980; 1983)

$\delta^{15}N_{control\ plant}$: isotopic composition of the seed of the non-fixing control plant (‰).

Statistical analysis

Analysis of variance was performed on the data and means were classified according to Duncan's test.

Table 2. Characteristics of fababean seed from two field locations, that relate to yield, quantity of biologically fixed dinitrogen and soil derived nitrogen (1982)

Name of genotypes	Location 1						Location 2					
	Protein		$\delta^{15}N$ ‰[a]	N source			Protein		$\delta^{15}N$ ‰[a]	N source		
	%	t ha^{-1}		Fixation		Soil	%	t ha^{-1}		Fixation		Soil
				%	kg ha^{-1}	kg ha^{-1}				%	kg ha^{-1}	kg ha^{-1}
1. 370	30.7	1.07	14.9bc	53	91	80	30.6	0.32	24.3c	53	27	24
2. 240	28.8	0.67	14.7bc	54	58	50	27.6	0.24	31.1d	40	16	22
3. PK	37.4	1.12	9.9ab	68	123	56	34.8	0.39	16.5ab	67	42	21
4. 127	34.4	1.09	7.1a	77	134	40	32.7	0.50	18.8b	63	51	29
5. Ascott	35.0	1.49	13.2bc	59	141	98	35.2	0.66	13.4a	73	77	29
6. 316	30.7	0.59	18.7c	42	40	54	29.0	0.23	24.9c	52	19	18
7. Deiniol	34.8	1.33	10.2ab	68	144	69	31.9	0.64	14.5a	71	72	30
8. 319	32.0	1.13	13.4bc	58	106	75	27.5	0.30	29.9d	42	21	27
9. 196	36.2	1.10	12.2ab	62	109	67	32.1	0.54	13.6a	62	62	24
10. FlAd23 × HG115	31.2	1.50	9.0ab	71	171	69	31.5	0.82	14.8a	71	93	38
Mean	33.1	1.10	12.3	61	112	66	31.3	0.46	20.2	61	48	26
LSD 0.05	3.9	0.14	3.4	11	30	20	1.4	0.10	2.5	5	10	9

[a]Duncan test at 5% level

Results

The seed protein content and yield, the seed ^{15}N content and the amounts of nitrogen derived from the atmosphere and from the soil, are given for each trial and genotype in Tables 1 and 2. Significant effects for genotype, location and their interaction on protein content of seed (Table 3), were detected. The range in protein content was quite large: from 22.2 to 37.4% (Tables 1 and 2). A significant effect of genotype and location also occurred for protein yield (Table 3) but the interaction was not significant. Protein yield at location 2 was lower that at location 1, in both years, but the largest difference occured in 1982 when the irrigation frequency was not adequate to maintain sufficient plant available water in the sand.

A significant effect of genotypes, for the two years at the two locations, was detected for ^{15}N enrichment by variance analysis (column 3, Table 3). For this character, the coefficients of variation were 23 and 14% in 1981 for locations 1 and 2 respectively, 19 and 8% in 1982, respectively. There was a location (soil) effect in both years for the ^{15}N enrichment of seed (Table 3) which was higher at location 2 (sandy soil, Tables 1 and 2), but no significant interaction between genotypes and locations (Table 3). Figures 1 and 2 illustrate the good agreement between the two locations (soils) for the classification of genotypes. Considering the 5 genotypes which were common for the two years (127, 360, Ascott, Deiniol, PK), no significant year × location interaction could be detected.

Using oat as a common control plant, calcu-

Table 3. Statistical significance of treatment effects on various parameters related to protein content of fababean seed and source of N in seed.

Year	Source of variation	Protein		$\delta^{15}N$ ‰	N source		
		%	t ha^{-1}		Fixation		Soil
					%	kg ha^{-1}	kg ha^{-1}
1981	Genotypes	S	S	S	S	S	S
	Locations	S	S	S	NS	NS	NS
	Genotype × location	S	NS	NS	NS	NS	NS
1982	Genotypes	S	S	S	S	S	S
	Locations	S	S	S	NS	S	S
	Genotype × location	S	NS	NS	NS	NS	NS

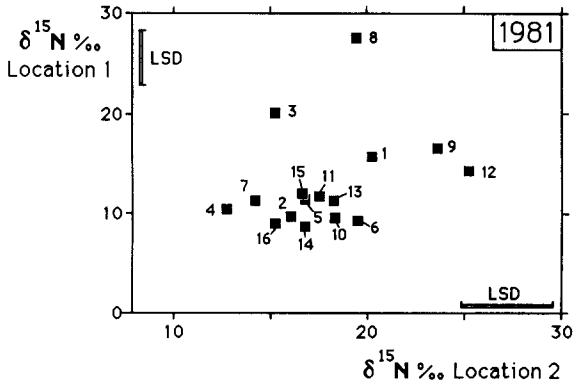

Fig. 1. Correlation between the two locations for isotopic composition ($\delta^{15}N$) of the 16 genotypes studied in 1981 trial. Code of genotypes: see Table 1.

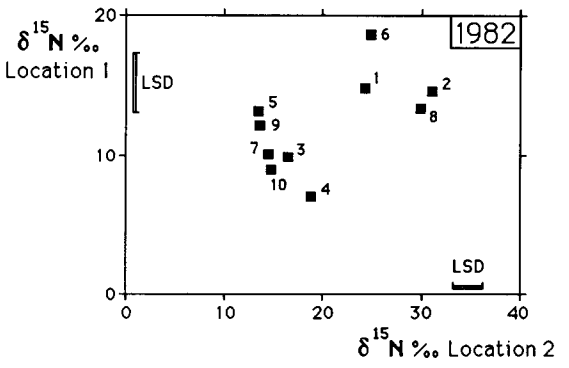

Fig. 2. Correlation between the locations for isotopic composition ($\delta^{15}N$) of the 10 genotypes studied in 1982 trial. Code of genotypes: see Table 2.

lations were made on the quantities of both fixed and soil nitrogen taken up by the different genotypes (columns 5 and 6, Tables 1, 2). A broad range involving significant differences among genotypes was evident.

The quantity of fixed dinitrogen per hectare in 1982 reached 170 kg·ha^{-1} for the best genotype under classical agronomic practices. In small plots with low plant densities at location 1 in 1981, the seed yield was probably overestimated (due to a high border effect) with values approaching 250 kg ha^{-1}. Differences between high and low quantities of dinitrogen fixed among genotypes were seven fold, whereas the extremes for quantity of soil nitrogen in seed were only two fold.

The mean percentage of nitrogen in seed from N_2 fixation was close to 70% in 1981 and 60% in 1982 (column 4). There was no difference between the two locations. Differences between genotypes in the percentage of nitrogen from N_2 fixation were significant and the values ranged from 40 to 83%.

Table 4 reports correlations between the different parameters studied with the following being of special interest:

— For the four trials, the correlation of ^{15}N enrichment with nitrogen yield was negative and highly significant. Also, highly significant correlations occurred between the protein yield and the quantity of fixed nitrogen.

— The positive correlation between the quantity of dinitrogen fixed and soil nitrogen in three out of four experiments, suggests that higher fixing genotypes also assimilate more soil nitrogen.

— Only for the sandy soil (location 2) were significant and positive correlations observed between protein content and the quantity of nitrogen derived from fixation.

In order to quantify absolute values of fixed dinitrogen, several control plants were tested in 1982. These were oat, wheat, non-nodulated chickpea and a non-fixing mutant of *Vicia faba* selected in our laboratory. Significant differences in the ^{15}N enrichment of these different crops are shown in Table 5. Chickpea (uninoculated) and *Vicia faba* (fix$^-$ mutant) gave a very low seed yield and their maturity occurred 15 days earlier than other *Vicia faba* genotypes involved in the trial. Calculations of the percentage of nitrogen derived from fixation when using wheat, oat, chickpea and fababean fix$^-$ as the control plant indicated 73, 74, 79 and 84% respectively for the higher N_2 fixing genotype and 378, 41, 52 and 60% for the lower N_2 fixing genotype.

Discussion

All genotypes were nodulated by the indigenous Rhizobium populations which were abundant in the two soils.

Genotypes of very diverse geographic origins with unknown parental relationship were used and all were adapted to our local environment. Line 33 was a primitive form of *Vicia faba* of Indian origin and differed from all the plant material studied by its earliness and low yield potential. This fact could have explained its particular behaviour in the 1981 trials and illustrated in Figure 1. Location 2 had a

Table 4. Correlations between various parameters related to protein content of fababean seed and source of N in seed (‰)

Year and location		Protein		$\delta^{15}N$ ‰	N source		
		%	t ha^{-1}		Fixation		Soil
					%	kg ha^{-1}	kg ha^{-1}
		1	*2*	*3*	*4*	*5*	*6*
1981 Location 1	*1*	1000					
	2	−316	1000				
	3	280	−858**	1000			
	4	−280	858**	−1000**	1000		
	5	−273	996**	−877**	877**	1000	
	6	−562*	659**	−363	363	590*	1000
1981 Location 2	*1*	1000					
	2	522*	1000				
	3	−426	−728**	1000			
	4	427	730**	−1000**	1000		
	5	532*	988**	−815**	817**	1000	
	6	346	775**	−176	179	666*	1000
1982 Location 1	*1*	1000					
	2	468	1000				
	3	−537	−632*	1000			
	4	546	638*	−999**	1000		
	5	517	945**	−838**	842**	1000	
	6	96	607	202	−193	315	1000
1982 Location 2	*1*	1000					
	2	597	1000				
	3	−867**	−834**	1000			
	4	861**	836**	−1000**	1000		
	5	658*	994**	−885**	887**	1000	
	6	235	856**	−458	461	792**	1000

* significant at 0.05% level.
**significant at 0.01% level.

lower yield and this was especially the case in 1982 which was marked by a severe drought period at the pod filling stage and a consequently reduced yield.

Problems in measuring dinitrogen fixation using the ^{15}N enrichment technique have been discussed by previous authors such as Witty (1983). These problems may have resulted from the interaction between uneven labelling of the soil (variations of concentration of ^{15}N along time and zonation due to nitrogen uptake, leaching and immobilization), and the fluctuating pattern of nitrogen assimilation by the plant. It should be the same for all compared genotypes and control plants (same kinetics of assimilation and the same root assimilation zone). The only control we had on these different parameters was a measure of maturity. Through it, Line 33 of *Vicia faba*, the non-nodulated chickpea and the fix$^-$ mutant of *Vicia faba* reached maturity 15 days ahead of the remaining genotypes, an occurrence which could have invalidated the calculations of fixed dinitrogen made with them, whereas oat appeared more adequate according to this criteria. We must also consider that the plant breeder has a primary interest in the comparison between two N_2 fixing cultivars of the same species, which according to the previous discussion, should be more accurate if they ripen at the same time rather than a comparison with an earlier maturing, non fixing control plant. The absence of an absolute reference plant for the quantity of fixed N_2 makes our choice of control plant somewhat arbitrary.

Using the isotope dilution technique with a low level of ^{15}N enrichment in the soil, the precision we obtained for estimating the quantity of fixed dinitrogen was slightly lower (coefficient of variation

Table 5. Seed yield, protein content and $\delta^{15}N$ of four reference plants grown at field location 1 during 1982

Plant	Yield t ha^{-1}	Protein content %	$\delta^{15}N$ ‰
Wheat	7.5	13.4	31 a[a]
Oat	5.2	16.6	33 a[a]
Chickpea (uninoculated)	1.8	20.1	41 b[a]
Vicia faba (Fix^- mutant)	1.9	25.3	52 c[a]

[a] Duncan test at 5% level.

ranged from 15 to 20%) than the precision for protein yield measurements (CV ranged from 9 to 17%) and much higher than the precision we generally obtained by the acetylene reduction assay for estimating nitrogenase activity (CV frequently higher than 40%). Consequently, we were able to measure significant differences between genotypes with regard to the quantity of fixed dinitrogen. However the least significant difference, ranging from 10 to 40 kg for the fixed N_2 ha^{-1}, needed to be decreased to provide greater precision. The absence of significant interactions between genotypes and soils for ^{15}N enrichment in the two years (Figures 1 and 2), indicated that the plant breeder could reliably compare the genotypes using this technique, although the cost of sample analysis may be prohibitive and for this reason may only be applied in the final characterization of genotypes.

Only the nitrogen in the seed was measured in this experiment and although it was known to represent 80 to 85% of the above ground nitrogen yield of fababean at maturity, we had no information on the origin of the nitrogen present in the roots and stems at harvest time. Regarding nitrogen in the seed, it appeared that a very large genetic variability was introduced into the trial (Table 1 and 2). This variability was best expressed by the quantity of fixed dinitrogen and not in the amount of nitrogen derived from the soil. The limited available soil nitrogen or some limit of the plant itself may have been the reasons for the small differences associated with assimilation of soil nitrogen.

The percentages of nitrogen derived from fixation were found to range from 40 to 83% and these values are smaller and more wider ranging that those found by Brunner and Zapata (1979) in *Vicia faba* (82 to 93%). The use in our investigations of genotypes differing from theirs, in origin and yield potential, may explain the contrast.

Measurements indicated that the higher yielding genotypes fixed more nitrogen (Table 4) and this observation was in agreement with those made by other authors for several crops (Barnes *et al.*, 1981; Fried *et al.*, 1983; Hobbs and Mahon, 1982). Presumably dinitrogen fixation regulated the yield, or some common factor such as a limit in carbon source regulated both yield and fixation. In either case, a breeding procedure aimed at improving nitrogen fixation could start with a selection for yield in an environmental condition that allows fixation (*i.e.* presence of compatible Rhizobium and a moderate level of soil mineral nitrogen). Admittedly, this conclusion is linked to our choice of genotypes and has to be tested on a larger sampling.

Only in the sandy soil (location 2) was a significant and positive correlation found between protein percentage in the seed and the quantity of fixed nitrogen. Probably the lower dinitrogen fixing genotypes provided less nitrogen to the seed because combined available nitrogen was very limited. This suggested that protein percentage could serve as an easy and likely breeding criteria to evaluate the efficiency of dinitrogen fixation when soil nitrogen is low. However this result needs to be verified in further experiments and the level of significance of the correlation leaves some doubt about the possibility of using it.

Conclusion

The ^{15}N technique using a low level of isotope enrichment of the soil was demonstrated to be useful in measuring dinitrogen fixation under field conditions and to facilitate the discrimination between genotypes. Major differences among genotypes were observed for percentages of nitrogen fixed and a much smaller variability was expressed for the quantity of nitrogen derived from the soil. The ^{15}N technique we employed still required improved precision and reduced cost in order to be used effectively in a breeding program. Admittedly, the work on these quantitative characters is not the only way to select legumes for increased dinitrogen fixation and other developing methods include the use of single mutated genes now available in this species which could be of value in agronomic research (such as nitrate tolerant mutants, hyper-

nodulating mutants, mutants of recognition for specific strains of Rhizobium....).

Acknowledgements

The authors thank J Picard, R Weaver, G H Wagner and J Kough for the fruitful discussions we had on this work and improvements of the English version of this text, and H Delarambergue, F Dhainaud, M Grably and J Guillemot for their technical assistance.

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