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Nitrogen nutrition, yield and protein content in soybean

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Abstract

Soybean recombinant inbred lines derived from a cross between two cultivars of different genetic origins, each with a high seed protein content, were analyzed for their dinitrogen fixation and nitrate assimilation abilities and for their seed traits. The influence of both nitrogen sources on yield and protein content was evaluated. The importance of symbiotic N_2 fixation in yield and seed protein content was corroborated. Dinitrogen fixation efficiency during the reproductive growth period until late stages (R2–R6 + 10 d) is involved in seed protein content. The yield is more directly related to mineral nitrogen assimilation in the first stages of the reproductive growth period (R2) and to high dinitrogen fixation rates at stage R6. Nitrogen nutrition abilities are independent and can be used as complementary criteria when selecting for agronomic seed traits.

Keywords: Dinitrogen fixation; Nitrate assimilation; Yield; Protein content; Soybean

1. Introduction

Nitrogen nutrition in soybean, as in other legumes, is ensured both by dinitrogen fixation and mineral nitrogen assimilation. These two sources can be complementary or antagonistic in relation to the environmental factors or developmental stages [1]. In most soils where the nitrate content is moderate, the proportion of nitrogen which is derived from symbiotic fixation in soybean is about 50% [2,3] but can reach 75% in sandy loamy soils [4]. The highest rate of nitrogen fixation occurs at the end of flowering and during pod fill [5–8]. The nitrogen assimilated between the start of pod development (stage R3) and the start of maturity (stage R7) seems to be the predominant source of nitrogen for seed development [9,10].

Improving N_2 fixation could even facilitate high productivity [11] and high seed protein content [12]. Indeed, rapid N_2 fixation during pod fill (stages R5–R6) was shown to contribute to increased seed yield and seed protein content [13]. Dinitrogen fixation is thus a decisive physiological parameter both for enhanced productivity and higher seed quality [14], traits often reported as negatively correlated [15]. In spite of the high energy cost of N_2 fixation, some investigations [5,7,16] suggested that photosynthetic efficiency in soybean could be adjusted to the photosynthate requirements of the nodules.

However, as a result of the common action of both nitrogen sources, NO_3 assimilation remains a major pathway of N nutrition [17]. Assimilation reaches an earlier maximum than fixation, usually at full bloom (stage R2), and declines thereafter [6,8]. NO_3 assimilation is associated with plant biomass gain during vegetative stages and until flowering. In spite of its lower efficiency during

Abbreviations: ARA, acetylene reduction activity; sNRA, specific nitrate reductase activity; PP, seed percent protein; YLD, yield.

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pod fill, NO_3 assimilation is also necessary to achieve higher seed yield and seed protein content [13,18].

The aims of this study were (i) to estimate the influence of both nitrogen sources on yield and protein content, and (ii) to evaluate the balance between the nitrogen sources at different reproductive growth stages. The investigations were carried out on soybean recombinant inbred lines. The lines were expected to segregate for all traits and to express a wide genetic variability.

2. Materials and methods

2.1. Plant material

Fourteen F6 recombinant inbred lines derived by single seed descent from a cross between Provar (female) and X514-95 were used. Each parental line is of group maturity well-adapted to temperate areas. Provar is of US origin and is characterized by a high protein content, combined with a reasonably good yield. X514-95 was obtained from a French selection program using Canadian soybean cultivars. X514-95 protein content is high but its yield is lower.

The recombinant inbred lines were evaluated at different growth stages for their dinitrogen fixation and nitrate assimilation abilities, and on mature seed for their yield and total protein content.

2.2. Experimental conditions

The investigations were carried out in a greenhouse under natural lighting, hydrometric and temperature conditions. Plants were grown in pots (18-cm in diameter and 16-cm deep) filled with a 1:1:1 sand:soil:peat mixture (typical Udifluvent loamy sandy soil) that contained total N of 1.5 g kg^{-1}. Seeds were germinated in vermiculite for 3 days at 25°C and seedlings (one per pot) were transplanted on soil. Seed and soil were inoculated (10^6 bacteria per seed or per dm^3 of soil) with *Bradyrhizobium japonicum* strain G49 (Lipha, Lyon, France). Plants were watered daily and fertilized twice a week with the IRAT solution (Institut de Recherche en Agronomie Tropicale, France) supplied in magnesium [19] and containing 3.2 mM $Ca(NO_3)_2$.

2.3. ARA measurements

Dinitrogen fixation was estimated by measuring the Acetylene Reduction Activity (ARA) using an in situ method [20]. The pot was tightly sealed and acetylene was injected into the soil around the root system; the acetylene volume amounted to 10% of total porosity of the mixture in the pot. After a 7-min incubation, samples were removed to determine ethylene concentration by gas chromatography (Delsi Model DI 200, Paris, France). The measurements were carried out at reproductive stages [21] R2 (full bloom), R5 (start seed), R6 (full green seed) and 10 days after R6. The results are expressed in μmol C_2H_4 h^{-1} $plant^{-1}$. The integrated ARA (iARA) was calculated according to Patterson and LaRue [22] through the period R2–R6 + 10 d and is expressed in mmol C_2H_4 $plant^{-1}$.

2.4. NRA measurements

Nitrate assimilation was estimated at reproductive stages R2, R5 and R6 only, from Nitrate Reductase Activity (NRA) measurements, using the in situ method described by Robin et al. [23]. The sample (last fully developed leaf) was bathed in a 100 mM KNO_3 solution and placed in darkness and in anoxic conditions created by vacuum. After 3 h incubation and extraction by boiling water, the accumulated nitrite in the tissues was measured colorimetrically. The activity expressed in μmol NO_2^- h^{-1} gDW^{-1} is termed specific NRA (sNRA).

2.5. Seed traits measurements

Plants were harvested at complete maturity. Seed percent protein (PP) was evaluated by Kjeldahl method (Tecator Kjeltec, Auto 1030, Höganäs, Sweden). Seed yield (YLD) was also determined.

2.6. Statistical analysis

The experiment consisted of a complete factorial design of 14 genotypes with six replications. Genotypes were randomly allocated in three blocks with two replicates. Data were subjected to a one-way analysis of variance [24] with STAT-ITCF (Institut Technique des Céréales et des Fourrages, Paris,

Table 1
Analysis of variance for nitrogen nutrition components (N_2 fixation and NO_3 assimilation) and seed traits (PP, seed percent protein; YLD, yield) in the 14 soybean genotypes[a]

Source of Variation	Df[b]	Mean squares[c]										
		N_2 fixation						NO_3 assimilation			Seed traits	
		ARA R2	ARA R5	ARA R6	ARA R6+10 d	iARA	ARA R5/R6	SNRA R2	sNRA R5	sNRA R6	PP	YLD
Genotype	13	1.36^{NS}	4.78***	6.23***	2.45**	1.84**	0.27***	28.83***	5.84***	1.03**	38.99***	12.68***
Block	2	1.64^{NS}	0.07^{NS}	0.26^{NS}	0.06^{NS}	0.16^{NS}	0.01^{NS}	0.08^{NS}	0.03^{NS}	0.02^{NS}	0.38^{NS}	0.75^{NS}
Residual	26	0.81	0.98	0.72	0.10	0.30	0.03	0.90	0.03	0.15	0.84	1.22

[a] NS, ** and ***: Not significant and significant at the 0.01 and 0.001 levels, respectively, as determined by an *F*-test of the analysis of variance.
[b] Degrees of freedom.
[c] Mean squares correspond to variances.

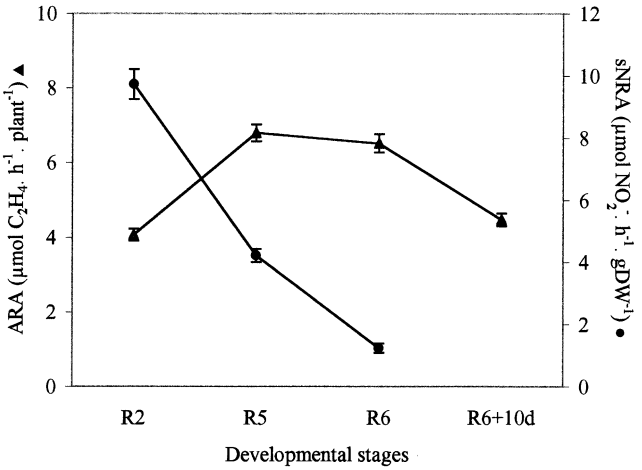

Fig. 1. N_2 fixation (ARA) and NO_3 assimilation (sNRA) for the 14 genotypes at each developmental stage. Vertical bars represent SE (standard error).

France). The significance of mean differences was tested by an F-test using the F value = (factor mean square/residual mean square). Phenotypic correlations were calculated using the mean of the six replications.

3. Results

3.1. N_2 fixation

The genotypes displayed significantly different N_2 fixation activities during pod filling (stages R5–R6 + 10 d) whereas these differences were not expressed at flowering (stage R2) (Table 1). At this stage, the nodules were just functional and the genotypes displayed a low fixation activity. The genotypes could also be distinguished by their fixation ability over the whole reproductive cycle from R2 to R6 + 10 d (iARA) (Table 1).

The nitrogenase activity usually reaches a maximum at R5 before it declines. In the investigation reported here, the overall analysis of the 14 genotypes showed the maintenance of high fixation values beyond stage R5 until R6 (Fig. 1). Therefore, the ratio of the ARA values observed at these two stages (R5/R6 ARA) was close to unit. However, the genotypes differed significantly in their ability to maintain a high activity at stage R6, as shown by the analysis of variance of the R5/R6 ARA ratio (Table 1).

The analysis of phenotypic correlations (Table 2) corroborated this result. The absence of link between ARA values at stages R5 and R6 ($r = 0.038^{NS}$) suggested that the symbiotic fixation activity did not follow an identical profile in all genotypes. The R5/R6 ARA ratio demonstrated the involvement of two differing behaviour patterns: some genotypes reached a maximum fixation activity at stage R5 (R5/R6 ARA > 1) whereas other genotypes were characterized by a highest fixation activity at stage R6 (R5/R6 ARA < 1).

In contrast, the occurrence of a positive link between the ARA values measured at stages R2 and R5 ($r = 0.376$*) suggested that all genotypes followed a similar profile (Table 2). The correlations between the integrated fixation activity (iARA) over the whole reproductive cycle (R2–R6 + 10 d) and the ARA values at all developmental stages showed that the iARA value reflected the fixation potential of genotypes (Table 2).

3.2. NO_3 assimilation

The genotypes displayed significantly different nitrate assimilation activities at all developmental stages (Table 1). The Nitrate Reductase (NR)

Table 2
Phenotypic correlations between N_2 fixation and NO_3 assimilation components at different reproductive growth stages[a]

	ARA R2	ARA R5	ARA R6	ARA R6+10 d	iARA	sNRA R2	sNRA R5
ARA R5	0.376*						
ARA R6	0.217^{NS}	0.038^{NS}					
ARA R6+10 d	0.406**	0.248^{NS}	0.383*				
iARA	0.441**	0.622***	0.719***	0.424**			
sNRA R2	-0.083^{NS}	-0.139^{NS}	0.110^{NS}	-0.199^{NS}	-0.045^{NS}		
sNRA R5	-0.110^{NS}	-0.149^{NS}	-0.287^{NS}	-0.247^{NS}	0.230^{NS}	0.593***	
sNRA R6	0.068^{NS}	-0.141^{NS}	-0.061^{NS}	-0.118^{NS}	0.050^{NS}	0.070^{NS}	0.320*

[a] NS: Not significant. *, ** and ***: significant at the 0.05, 0.01 and 0.001 levels, respectively.

Table 3
Phenotypic correlations between seed traits (PP: Seed percent protein and YLD: Yield) and N_2 fixation or NO_3 assimilation components at different reproductive growth stages[a]

Seed traits	N_2 fixation						NO_3 assimilation		
	ARA R2	ARA R5	ARA R6	ARA R6+10 d	iARA	ARA R5/R6	sNRA R2	sNRA R5	sNRA R6
PP	0.378*	0.389*	0.214^{NS}	0.483**	0.324*	0.131^{NS}	-0.143^{NS}	-0.171^{NS}	-0.222^{NS}
YLD	-0.113^{NS}	−0.440**	0.549**	0.011^{NS}	0.216^{NS}	−0.673***	0.423**	0.055^{NS}	0.001^{NS}

[a] NS: Not significant. *, ** and ***: significant at the 0.05, 0.01 and 0.001 levels, respectively.

activity declined rapidly from stage R2 as the nitrogenase activity increased (Fig. 1), which confirmed the inverse relationship between these two enzyme profiles during plant development.

Based on the positive links for sNRA between stages R2–R5 and R5–R6, it would be appear that the 14 genotypes presented a similar nitrate assimilation profile (Table 2).

3.3. Relationships between N_2 fixation or NO_3 assimilation and seed traits

The differences among genotypes for protein content and yield were significant (Table 1), showing a large variability for these traits. Protein content was positively associated with the N_2 fixation activity at all stages of the reproductive cycle (Table 3). However, a high symbiotic activity at stage R5 was more involved in protein production ($r = 0.389$*) than in other seed compounds biosynthesis. Thus, the R5/R6 ARA ratio was positively linked to protein content even though the relation was not significant, which corroborated the predominance for protein biosynthesis of N_2 fixation efficiency at stage R5 compared to the one at stage R6 (Table 3). A late symbiotic activity contributed to other compounds biosynthesis involved in seed yield, as shown by the relations between yield and N_2 fixation activities at stage R6 ($r = 0.549$**) and at stage R5 ($r = -0.440$**).

The sNRA level did not have any significant effect on seed protein content (Table 3). In contrast, yield was significantly associated with sNRA at stage R2 which corresponds to the maximum activity. The occurrence of an efficient nitrogen nutrition during the vegetative period is required to ensure a satisfactory biomass production.

3.4. Comparison of the two symbiotic fixation behaviours

Table 4 illustrates the comparison between the two groups of genotypes according to their N_2 fixation behaviour between stages R5 and R6. The ARA values were significantly different at stages R5 and R6 in the two groups whereas they were similar at the other stages.

The two behaviour patterns were not associated with the NR activity, even though genotypes with a high N_2 fixation at stage R6 exhibited a superior nitrate assimilation activity at stage R2 (Table 4).

The effect of a late fixation activity on yield (11.08 vs. 8.03 g plant^{-1}) was corroborated, but

Table 4
Comparison of the two symbiotic fixation behaviours at stages R5 and R6[a]

Agronomic Parameters	R5/R6 ARA ratio		Significance
	>1	<1	
N_2 fixation			
ARA R2	4.06	4.05	NS
ARA R5	7.22	6.02	**
ARA R6	5.69	8.02	***
ARA R6+10 d	4.35	4.68	NS
iARA	5.24	5.66	NS
NO_3 assimilation			
NRA R2	9.27	10.52	NS
NRA R5	4.23	4.20	NS
NRA R6	1.29	1.16	NS
Seed traits			
PP	45.41	46.42	NS
YLD	8.03	11.08	***

[a] NS, ** and ***: Not significant and significant at the 0.01 and 0.001 levels, respectively, between the two groups as determined by an *F*-test of the analysis of variance.

the two groups of genotypes did not differ by their protein content.

3.5. Relationships between N_2 fixation and NO_3 assimilation

The correlations between the enzyme activities of the two nitrogen nutrition pathways were negative and not significant (Table 2). The abilities of the genotypes for their mineral nitrogen assimilation and their symbiotic N_2 fixation activities would be independent. The sNRA at stage R2 and ARA at stage R6, which were positively linked to yield, were not correlated. Thus, the genotypes characterized by satisfactory nitrate assimilation during the vegetative period and high N_2 fixation during seed filling would be the most productive.

4. Discussion

Symbiotic dinitrogen fixation is the main nitrogen source in soybean and is considered as the major parameter of seed protein content. N_2 fixation efficiency through R2–R6 + 10 d allows high seed protein contents to be reached, as this period is the main phase of protein accumulation. In fact, high metabolic activity starts 18 days after flowering (DAF) (stages R3–R4) with the proliferation of the first protein and lipid bodies. The number and the size of the protein bodies greatly increase between 26 and 36 DAF, around stage R5. From stage R6, the accumulation of the protein and lipid bodies proceeds until stage R7 [25]. A vigorous N_2 fixation during the reproductive growth stages, until R6 + 10 d, contributes to higher seed protein concentration. These results are in agreement with those of Leffel et al. [12] who reported that a later time of initiation of N remobilization and maintenance of N_2 fixation until late R6 were associated with the high-protein content.

At stage R6, an intense phase of carbohydrate synthesis, N_2 fixation seems to affect yield essentially. The relationship between yield and symbiotic fixation efficiency has often been reported [11,19,26].

Increasing N_2 fixation rates during the pod-fill period, especially between stages R5 and R6, leads to the possibility of improving simultaneously yield and protein content. Prolonging the pod-fill period is thought to increase both seed yield [13,27] and seed protein content [13]. The latter author showed that a higher and relatively rapid fixation rate retards mobilization of N from the vegetative material and thus prolongs the stages R5 and R6.

However, because of the high N demand from the seed, a soybean plant may become N deficient during pod fill [13,28]. Indeed, it is frequently reported that seed yield and seed protein content are highest when N is obtained from both N fertilizer and N_2 fixation [5,13]. In order to increase seed yield, seed N content and plant biomass, some N must be derived from the soil during pod fill [18].

The impact of NO_3 assimilation on seed traits is thus essential. The initial step of nitrate reduction, which is catalyzed by the nitrate reductase (NR) enzyme, is the rate limiting step [29]. Seasonal profiles of N_2 fixation and NO_3 assimilation were reported by several authors [5,6,8,22,30]. Fixation and assimilation participated in N nutrition in a complementary manner. Under field conditions, NR activity reaches a maximum near the full-bloom stage (R2) and declines thereafter [6,8]. Nitrogenase activity starts to contribute to the overall N input from the end of the vegetative growth phase, and increases until the pod-fill stages (R5–R6) [6,8,22,30]. The decline in NR activity during the pod-fill stages is most likely due to decreasing soil nitrate availability [31] and to moisture conditions which also affect nitrate uptake by the plant root [5]. Thus, N fertilizer application may then be required.

These profiles of enzyme activities were corroborated by the data presented above. However, no significant correlation was found between NR activity and the other parameters measured. Only the yield was associated with NR activity during the early stages of the reproductive growth period (R2). This relationship can be accounted for in terms of the effect of NO_3 assimilation on plant growth. Nitrate reductase, which is functional from the beginning of the developmental cycle, is the key enzyme involved in the reduction of nitrate to ammonium. Ammonium incorporation into protein then requires carbohydrates issued from photosynthesis. Proteins are initially accumulated in leaves and roots, and contribute to biomass production before breakdown and redistribution to reproductive plant parts [32]. Thus, NO_3 assimilation appears to be a nitrogen nutrition pathway of the most importance. During periods of development when N_2 fixation alone would not ensure adequate plant nitrogen supply, as after a drought

stress [1,8], NO_3 assimilation can provide the N complement required by plant growth. According to its importance and to its relationship with yield, NO_3 assimilation efficiency appears to be valuable criterion of indirect selection. The possible use of the positive correlations between NR activity and agronomic traits, such as productivity and seed protein content, was reported for legumes [33,34] and cereals [35,36]. In maize hybrids, the attempts to increase the yield potential by selecting for enhanced NR activity [37] led to some positive responses [36].

No association was found between NR activity at stage R2 and ARA at stage R6 although both activities are involved in the yield. Thus, indirect selection based simultaneously on both nitrogen nutrition pathways might improve the yield. A selection based on several criteria is thus likely to be more efficient than one relying on a single parameter. Moreover, selecting for enhanced symbiotic N_2 fixation led to simultaneously improve yield and protein content.

In conclusion, results presented above confirm the importance of symbiotic N_2 fixation in yield and seed protein content. Dinitrogen fixation efficiency during the whole reproductive growth period until late stages is involved in seed protein content. Yield is more directly related to mineral nitrogen assimilation in the first stages of the reproductive growth period and to high dinitrogen fixation rates at stage R6. These results suggest that both nitrogen nutrition pathways can be used as indirect criteria when selecting for agronomic seed traits.

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