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CROP PRODUCTION

Di-nitrogen fixation at the early and late growth stages of soybean

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ABSTRACT. Soybean derives a significant portion of the required nitrogen (N) from the symbiosis with rhizobia bacteria. However, information on the available genetic variation for N_2 fixation capacity in different growth stages of soybean is limited. The objective of this study was to investigate the N_2 fixation capacity of 22 soybean lines compared with that of non-nodulating and supernodulating checks at the early and late growth stages and identify the most informative traits for selection. Two cycles of greenhouse experiments were carried out to estimate the percentage of N derived from the atmosphere (%Ndfa) as well as 10 different traits related to N_2 fixation. The results showed that %Ndfa was significantly different among the lines at the early and late growth stages. SPAD readings showed the highest correlation with the early N_2 fixation, whereas shoot dry weight with the late N_2 fixation. Early and late %Ndfa could be used to select superior lines for N_2 fixation and study the underlying physiological and molecular mechanism.

Keywords: *Glycine max*; %Ndfa; ^{15}N dilution technique; nodulation; symbiosis.

Fixação de nitrogênio na fase inicial e final de desenvolvimento da soja

RESUMO. Na soja uma parcela significativa do nitrogênio (N) requerido deriva de sua simbiose com a bactéria do gênero rhizóbio. No entanto, informações sobre variabilidade genética disponível para a capacidade de fixação de N_2 em diferentes estádios fenológicos é limitada. O objetivo deste estudo foi investigar a capacidade de fixação de N_2 durante a fase inicial e final de desenvolvimento de 22 genótipos de soja comparada a de genótipos não nodulantes e supernodulante utilizados como testemunhas, e também identificar os caracteres mais informativos para fins de seleção. Dois ciclos de experimentos foram conduzidos em casa de vegetação para estimar o percentual de N derivado da atmosfera (%Ndda), assim como outros 10 caracteres relacionados a fixação de N_2. Os resultados demonstraram que o %Ndda foi significativamente diferente entre os genótipos nas duas avaliações. Leituras de fotossíntese com SPAD apresentaram a maior correlação com a avaliação inicial de fixação de N_2, enquanto que a massa seca da parte aérea demonstrou a mais alta correlação com a avaliação de fixação de N_2 na fase final de desenvolvimento. Avaliações do %Ndda nas fases inicial e final de desenvolvimento da cultura podem ser utilizadas para seleção de genótipos superiores de soja para fixação de N_2, assim como para o estudo dos mecanismos de controle nos âmbitos fisiológico e molecular.

Palavras-chave: *Glycine Max*; %Ndda; técnica de diluição do nitrogênio-15; nodulação; simbiose.

Introduction

The nitrogen (N) requirements of soybean are probably the highest among all major crops, since it demands 29 mg of N g^{-1} photosynthate compared with 11 mg of N g^{-1} photosynthate in corn and 26 mg of N g^{-1} photosynthate in cowpea (Sinclair & Wit, 1975). The crop derives 36–69% of its total N through its symbiotic relationship with rhizobia bacteria (Salvagiotti et al., 2008) and the rest from the available soil N.

Yield improvement in soybean is relatively high and averages 31.2 kg ha^{-1} $year^{-1}$. Thus, some concerns have been raised regarding the capacity of the new high-yielding cultivars to reach their yield potential without the input of nitrogenous fertilizers (Specht, Hume, & Kumudini, 1999; Salvagiotti et al., 2008). Moreover, previous studies have suggested a reduction in the N_2 fixation capacity of new cultivars compared with that of old ones (Nicolás, Arias, & Hungria, 2002; Van Kessel & Hartly, 2000). Therefore, the evaluation of lines and traits, aiming to improve the N_2 fixation capacity, is considered crucial (Nicolás et al., 2002).

In soybean, N_2 fixation varies among different growth stages, reaching the highest rate at the R2–R4 (Keyser & Li, 1992; Imsande, 1989; Latimore, Giddens, & Ashley, 1977; Lawn & Brun, 1974; Thibodeau & Jaworski, 1975). However, N accumulation follows a different pattern during the

soybean growth cycle, suggesting a limitation in the N supplied through symbiosis (Keyser & Li, 1992; Phillips & DeJong, 1984).

Multiple evaluations of the N_2 fixation activity of the nodulating line Chippewa and its non-nodulating isoline were performed and unveiled significant variation in the proportion of N derived from fixation at different growth stages (Zapata, Danso, Hardarson, & Fried, 1987). Using the acetylene reduction activity assay for evaluating nitrogenase activity, Pazdernik, Graham, Vance, and Orf (1996) screened 20 soybean lines and identified considerable variation in early nodulation traits and N_2 fixation activity, whereas Fabre and Planchon (2000) concluded that the studied lines differed in N_2 fixation activity at the R5 and R6 growth stages, but not at the R2 growth stage. Herridge, Bergersen, and Peoples, (1990) used the ureide abundance and ^{15}N natural abundance methods, collecting data at the V8–R6.5 growth stages of seven soybean lines and demonstrated an overall increase in N_2 fixation with the advance of growth cycle; however, the pattern of fixation was genotype- and environment-dependent.

Variations in the N_2 fixation activity throughout the growth cycle of soybean have been attributed to differences in the expression of genes that control nodule development (Kaewsuralikhit, Yokoyama, Kouchi, & Arima, 2005). However, information on the underlying genetic mechanism of symbiosis in soybean is still limited mainly due to the difficulties (Santos et al., 2013) and relatively high cost of accurately measuring the N_2 fixation rate. In this context, a limited number of studies have focused on the study of traits related to N_2 fixation (Nicolás et al., 2002). In addition to nodulation, some other traits, such as photosynthesis parameters, seed weight, seed protein, plant height, and time to maturity, have been used to discriminate soybean genotypes regarding their N_2 fixation capacity (Vollmann, Walter, Sato, & Schweiger, 2011).

Therefore, information on measurements that can help to identify soybean lines with enhanced N_2 fixation capacity throughout the soybean growth cycle is needed. Furthermore, evaluations of different soybean lines at different growth stages are critical for identifying genetic variation for high N_2 fixation. The objectives of this study were to: (1) assess the value of early and late evaluations of soybean lines for high N_2 fixation capacity; (2) test the association of measurements with traits directly and indirectly related to N_2 fixation; and (3) select lines that can be used for studying the underlying mechanisms involved in the genetic control and inheritance of traits responsible for high N_2 fixation capacity.

Material and methods

Plant material

Twenty-two soybean lines of different maturity groups (II–IX) and genetic backgrounds were selected for this study based on their potential N_2 fixation, nodulation, and higher yield in order to investigate the association of growth stages and N_2 fixation (Table 1). Additionally, three checks were included; the non-nodulating soybean cultivars Nitrasoy (Burton, Israel, & Bishop, 2006) and D68-099 (Hartwig, 1994) for estimating the amount of N derived from fixation as well as the supernodulating mutant SS2-2 (Youn et al., 2008; Youn et al., 2009) for evaluating the levels of N_2 fixation.

Greenhouse Experiments

The study was conducted under greenhouse conditions at Southern Illinois University, Carbondale, IL, USA. All the lines were sown in plastic pots (15 cm in diameter; 14 cm in depth) filled with Fafard Growing Mix 2 (Conrad Fafard Inc., Agawam, MA, USA), consisting of 70% peat moss, 20% perlite, and 10% vermiculite. A solution, containing 14 mg of P and 18 mg of K, was applied to each pot prior to sowing. The first cycle of the study was sown on February 6, 2015 and the second on December 12, 2015; plants were then grown until they reached the R7 growth stage (Fehr, Caviness, Burmood, & Pennington, 1971). Three seeds were planted per pot, and two weeks later thinned to a single plant per pot. The lines were assigned to the experimental units following a randomized complete block design with four blocks and four replications within each block. Plants were kept throughout the cycle in a 16-h photoperiod at 26/22ºC day/night. All the pots were watered once a day with a volume of water to prevent leaching of the ^{15}N fertilizer.

Inoculation with *Bradyrhizobium japonicum*

The *B. japonicum* strain USDA 110 was selected to inoculate soybean seeds due to its vastly and long term use in N_2 fixation soybean studies, and no additional strains were applied to prevent any interaction effects. USDA 110 was kindly provided by the USDA Soybean Genomics and Improvement Lab in Beltsville, MD, USA. The supplied sample was grown in liquid modified arabinose gluconate medium, pH 6.6 (Van Berkum, 1990) for 7 days in a shaking incubator until reaching the stationary phase. The inoculation was performed on the seed prior to sowing at a rate of 2×10^5 cells $seed^{-1}$.

^{15}N labelling

A solution of ^{15}N labeled urea with 10% atom ^{15}N excess was carefully applied to the growing medium of

each pot prior to sowing. The medium was removed individually from each pot and mixed with the labeled solution using a plastic bag to achieve uniform distribution, and then, the labeled medium was returned to each pot. The total amount of labeled N was 7.82 mg N kg^{-1} of potting medium. A plastic tray was placed underneath each pot to collect any leaching and prevent the ^{15}N labeled material to spread on the greenhouse benches.

Sampling

Sampling was performed at 35 d after emergence (DAE; early growth stage) and R7 (late growth stage). The upper most fully developed leaf was collected at the early growth stage and pod walls at the late growth stage. These samples were then oven dried at 70°C for 72h in a convection oven (Isotemp 500; Fisher Scientific, Waltham, MA, USA) and ball milled in steel vials using a 5100 Mixer/Mill (SPEX SamplePrep LLC, Metuchen, NJ, USA) for 15 min. After weighing and packing in tin capsules, the samples were placed in 96-well plates and sent to the Mass Spectrometry Facility of Southern Illinois University for the analysis of ^{15}N enrichment. Prior to the collection of leaf samples at the early growth stage, three measurements of chlorophyll concentration were obtained from the center of the leaflets using a Minolta SPAD-502 chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan), and the mean value was recorded as SPAD meter readings (SPD). Shoot, nodule, and seed samples were collected at the late growth stage and oven dried at 70°C for 72h prior to weighing and recording of shoot dry weight (SDW), nodule number (NN), nodule dry weight (NDW), total seed number (TSN), total seed weight (TSW), and mean seed weight (MSW). The number of days to maturity was recorded as DTM.

^{15}N Analysis and Total %N

For estimating ^{15}N enrichment and total %N, leaf and pod wall samples were analyzed using continuous flow elemental analysis isotope ratio mass spectrometry (CF-EA-IRMS) with a Thermo-Scientific Delta V Plus isotope-ratio mass spectrometer (Bremen, Germany) connected to a Costech 4010 Elemental Combustion System (Costech Analytical Technologies Inc., Valencia, CA, USA) via a Conflo IV unit. The measured ^{15}N abundance of each sample represented by the parameter atom% ^{15}N was subtracted from the natural abundance of ^{15}N in the atmosphere (0.3663 atom% ^{15}N) to obtain the atom% ^{15}N excess (Unkovich et al., 2008) in the leaf (LAT%) and pod wall samples (PAT%); the analysis also provided the percentage of N in the leaf (%LN) and pod wall (%PN) samples. The percentage of N derived from the atmosphere (%Ndfa) was estimated as follows (Unkovich et al., 2008):

$$\%\text{Ndfa} = \left(1 - \frac{\text{atom\%}^{15}\text{N excess } N_2 - \text{fixing plant}}{\text{atom\%}^{15}\text{N excess reference plant}}\right) \times 100$$

The non-nodulating lines Nitrasoy and D68-099 were used as references.

Statistical Analysis

All statistical analyses were performed using JMP 13 (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was performed in conjunction with Tukey's test to identify differences among lines or Dunnett's multiple comparison test for comparing the %Ndfa of each line at the early and late growth stages with that of the supernodulating check SS2-2. Evaluation cycle (EC) and the interaction between genotype and EC (G × EC) were considered as fixed effects, whereas block as a random effect.

Pearson's correlation was used to determine the relations among measurements and traits. A correlation coefficient was estimated for each pair-wise combination of traits related to N_2 fixation (Table 3) using JMP 13. To analyze the association of these traits with early and late fixation activity, the estimates of leaf (-LAT%) and pod (-PAT%) atom% ^{15}N excess were included. For these two parameters, the data were transformed using the negative value of each data point. This approach allowed to have an estimation of N_2 fixation activity for all genotypes, including the non-nodulating checks. Since the negative values of atom% ^{15}N excess were used, all the traits associated with the fixation activity yielded a positive correlation with -LAT% and -PAT%.

The Ward's minimum variance method (Ward, 1963) was used for clustering analysis. ANOVA was performed using the minimum variance criterion in the sum of squares to separate the lines in different clusters. These analyses were performed separately for the early and late growth stage and consequently, two dendrograms were constructed.

Results and discussion

Assessment of Di-nitrogen Fixation

Measurements of di-nitrogen fixation parameters at the early and late growth stages are presented in Table 1. The estimates of %Ndfa using D68-099 as a check were significantly different from those obtained using Nitrasoy as a check at the early (t = -9.5; $p < 0.01$) and late growth stages (t = 5.5; $p < 0.01$).

Table 1. Leaf (LAT%) and pod (PAT%) enrichment of ^{15}N and least squares means for early and late percentage of nitrogen derived from atmosphere (%Ndfa) for 22 lines, one supernodulating line, and two non-nodulating lines grown under greenhouse conditions for two growing seasons in ^{15}N-labelled soil.

Soybean line	Early (35 DAE) %Ndfa			Late (R7) %Ndfa		
	LAT%	D68-099	Nitrasoy	PAT%	D68-099	Nitrasoy
JTN-4307	0.1642	20	18	0.0121	85	88
JTN-5203	0.1759	14	12	0.0157	82	85
Osage	0.1469	27	25	0.0207	74	81
Ozark	0.1517	27	24	0.0138	84	87
R05-3239	0.1671	20	18	0.0216	74	79
Jake	0.1727	16	14	0.0149	83	86
Saluki 4910	0.1837	12	9	0.0148	83	85
Bragg	0.1784	14	12	0.0091	90	91
Davis	0.1398	31	29	0.0098	89	91
Enrei	0.1498	26	25	0.0679	20	32
Williams	0.1512	27	25	0.0180	80	82
PI 471938	0.1793	10	9	0.0139	82	86
Clark	0.1771	12	10	0.0118	87	88
Bossier	0.1537	25	24	0.0091	89	91
Centennial	0.1618	22	20	0.0124	85	88
Hardee	0.1546	26	24	0.0109	87	90
Jackson	0.1822	11	9	0.0267	67	73
S.J.2	0.1968	4	2	0.0105	88	90
J-200	0.1724	14	13	0.0161	83	84
R01-416F	0.1529	25	23	0.0128	85	87
PI 96169B	0.1890	7	6	0.1322	0	0
PI 96171	0.1469	27	25	0.0632	26	34
Supernodulating check						
SS2-2	0.1255	38	38	0.0129	86	88
Non-nodulating checks						
Nitrasoy	0.2023			0.1071		
D68-099	0.2080			0.0930		
Analysis of variance						
Genotype (G)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Evaluation Cycle (EC)	< 0.0001	< 0.0001	< 0.0001	ns	ns	ns
G x EC	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Multiple comparison test						
Tukey HSD 95%	0.0443	22	21	0.0277	32	27

However, differences in ranking were minor, and the estimates for %Ndfa obtained from the two checks were highly correlated at both the early (r = 0.97; $p < 0.0001$) and late growth stages (r = 0.91; $p < 0.0001$). The LSmean of atom% ^{15}N excess was used as an auxiliary criterion for distinguishing the capacity of N_2 fixation of each line (Table 1).

All the estimates of %Ndfa were significantly affected by G ($p < 0.0001$), EC ($p < 0.0001$), and G×EC ($p < 0.0001$), except for that at the late growth stage that was not affected by EC. These results showed that the host control of N_2 fixation in soybean has an important contribution to the overall symbiotic N_2 fixation, but also that this trait is strongly affected by environmental factors.

At the early growth stage, S.J.2 and Davis showed the lowest and highest %Ndfa, respectively, and the estimates varied from 4–31% and 2–29% when using D68-099 and Nitrasoy as a check, respectively. The proportion of N derived from fixation at the early growth stage was in accordance to that reported by George and Singleton (1992), in which the average was 28% for the total N fixed during all the vegetative growth stages (George and Singleton, 1992). The limitation of soybean to supply N through N_2 fixation during the early growth stages has been well documented and can be attributed to the inability of the symbiotic rhizobia to supply all the required N by the crop (Phillips & DeJong, 1984; Keyser & Li, 1992).

At the late growth stage, PI96169B and Bragg showed the lowest and highest %Ndfa, respectively, and the estimates varied from 0–90% and 0–91% when using D68-099 and Nitrasoy as a check, respectively. Except for PI96169B that did not form any nodules with USDA 110, %Ndfa values estimated in the present study were in accordance to those reported by Salvagiotti et al. (2008) that in a review paper of 61 studies on soybean N_2 fixation reported a range of 58–98%.

Our results showed that atom% ^{15}N excess in leaf (LAT%) and pod walls (PAT%) were significantly influenced by G ($p < 0.0001$), EC ($p < 0.0001$), and G × EC ($p < 0.0001$), except for PAT% that was not affected by EC. Of these parameters, the high values for atom% ^{15}N excess indicated a reliance of the line on the N derived from the soil instead of that obtained from N_2 fixation. This pattern was confirmed by the higher enrichment of leaf and pod wall samples in both non-nodulating

lines. The only exception was LAT% and PAT% of PI96169B, probably due to an incompatibility between the line and USDA 110; however, further studies need to be conducted to confirm this assumption. Davis and S.J.2 showed the lowest and highest LAT%, whereas Bossier and PI96169B showed the lowest and highest PAT%, respectively.

The overall high proportions of N derived from fixation identified at the late growth stage were consistent with those found by Harper (1987) that studied N_2 fixation under low soil-N conditions and reported that N derived from fixation was 80–94%. Although the average contribution of N_2 fixation at the late growth stage ($mean_{\%Ndfa}$ = 66.3; data not shown) was markedly higher than that at the early growth stage ($mean_{\%Ndfa}$ = 17.3; data not shown), the level of genetic variation followed the opposite trend. These results were in agreement with those reported by Pazdernik et al. (1996) that analyzed 20 soybean lines for early N_2 fixation and nodulation efficiency and suggested that early nodule formation can markedly improve N_2 fixation capacity.

We used the Dunnett's test to compare the N_2 fixation capacity of each line with that of the supernodulating check SS2-2. At the early growth stage, the %Ndfa of nine lines (Davis, Enrei, R01-416F, Williams, PI96171, Osage, Hardee, Bossier, and Ozark) did not show any significant differences from that of SS2-2 (Figure 1), indicating their high N_2 fixation capacity at the early growth stage. The remaining 13 lines yielded a significantly lower %Ndfa compared with that of SS2-2, demonstrating a limited N_2 fixation capacity at the early growth stage.

At the late growth stage, the %Ndfa of 19 lines (Bossier, S.J.2, Davis, Bragg, Hardee, Centennial, JTN-4307, Clark, Ozark, R01-416F, Jake, Saluki 4910, PI471938, JTN-5203, J-200, Williams, Osage, R05-3239, and Jackson) did not show any significant differences from that of SS2-2 (Figure 2). PI96169B, PI96171, and Enrei showed %Ndfa significantly different from that of the supernodulating check, indicating their poor performance in N_2 fixation at the late growth stage.

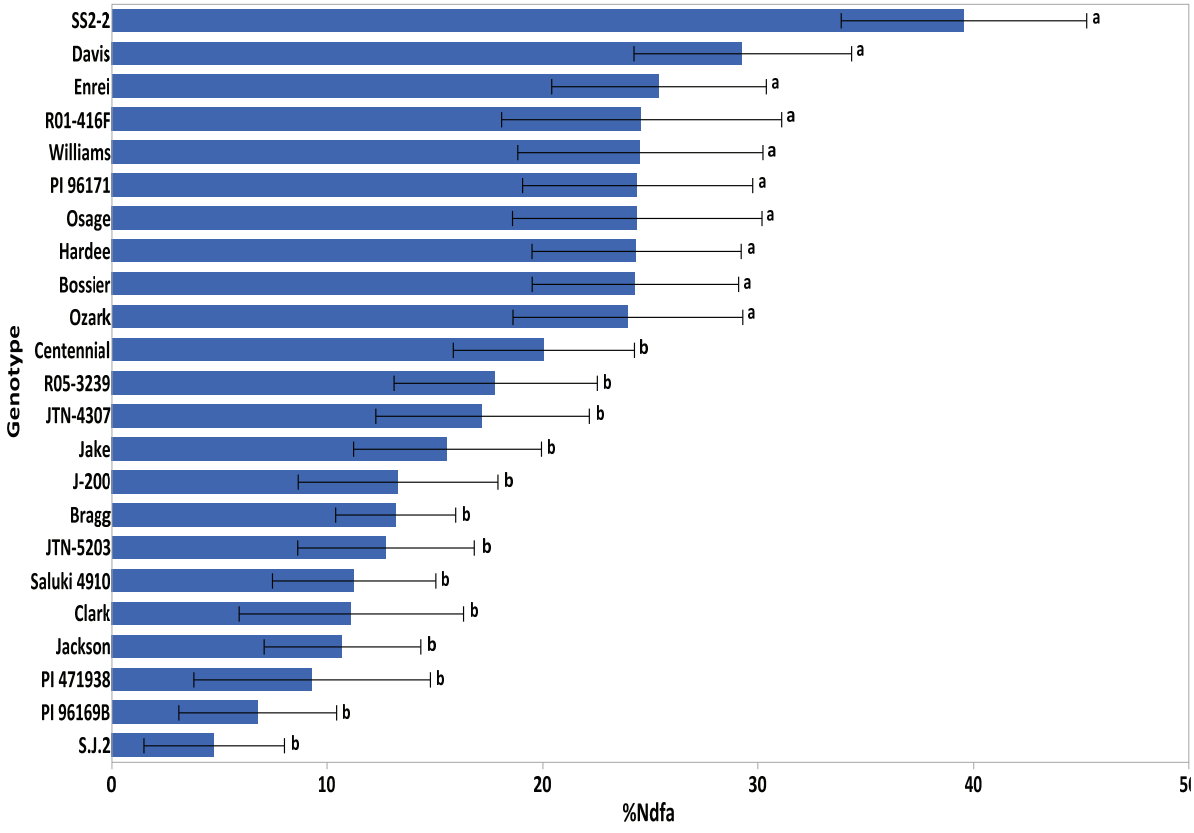

Figure 1. Comparison of %Ndfa at the early growth stage (35 d after emergence) between each of the 22 lines and the supernodulating line, SS2-2, using the Dunnett's multiple comparison test. Different letters indicate significant differences between the line and the check. Error bars indicate one standard error of the mean.

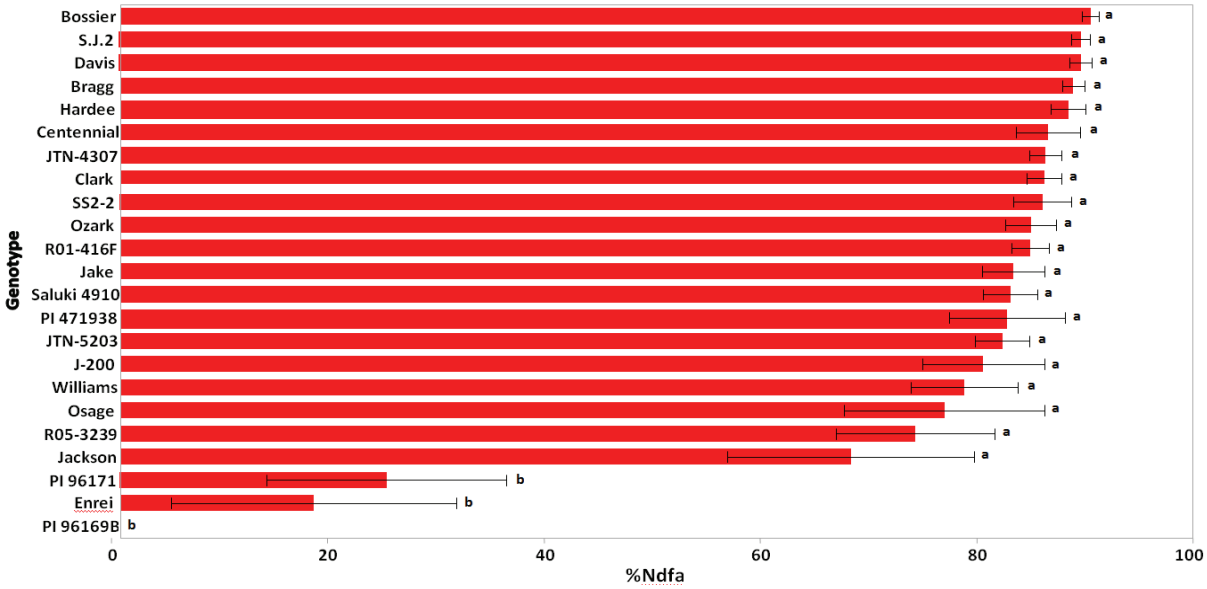

Figure 2. Comparison of %Ndfa at the late growth stage (R7) between each of the 22 lines and the supernodulating line, SS2-2, using the Dunnett's multiple comparison test. Different letters indicate significant differences between the line and the check. Error bars indicate one standard error of the mean.

The contribution of N_2 fixation to the total N accumulated during the vegetative period is estimated to be 21%, markedly lower than that at the R3–R7 growth stages that is approximately 56% (Zapata et al., 1987). In the present study, given the substantial variability found among the lines in the measurement of early N_2 fixation, we compared the mean of the two groups of lines discriminated using the Dunnett's test, and a difference of 14% in %Ndfa was found (data not shown); this difference in early N_2 fixation capacity might help to increment the accumulation of N derived from fixation during the vegetative period.

Further studies are needed to investigate the identified restriction of N_2 fixation in the vegetative phase. This limitation is markedly higher in the beginning of growth cycle, and thus, various studies have reported the benefits of N application at the early growth stages of soybean, a practice that is commonly known as 'starter N'. Osborne and Riedell (2006) obtained a 6% yield increase when 16 kg N ha^{-1} was applied at planting.

Traits related to N_2 fixation

The mean values of the 10 N_2 fixation-related traits (SPD, SDW, TSW, TSN, MSW, L%N, P%N, NN, NDW, and DTM) assessed in the 22 studied lines as well as the non-nodulating and supernodulating checks are presented in Table 2. The results showed that all the 10 N_2 fixation-related traits assessed in this study were significantly ($p < 0.001$) affected by G, EC, and G×EC, except for MSW that was not affected by G×EC or L%N that was not affected by EC. Among the 10 N_2 fixation-related traits, NN and NDW were the most directly related to nodulation; and the supernodulating check was significantly different from all the studied lines ($p < 0.0001$) for these two nodulation traits. This indicates that the studied lines had an adequate nodulation, but none of them reached the nodulation pattern of the supernodulating check. Although a superior nodulation ability is desirable for breeding lines with higher N_2 fixation capacity, the level of nodulation displayed by supernodulating mutants can restrict root growth and lead to a 30–40% yield reduction (Day, Lambers, Bateman, Carroll, & Gresshoff, 1986; Gremaud & Harper, 1989; Wu & Harper, 1991; Herridge, 2003). The MSW of Enrei was significantly ($p < 0.0001$) higher than that of the supernodulating check and all the other lines. The non-nodulating checks Nitrasoy and D68-099 performed poorly or showed the lowest values for P%N, SDW, MSW, L%N, TSW, and TSN. Additionally, Bossier showed the highest SDW, Enrei the highest SPD, and R01-416F the highest P%N.

A previous study that assessed soybean plants under controlled conditions demonstrated that the mean seed weight, total biomass, net photosynthetic output, and total plant N are related to both N_2 fixation and seed yield and that these effects were more pronounced at the pod-fill stage (Imsande, 1989). Analogous traits studied herein also accompanied the N_2 fixation activity among the studied lines; the supernodulating check showed the highest values of TSW, TSN, and L%N, whereas the non-nodulating checks the lowest values, indicating an overall robust relationship of these traits with the N_2 fixation activity.

Table 2. Comparison of nitrogen fixation-related traits among 22 lines, one supernodulating line, and two non-nodulating lines grown under greenhouse conditions for two growing seasons in ^{15}N-labelled soil.

	SPD	SDW	TSW	TSN	MSW	L%N	P%N	NN	NDW	DTM
		g plant^{-1}	g plant^{-1}	number plant^{-1}	mg seed^{-1}	%	%	number plant^{-1}	mg plant^{-1}	number of days
JTN-4307	28.3	10.3	4.6	48.8	98	2.59	0.99	107.5	192	170
JTN-5203	26.6	7.2	2.8	34.8	76	2.51	1.58	55.8	139	164
Osage	25.5	12.9	3.6	42.4	82	2.86	1.10	89.7	205	176
Ozark	25.1	7.2	2.1	24.3	87	2.21	1.65	54.5	101	166
R05-3239	28.0	7.5	2.4	28.2	85	2.45	1.48	70.3	128	162
Jake	26.6	8.6	3.0	29.2	96	2.46	1.28	60.4	105	163
Saluki 4910	26.2	9.1	3.2	34.6	88	2.60	1.39	103.9	159	171
Bragg	20.1	14.0	2.7	30.8	108	1.88	1.16	136.1	245	180
Davis	24.8	10.9	1.9	20.9	73	2.63	1.64	56.4	100	175
Enrei	34.3	3.6	3.7	14.2	250	2.85	0.61	43.7	190	97
Williams	22.4	7.9	4.6	36.7	125	2.18	0.94	119.0	235	152
PI 471938	23.7	9.8	3.1	31.9	85	2.05	1.51	68.5	107	161
Clark	21.2	9.8	3.8	37.9	96	2.01	1.26	71.7	162	161
Bossier	23.4	15.6	2.6	31.7	77	2.38	1.32	154.0	294	186
Centennial	24.3	12.2	3.4	35.5	90	1.93	1.24	128.0	249	177
Hardee	25.5	12.2	2.1	26.9	68	2.25	1.45	81.4	156	189
Jackson	22.6	13.3	3.2	31.3	95	2.13	0.81	95.1	184	169
S.J.2	23.1	12.7	1.2	17.6	45	2.11	1.31	94.5	213	196
J-200	23.7	14.6	1.3	15.5	70	1.96	1.69	88.6	132	196
R01-416F	26.2	8.0	2.5	27.1	80	2.44	1.84	92.9	167	159
PI 96169B	27.7	3.0	1.6	10.3	163	1.85	0.47	2.9	32	107
PI 96171	30.9	3.5	1.9	16.4	117	2.47	0.85	40.1	139	108
Supernodulating check										
SS2-2	31.3	9.8	4.6	48.8	112	3.49	1.58	294.1	541	157
Non-nodulating checks										
Nitrasoy	21.7	4.0	0.4	7.3	54	1.58	0.73	0	0	179
D68-099	23.9	2.9	0.5	8.5	50	1.92	0.78	0	0	184
Analysis of variance										
Genotype (G)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Evaluation Cycle (EC)	< 0.0001	< 0.0001	< 0.0001	<0.0002	< 0.0001	ns	< 0.0001	< 0.0001	< 0.0001	< 0.0001
G x EC	< 0.0001	< 0.0001	< 0.0001	< 0.0001	ns	0.0437	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Multiple comparison test										
Test mean	25.5	9.2	2.9	28.8	100	2.32	1.19	84.8	168	165
Tukey 95%	5.4	3.2	1.8	18.2	70	0.86	0.60	79.9	151	21

SPD, SPAD readings (mg chlorophyll m^{-2}); SDW, shoot dry weight (g plant^{-1}); TSW, total seed weight (g plant^{-1}); TSN, total seed number per plant; MSW, mean seed weight (mg seed^{-1}); L%N, leaf % nitrogen; P%N, pod % nitrogen, NN, number of nodules per plant; NDW, nodule dry weight (mg plant^{-1}); DTM, d to maturity.

Table 3. Correlation matrix of ^{15}N enrichment and N fixation related traits for 22 lines, one supernodulating line, and two non-nodulating lines grown under greenhouse conditions for two growing seasons in ^{15}N-labelled soil.

	NN	NDW	TSN	TSW	MSW	DTM	SPD	SDW	L%N	P%N	-LAT%	-PAT%
NN	1.00	0.88**	0.33**	0.20**	-0.10**	-0.12**	-0.18**	0.39**	0.07^{ns}	0.20**	0.07^{ns}	0.27**
NDW		1.00	0.39**	0.30**	-0.01^{ns}	-0.16**	-0.11**	0.39**	0.08*	0.11**	0.09*	0.26**
TSN			1.00	0.78**	-0.02^{ns}	0.06^{ns}	-0.01^{ns}	0.31**	0.10*	-0.13**	0.20**	0.34**
TSW				1.00	0.40**	-0.06^{ns}	0.10*	0.12**	0.09*	-0.29**	0.24**	0.26**
MSW					1.00	-0.31**	0.23**	-0.27**	0.09*	-0.32**	0.08*	-0.18**
DTM						1.00	0.16**	0.26**	0.01^{ns}	-0.04^{ns}	0.16**	0.35**
SPD							1.00	-0.28**	0.44**	-0.23**	0.43**	-0.10**
SDW								1.00	0.00^{ns}	0.21**	0.03^{ns}	0.47**
L%N									1.00	0.10*	0.12**	0.09*
P%N										1.00	-0.18**	0.35**
-LAT%											1.00	0.15**
-PAT%												1.00

*, **. Significance at $p < 0.05$ and $p < 0.01$, respectively. NN, number of nodules per plant; NDW, nodule dry weight (mg plant^{-1}); TSW, total seed weight (g plant^{-1}); TSN, total seed number per plant; MSW, mean seed weight (mg seed^{-1}); DTM, d to maturity; SPD, SPAD readings (mg chlorophyll m^{-2}); SDW, shoot dry weight (g plant^{-1}); L%N, leaf % nitrogen; P%N, pod % nitrogen; -LAT%, negative leaf atom% ^{15}N excess; -PAT%, negative pod atom% ^{15}N excess.

Correlation analysis

A weak, but significant, correlation between the early -LAT% and the late -PAT% ($r = 0.15$; $p < 0.01$) suggested significant differences in N_2 fixation profile among the lines, showing that the superior N_2 fixation during the early growth stages did not last until the late growth stages. The strongest correlation was identified between -LAT% and SPD as well between -PAT% and SDW (Table 3). Since N_2 fixation was related with different traits at the early and late growth stages this confirmed the independence of the two evaluations. The variability in the N_2 fixation pattern of each line and the traits related to each of the measurements might be a promising tool for selecting lines with improved N_2 fixation capacity in breeding programs.

The nodulation parameters NN and NDW were found to be highly correlated ($r = 0.88$; $p < 0.01$), indicating that only one of those needs to be used for

screening lines for N_2 fixation capacity. Additionally, both NN and NDW were moderately correlated with SDW (r =0.39; $p < 0.01$). This result might reflect the influence of the N derived from the nodules in the biomass accumulation. Mastrodomenico and Purcell (2012) reported that the N derived from fixation is largely accumulated into the biomass; however, a large portion of this N is not remobilized to the seed.

At the late growth stage, -PAT% was moderately correlated with SDW (r = 0.47; $p < 0.01$), similarly as reported by Herridge et al. (1990), in which *P* that represented the proportion of N derived from fixation was significantly correlated with the crop dry matter (r = 0.41; $p < 0.05$); however, the coefficient of correlation between NDW and -PAT% (r = 0.26; $p < 0.01$) found in the present study was markedly lower than that between nodule weight and *P* (r = 0.79; $p < 0.001$).

The coefficient of correlation between NN and -PAT% (r = 0.27; $p < 0.01$) was similar to that reported by Houngnandan et al. (2008) between nodule number and $\delta^{15}N$ (r = 0.325*) in a diverse group of soybean cultivars. Additionally, Pazdernik, Graham, and Orf (1997) studied a soybean population to investigate early nodulation traits and found that %Ndfa at R5 was significantly correlated with nodule fresh weight (r = 0.33; $p < 0.01$).

Correlation analysis also revealed associations with traits that might be used to reduce the cost of population screening for N_2 fixation capacity. SDW was moderately correlated with NN (r = 0.39; $p < 0.01$) and NDW (r = 0.39; $p < 0.01$), which are commonly used to improve N_2 fixation in soybean, but can impose practical and economical limitations in the screening of large populations. The correlation between SPD and L%N (r = 0.44; $p < 0.01$) might also be useful due to the lower cost to assess the former. Additionally, the correlation of TSN with NN (r = 0.33; $p < 0.01$) and NDW (r = 0.39; $p < 0.01$) could be advantageous, since the screening of the former is less laborious than that of the latter two.

Cluster analysis

Considerable variation was identified among the 22 soybean lines and the supernodulating check for N_2 fixation at the early and late growth stages, which was also demonstrated by the weak correlation between %Ndfa at the early and late growth stage (r = 0.14; $p < 0.001$) using both checks. These results confirmed the overall different performance of lines regarding the N_2 fixation capacity at different growth stages.

At the early growth stage, cluster analysis classified the 22 lines and SS2-2 into three different clusters (Figure 3): Cluster 1 included 10 lines with a high N_2 fixation capacity; Cluster 2 included 12 lines with a low N_2 fixation capacity; and Cluster 3 included only SS2-2 that had the highest N_2 fixation activity.

At the late growth stage, the 22 lines and the check SS2-2 were also classified into three different clusters: Cluster 1 included 20 lines with a high N_2 fixation capacity; Cluster 2 included Enrei and PI96171 with a low N_2 fixation capacity; and Cluster 3 included PI96169B that had the lowest N_2 fixation activity.

Soybean is estimated to spend 5.2–18.8 g of C g^{-1} of fixed N_2 (Minchin & Witty, 2005). To meet both the requirements of photosynthate from the plant and the N_2-fixing rhizobia, the elevated N_2 fixation activity is likely to be accompanied by a higher photosynthetic rate (Kaschuk, Hungria, Leffelaar, Giller, & Kuyper, 2010). Thus, the identification of this pattern may allow to develop new lines without compromising the photosynthate required for other physiological processes.

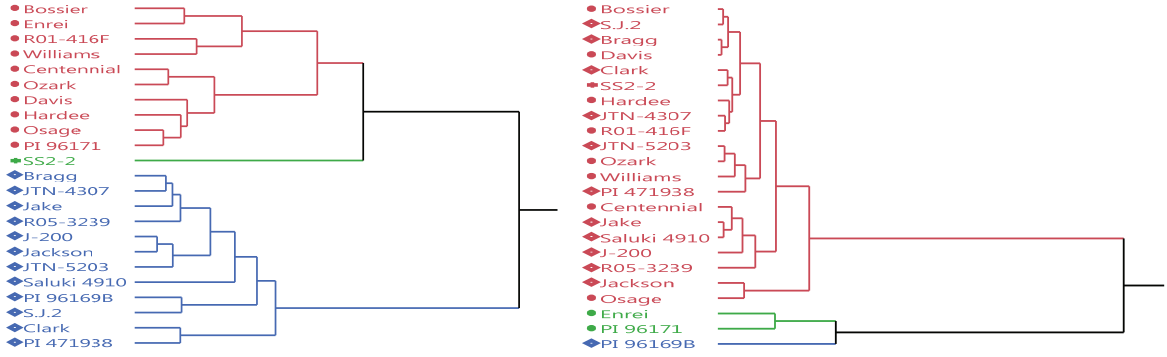

Figure 3. Clustering of 23 soybean lines and the two non-nodulating lines, Nitrasoy and D68-099, based on %Ndfa at the early (35 days after emergence; left) and late (R7; right) growth stages using the Ward's method.

In the present study, the identified variation in N_2 fixation activity during the plant cycle might be associated with the time and pattern of nodule formation on soybean roots. Nodules that are formed during the early growth stages are usually located at the top of the main root and are known to last on average 65 days. Later in the growth cycle, the N_2 fixation activity is maintained by a secondary set of nodules that is usually located on deep and lateral roots (Keyser & Li, 1992; Zapata et al., 1987). In the nodule aging process, the peak of N_2 fixation activity occurs at 4–5 weeks after the infection of plant cells by rhizobia; then, the nodules start to senesce and the rate of N_2 fixation is decreased (Dupont et al., 2012). Nonetheless, further research is needed on the formation and aging of nodules in soybean and its effect on N_2 fixation activity.

Conclusion

Overall, our results revealed significant variation in N_2 fixation capacity among the studied lines and also, between and within the early and late growth stages. The traits that were found closely related to N_2 fixation as well as the lines with contrasting N_2 fixation capacity at the early and late growth stages could be used in future studies to better understand the underlying physiological and molecular mechanisms of N_2 fixation in soybean.

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