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Differences in photosynthetic behaviour and leaf senescence of soybean (*Glycine max* [L.] Merrill) dependent on N_2 fixation or nitrate supply

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ABSTRACT

Biological N_2 fixation can fulfil the N demand of legumes but may cost as much as 14% of current photosynthate. This photosynthate (C) sink strength would result in loss of productivity if rates of photosynthesis did not increase to compensate for the costs. We measured rates of leaf photosynthesis, concentrations of N, ureides and protein in leaves of two soybean cultivars (*Glycine max* [L.] Merrill) differing in potential shoot biomass production, either associated with *Bradyrhizobium japonicum* strains, or amended with nitrate. Our results show that the C costs of biological N_2 fixation can be compensated by increased photosynthesis. Nodulated plants shifted N metabolism towards ureide accumulation at the start of the reproductive stage, at which time leaf N concentration of nodulated plants was greater than that of N-fertilized plants. The C sink strength of N_2 fixation increased photosynthetic N use efficiency at the beginning of plant development. At later stages, although average protein concentrations were similar between the groups of plants, maximum leaf protein of nodulated plants occurred a few days later than in N-fertilized plants. The chlorophyll content of nodulated plants remained high until the pod-filling stage, whereas the chlorophyll content of N-fertilized plants started to decrease as early as the flowering stage. These results suggest that, due to higher C sink strength and efficient N_2 fixation, nodulated plants achieve higher rates of photosynthesis and have delayed leaf senescence.

INTRODUCTION

Biological N_2 fixation can fulfil the N demand of legume crops such as soybean (*Glycine max* [L.] Merrill), resulting in a significant increase in plant total N accumulation and a higher N concentration in seeds (Imsande 1988) compared with N-fertilized plants. However, in terms of N acquisition, these benefits are accompanied by an increase in respiration costs of 14% or more of current photosynthesis when compared with N-fertilized soybean (Finke *et al.* 1982; Kaschuk *et al.* 2009). Nitrate assimilation results in costs of up to 2.5 g C g^{-1} N assimilated, whereas N_2 fixation costs 5.2–18.8 g C g^{-1} N (Minchin & Witty 2005). Therefore, N_2 fixation will be limited by photosynthate availability if there is no simultaneous increase in rates of photosynthesis (Lawn & Brun 1974; Abu-Shakra *et al.* 1978; Fujita *et al.* 1988; Imsande 1988).

As N is essential for synthesis of Rubisco – the main enzyme in CO_2 fixation – and for synthesis of light-harvesting chlorophylls, N_2 fixation could enhance leaf N concentration and therefore stimulate photosynthesis (Evans 1989; Hikosaka & Terashima 1995). However, leaf N concentration and photosynthesis increase linearly only until a critical N concentration is reached in the leaves (*e.g.* Robertson *et al.* 2002). Beyond that, it is likely that a further increase in leaf N concentration will result in partial deactivation of the photosynthetic machinery (Mächler *et al.* 1988; Hikosaka & Terashima 1995; Cheng & Fuchigami 2000). Furthermore, the rates of photosynthesis also respond to factors other than leaf N concentration, such as environmental conditions and changes in the source:sink ratio of the plant (Lawn & Brun 1974; Mondal *et al.* 1978; Wittenbach 1982, 1983; Crafts-Brandner & Egli

1987; Ainsworth *et al.* 2004). There are reports showing that a decrease in the sink:source ratio, by removing pods at the reproductive stage, decreases the rate of photosynthesis in soybean (Wittenbach 1982, 1983; Crafts-Brandner & Egli 1987). In addition, probably because of changes in the sink:source ratio, the absence of nodules decreases the response of photosynthesis to elevated CO_2 (Ainsworth *et al.* 2004). Therefore, increases in the sink:source ratio due to higher C costs of N_2 fixation compared with NO_3^- uptake (Minchin & Witty 2005) are likely to increase the rate of photosynthesis of soybean in symbiosis, regardless of the N effect, due to changes in the sink:source ratio.

We performed a study to examine the effect of soybean inoculation with efficient N_2-fixing rhizobia on photosynthesis on a leaf area basis in comparison with N-fertilized plants. Our first hypothesis is that increased C sink strength from N_2 fixation leads to an increase in rates of leaf photosynthesis, regardless of the N effect. We also predict that nodulated plants with a lower shoot biomass increase leaf photosynthesis more than nodulated plants with a higher shoot biomass. Our second hypothesis is that increased photosynthesis combined with efficient N_2 fixation increases the duration of leaf activity in photosynthesis and thereby delays leaf senescence.

MATERIAL AND METHODS

Experiment 1

Two different soybean cultivars were subjected to four treatments in the glasshouse: two rhizobial strains and two N treatments, each applied separately. In the inoculation treatments, two different strains of *Bradyrhizobium japonicum* [CPAC 7 (= SEMIA 5080) and CPAC 390] were used. The soybean cultivars were BRS 154 and BRS 262, both early cultivars with high yield potential but differing in potential harvest index. Plants were planted in 2.5-kg capacity plastic pots filled with a mixture of sand and vermiculite (1:1). Sand was soaked overnight in 5% hydrochloric acid, washed thoroughly with distilled water, mixed with vermiculite and autoclaved at 120 °C for 1 h. Seeds were surface-sterilized before sowing (soaked in 96% alcohol for 1 min; 0.25% sodium hypochlorite for 3 min; and rinsed with sterile distilled water four times). All plants received sterilized N-free nutrient solution (Broughton & Dilworth 1971) with the pH adjusted to 6.8. The non-inoculated plants received two different doses of KNO_3, consisting of 175 or 350 mg N, split into five applications until completion of stage R4 (45 days, pod 2 cm long at one of the four uppermost nodes with a completely unrolled leaf; Fehr *et al.* 1971). Rhizobia were grown in yeast medium according to Vincent (1970) to a density of 10^9 cells ml^{-1}, and 1 ml of this suspension was pipetted onto each seed of the inoculated treatments at sowing. Seven seeds were sown in each pot, and plants were thinned to one plant per pot at stage V1 (5 days, completely unrolled leaf at the unifoliolate node; Fehr *et al.* 1971). There were 16 replicates of each treatment at the beginning of the experiment. Four replicates of each treatment were harvested at V4 (25 days, four nodes on the main stem beginning with the unifoliate node; Fehr *et al.* 1971), four replicates at R2 (37 days, flower at node immediately below the uppermost nodes with a completely unrolled leaf), four at R4 and the last four at R5 (50 days, seeds beginning to develop; Fehr *et al.* 1971). Each set of treatments was arranged in a completely randomized design.

The experimental station is located in Londrina, Brazil (23°11′ S), where the daylength was 11 h·45 min on 17 May 2007, 11 h·31 min on 19 June 2007 and 11 h·33 min on 3 July 2007. The photosynthetically active radiation (PAR) in the greenhouse varied from 400 to 600 μmol photons $m^{-2}{\cdot}s^{-1}$ during the experimental period. Temperatures during the experiment were on average 32/21 °C (day/night).

Experiments 2 and 3

The above experiment was repeated in the following year and under similar conditions to experiment 1. Temperatures during the experimental period were on average 33/22 °C (day/night) and PAR varied from 400 to 600 μmol photons $m^{-2}{\cdot}s^{-1}$. The daylength was 13 h·34 min on 18 February 2008 and 11 h·58 min on 2 May 2008. Four replicates of each treatment were harvested at R2 to evaluate nodulation, starch and soluble sugar content (Experiment 2). Four other replicates of each treatment served for measurement of leaf chlorophyll content from the R2 stage onwards (from here onwards, considered as Experiment 3).

Analyses of photosynthesis

At the time of each measurement, plants were first removed from the glasshouse to open air in order to increase exposure to solar radiation, then, after 30 min of acclimatisation, photosynthetic rates were measured on the third expanded leaf between 10:00 and 11:00 h. Gas exchange was measured using a LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA), at saturating light of 1500 μmol photons $m^{-2}{\cdot}s^{-1}$. During the measurements, leaf-to-air vapour pressure deficit varied between 2.2 and 3.0 kPa, relative humidity between 32% and 41% and leaf temperature between 28 and 33 °C (measured with a thermocouple in the leaf chamber).

Shoot sampling

The leaves used for measurements of photosynthesis were detached from the stems, immediately weighed, frozen in liquid nitrogen and stored in a freezer at –80 °C. Parts of the leaves reserved for ureide analysis were used to estimate moisture content to express the data on a dry

weight basis. Roots and nodules were carefully washed with tap water and dried at 60 °C for 48 h. Nodules were then detached, counted and weighed. Shoots were dried, weighed and added to the weights of the leaves used in the above analyses.

Leaf N content and photosynthetic N use efficiency

Total N was extracted from 100 mg of dry ground leaves using the Kjeldahl method as described by Alves *et al.* (1994). Photosynthetic N use efficiency (PNUE) was calculated as the ratio of rate of photosynthesis [actual rate (μmol CO_2 $m^{-2}{\cdot}s^{-1}$) × 0.044 to obtain mg CO_2 $m^{-2}{\cdot}s^{-1}$] and N content in the leaves [N ($mg{\cdot}g^{-1}$) × 54.125 $g{\cdot}m^{-2}$ leaf]. The constant 54.125 $g{\cdot}m^{-2}$ was obtained by averaging the specific leaf weight of 24 soybean genotypes by Hesketh *et al.* (1981).

Leaf ureide-N

After weighing, frozen leaves were dried at 60 °C to constant mass, and ground. One hundred mg were used to extract ureides according to Hungria (1994). Ureides were determined according to the method of Vogels & van der Drift (1970).

Leaf protein content

About 1.3 g of frozen fresh leaves was ground using a mortar and pestle in liquid nitrogen, followed by extraction using 15 ml of buffer as described by Catt & Millard (1988). The homogenate was incubated on ice with rotation in a laminar flow hood for 2 h. The homogenate was centrifuged at 12,000 *g* for 20 min and the supernatant was filtered through 45-µm pore membranes. The soluble protein content was determined using a colorimetric assay (Bradford 1976).

Leaf soluble sugars and starch

Leaves were harvested in the afternoon (16:30 h), immediately frozen in liquid N, and stored at –80 °C until required, ground under liquid N and a 150–200 mg aliquot transferred to 2-ml tubes. The samples were washed with 100% acetone, stirred and centrifuged at 6000 *g* for 5 min several times until the supernatant was yellow-beige and clear. The tubes were opened and the samples dried in a laminar flow chamber. The pellets were suspended in 1.5 ml 80% ethanol, stirred for a few seconds, placed in boiling water for 20 min, and centrifuged three times each at 10,000 *g* for 10 min. The supernatants of each sample were combined and stored in a refrigerator. The supernatant contains the soluble sugars and the pellet contains the starch. The soluble sugars were determined based on the method described by Dubois *et al.* (1956). Total starch was analysed according to the enzymatic method of McClearly *et al.* (1997) using a commercial assay kit (K-TSTA; Megazyme International Ireland Ltd, Bray, Ireland).

Chlorophyll content

Chlorophyll was determined with a chlorophyll meter SPAD 502 (Konica Minolta Sensing, Inc., Osaka, Japan). Each replicate consisted of an average of three SPAD values, measured at different points on a leaf. The leaves were labelled for subsequent measurements. The average SPAD values were converted to chlorophyll content (mg chlorophyll m^{-2} leaf) based on a preliminary calibration. The calibration consisted of measuring SPAD values in three spots of 30 different soybean leaves. From each spot, a leaf disc (3.67 cm^2) was punched. Leaf disks were submerged in 25 ml 80% acetone and the flasks were covered with aluminium foil and incubated in the dark at 10 °C for 72 h. The absorbances of the samples were read at 645 and 663 nm, as recommended by Linder (1974). The chlorophyll content was calculated on a leaf area basis, with the equation provided by Arnon (1949), using the coefficients derived by McKinney (1941).

Maximum protein values and photosynthesis rates

The rates of photosynthesis and protein content were analysed by fitting quadratic regression equations as variables dependent on time (days after emergence) with SPSS 15.0.1 for Windows (SPSS Inc., Chicago, IL, USA, 1989–2006). The quadratic functions were differentiated to determine maximum values (days). The maximum values of photosynthesis and protein content were obtained after fitting the original quadratic functions with the maximum values of the derivative functions.

Statistical analyses

The experimental design was a split plot, with soybean cultivar as the main factor and N source (*i.e.* two rhizobia strains *versus* two rates of N fertilization) as the split-plot factor (n = 4). The data set was tested for homogeneity with Levene's test of equality of error variances and Q-Q plots to test for normality of the data. Data on N, protein and ureide-N content were log-transformed to achieve near-normal distributions. Shoot biomass, nodules, photosynthesis, N, protein, ureide-N, PNUE, sugars and starch were treated as independent measurements. The N-fertilized plants that eventually produced nodules were discarded from the analyses. The data set was analysed considering an unbalanced treatment structure using GENSTAT 11th edition (VSN International Ltd, Hemel Hempstead, UK, 2008). The square root of chlorophyll content (Experiment 3) against time was considered as repeated measurements. Each pairing of cultivar or N source was analysed independently with the *F*-test at $P < 0.05$.

Table 1. Nodule and shoot biomass, N-ureide and protein in leaves of two soybean cultivars inoculated with two *Bradyrhizobium japonicum* strains or receiving two rates of N-fertilizer (Experiment 1).

cultivar and N source	nodule (g DW/plant)				shoot (g DW/plant)			leaf N-ureide (μmol N-ureide g^{-1} DW)				leaf protein[b] (mg·g^{-1} DW)			
	V4	R2	R4	R5	V4	R2	R4	V4	R2	R4	R5	V4	R2	R4	R5
BRS 154															
B. japonicum CPAC 7	0.5	0.6	0.8	0.9	1.4	2.5	6.2	69.4	85.8	181.7	118.8	47.5	50.8	56.8	37.1
B. japonicum CPAC 390	0.6	0.7	0.7	0.8	1.3	2.3	6.4	67.9	145.3	135.9	160.7	59.1	65.5	53.4	35.2
KNO_3 (350 mg N)	0	0	0	0	1.7	2.6	10.9	114.3	73.4	25.0	14.5	65.0	61.1	40.2	38.7
KNO_3 (175 mg N)	0	0	0	0	1.3	2.9	8.2	150.5	53.4	22.2	11.5	76.7	83.6	50.1	49.1
BRS 262															
B. japonicum CPAC 7	0.8	0.8	0.8	0.8	1.5	3.1	7.5	95.6	72.2	106.9	11.8	47.0	60.8	63.2	23.1
B. japonicum CPAC 390	0.7	0.7	1.2	1.2	1.5	2.9	8.8	77.7	143.9	171.9	19.2	46.1	60.0	72.0	16.9
KNO_3 (350 mg N)	0	0	0	0	1.9	4.3	14.0	150.5	52.5	22.8	3.3	32.3	60.6	27.8	22.4
KNO_3 (175 mg N)	0	0	0	0	1.8	5.4	9.8	54.9	29.1	23.7	5.9	42.6	33.8	34.8	33.3
N source															
B. japonicum	0.7	0.7	0.9	0.9	1.4[a]	2.7[a]	7.2[a]	77.6[a]	111.8[a]	149.1[a]	152.2[a]	50.0	59.3	61.4[a]	28.1
KNO_3	0	0	0	0	1.7	3.8	10.1	117.6	52.1	23.4	24.0	54.1	59.8	39.7	36.2
cultivar															
BRS 154	0.5	0.6	0.8	0.9	1.4[a]	2.6[a]	7.7	100.5	89.4	95.6	129.7	62.1[a]	65.3	50.8	40.0[a]
BRS 262	0.8	0.7	1.0	1.0	1.7	3.9	9.4	94.7	74.4	89.7	49.8	42.0	53.8	52.6	26.6
rhizobial strain															
CPAC 7	0.7	0.7	0.8	0.8	1.5	2.8	6.9	82.5	79.0[a]	144.4	146.6	47.2	55.8	60.0	30.1
CPAC 390	0.6	0.7	1.0	1.0	1.4	2.6	7.6	72.8	144.6	153.9	157.9	52.6	62.8	62.7	26.1
KNO_3															
350 mg N	0	0	0	0	1.8	3.4	12.4	132.4	63.0[a]	24.1	24.0	48.6	60.9	60.0	30.6
175 mg N	0	0	0	0	1.6	4.2	9.0	104.3	41.2	22.9	24.1	59.7	58.7	62.7	43.9
P-value															
N source					0.006	<0.001	<0.001	n.s.	<0.001	<0.001	<0.001	n.s.	n.s.	0.010	n.s.
cultivar					0.002	<0.001	0.005	n.s.	n.s.	n.s.	0.008	0.016	n.s.	n.s.	0.003
N source × cultivar					n.s.	n.s.	n.s.	0.016	n.s.	n.s.	0.008	n.s.	0.053	n.s.	n.s.

[a]Indicates that differences between the two groups with N source, cultivar, rhizobial strain and rate of KNO_3 are significant at $P < 0.05$ by the *F*-test.

[b]Leaf protein concentration in this study is lower than that reported in other studies (*e.g.* Campbell *et al.* 1988; de Veau *et al.* 1992). Our underestimated values are attributed to a partial binding of sodium dodecyl sulphate to Rubisco (Catt & Millard 1988), however, they do not alter our conclusions.

RESULTS

Nodulation and shoot biomass

Both soybean cultivars, rhizobia-inoculated or N-fertilized, grew well under greenhouse conditions but shoot biomass of cultivar BRS 262 was 1.5 times larger than that of BRS 154 at the R2 stage, and 1.2-times larger at the R4 stage (Table 1). N-fertilized soybeans produced more shoot biomass than nodulated soybeans up to the R4 stage. Nodule biomass increased over time in both soybean cultivars. There was abundant nodulation in the roots of inoculated plants, but no nodulation in the N-fertilized plants up to the R4 stage. After the R4 stage, a few nodules formed on N-fertilized, non-inoculated plants (data not shown), and those plants were omitted from the analyses.

Leaf ureide-N, N and protein concentrations and photosynthetic N use efficiency

The ureide-N concentration in leaves of N-fertilized plants was higher than that of nodulated plants in the vegetative stage V4, but from R2 onwards nodulated plants always accumulated more ureides than N-fertilized plants (Table 1). There were no significant differences in leaf protein between nodulated and N-fertilized plants at V4, R2 and R5 stages (Table 1). At R4, the average leaf protein concentration of nodulated plants was

Table 2. Rates of leaf photosynthesis, leaf N, and photosynthetic N use efficiency (PNUE) of soybean plants inoculated with two *Bradyrhizobium japonicum* strains or receiving two levels of N fertilizer (Experiment 1).

cultivar and N source	photosynthesis (μmol CO_2 $m^{-2}·s^{-1}$)				leaf N (mg·g^{-1} DW)				PNUE (μg CO_2 mg^{-1} N s^{-1})			
	V4	R2	R4	R5	V4	R2	R4	R5	V4	R2	R4	R5
BRS 154												
B. japonicum CPAC 7	16.8	27.0	29.4	20.8	44.5	52.8	45.2	37.1	0.31	0.42	0.53	0.47
B. japonicum CPAC 390	16.2	24.4	30.4	24.3	45.9	51.5	47.3	46.0	0.29	0.38	0.52	0.44
KNO_3 (350 mg N)	10.8	21.0	17.5	17.4	58.8	60.0	29.9	28.5	0.15	0.29	0.47	0.52
KNO_3 (175 mg N)	8.5	20.8	14.7	15.9	53.4	44.8	21.2	28.5	0.13	0.42	0.64	0.46
BRS 262												
B. japonicum CPAC 7	14.6	21.0	23.5	23.4	49.1	47.4	46.7	36.6	0.25	0.37	0.41	0.54
B. japonicum CPAC 390	12.0	20.0	26.8	18.6	44.9	51.5	51.9	41.5	0.22	0.32	0.43	0.36
KNO_3 (350 mg N)	12.5	18.8	17.0	10.8	55.6	47.4	18.9	24.5	0.18	0.32	0.73	0.36
KNO_3 (175 mg N)	12.2	17.0	16.0	13.6	52.6	44.9	17.3	33.1	0.19	0.31	0.77	0.34
N source												
B. japonicum	14.9[a]	23.1[a]	27.5[a]	21.8[a]	46.1[a]	50.8	47.8[a]	40.1[a]	0.27[a]	0.37	0.47	0.45[a]
KNO_3	11.0	19.4	16.1	14.5	55.1	49.3	21.6	28.0	0.16	0.33	0.66	0.42
cultivar												
BRS 154	13.1	23.3[a]	23.4	19.6	50.7	52.3	36.3	35.0	0.21	0.38	0.55	0.47
BRS 262	12.8	19.2	21.3	17.0	50.6	47.8	35.8	33.6	0.21	0.33	0.57	0.41
rhizobial strain												
CPAC 7	15.7	24.0	26.5	22.1	46.8	50.1	46.0	36.4[a]	0.28	0.39	0.47	0.50
CPAC 390	14.1	22.2	28.6	21.5	45.5	51.5	49.7	43.8	0.25	0.35	0.48	0.40
KNO_3												
350 mg N	11.7	19.9	17.3	14.1	57.2	53.7	25.5	26.5	0.17	0.30	0.57	0.43
175 mg N	12.3	18.9	15.4	15.1	53.0	44.9	19.2	30.0	0.16	0.36	0.71	0.42
P-value												
cultivar	n.s.	0.002	n.s.	0.014	n.s.	n.s.	n.s.	0.020	n.s.	n.s.	n.s.	n.s.
N source	0.004	0.027	<0.001	0.002	0.001	0.028	<0.001	<0.001	<0.001	n.s.	n.s.	n.s.
cultivar × N source	0.038	n.s.	n.s.	0.050	n.s.	n.s.	n.s.	n.s.	0.017	n.s.	n.s.	n.s.

[a]Indicates that differences between the two groups with N source, cultivar, rhizobial strain and rate of KNO_3 are significant at $P < 0.05$ by the *F*-test.

significantly higher than that of N-fertilized plants. The soluble protein concentration in leaves ranged from 16.9 to 83.6 mg·g^{-1} leaf dry weight (Table 1).

At the beginning of the experiment (V4 stage), plants receiving N fertilizer accumulated more N in leaves than the nodulated plants, but later (from the R4 stage onwards), nodulated plants had a higher N concentration in the leaves (Table 2). At V4 stage, nodulated plants had higher PNUE than N-fertilized plants, but there were no differences in later stages.

Rates of photosynthesis

Nodulated plants had higher rates of photosynthesis than N-fertilized plants (Table 2). At the V4 stage, rates of photosynthesis were 12.0–16.8 μmol CO_2 $m^{-2}·s^{-1}$ in nodulated and 8.5–12.5 μmol CO_2 $m^{-2}·s^{-1}$ in N-fertilized plants. At the reproductive stages, rates of photosynthesis in both nodulated and N-fertilized plants increased. The highest rates of photosynthesis in N-fertilized plants were observed at stage R2 (17–21 μmol CO_2 $m^{-2}·s^{-1}$), whereas the highest rates of photosynthesis in nodulated plants were at stage R4 (23.5–30.4 μmol CO_2 $m^{-2}·s^{-1}$) (Table 2). Although the difference was only significant at the R2 stage, on average, plants with lower shoots (BRS 154) had higher rates of leaf photosynthesis (not canopy!) than those with higher shoots (BRS 262). Rates of photosynthesis were not correlated with leaf N concentration (data not shown).

Maximum values of leaf protein and rates of photosynthesis

We analysed the relationship between leaf protein and rates of photosynthesis using quadratic functions over the

Table 3. Date and estimated maximum values of photosynthesis and maximum leaf protein concentration of two soybean cultivars inoculated with two *Bradyrhizobium japonicum* strains or receiving two doses of N-fertilizer (Experiment 1).[a]

cultivar and N source	day	photosynthesis (μmol CO_2 m^{-2} leaf s^{-1})	adjusted R^2	P-value	day	protein (mg·g^{-1} leaf DW)	adjusted R^2	P-value
N source								
B. japonicum	40	27.2	0.55	<0.001	35	65.9	0.50	<0.001
KNO_3	38	18.9	0.19	0.001	29	49.2	0.09	0.028
cultivar								
BRS 154	40	25.0	0.27	<0.001	29	59.1	0.19	0.001
BRS 262	40	21.6	0.30	<0.001	35	55.9	0.41	<0.001
rhizobial strain								
CPAC 7	40	26.9	0.43	<0.001	35	62.7	0.43	<0.001
CPAC 390	42	27.9	0.74	<0.001	35	65.4	0.31	0.004
KNO_3								
350 mg N	38	20.1	0.17	0.036	32	50.9	0.23	0.013
175 mg N	39	18.0	0.19	0.022	68	35.8	0.02	n.s.

[a]The adjusted R^2 refers to goodness of quadratic regressions, with a P-value obtained after the *F*-test for the regressions. No statistical test was performed for the differences of pairs.

Table 4. Nodule biomass, sugar, starch and starch-to-sugar ratio in leaves of soybean inoculated with two *Bradyrhizobium japonicum* strains or receiving two levels of N fertilizer (Experiment 2).

cultivar and N source	nodules (g DW/plant)	soluble sugars (mg·g^{-1} FW)	starch (mg·g^{-1} FW)	starch-to-sugar ratio
BRS 154				
B. japonicum CPAC 7	0.6	9.2	7.7	0.9
B. japonicum CPAC 390	0.5	9.6	6.0	0.7
KNO_3 (350 mg N)	0	8.7	15.9	1.9
KNO_3 (175 mg N)	0	n.d.	n.d.	n.d.
BRS 262				
B. japonicum CPAC 7	0.5	9.1	9.9	1.1
B. japonicum CPAC 390	0.5	7.2	9.7	1.3
KNO_3 (350 mg N)	0	10.3	11.5	1.2
KNO_3 (175 mg N)	0	7.1	10.7	1.5
N source				
B. japonicum	0.5	8.8	8.4[a]	1.0[a]
KNO_3	0	8.8	12.4	1.5
cultivar				
BRS 154	0.5	9.3	9.5	1.1
BRS 262	0.5	8.4	10.5	1.3
rhizobial strain				
CPAC 7	0.6	9.2	8.8	1.0
CPAC 390	0.5	8.4	7.8	1.0
KNO_3				
350 mg N	0	9.8	13.8	1.5
175 mg N	0	7.2	10.7	1.5
P-value				
N source		n.s.	0.022	0.037
cultivar		n.s.	n.s.	n.s.
N source × cultivar		n.s.	0.045	0.009

n.s. = no significant difference at $P \geq 0.05$ by the *F*-test.
[a]Indicates that differences between the groups with N source, cultivar, rhizobial strain and rate of KNO_3 are significant at $P < 0.05$ by the *F*-test.

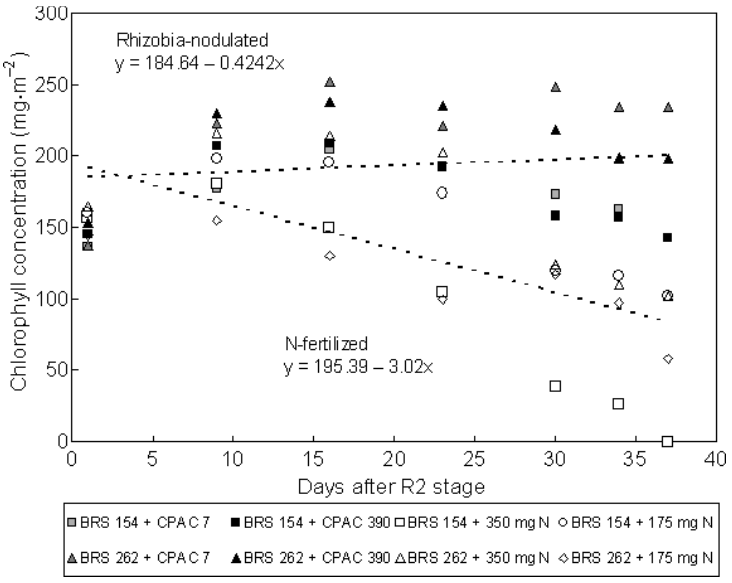

Fig. 1. Chlorophyll degradation in leaves of rhizobia-inoculated and N-fertilized soybeans (Experiment 3). The measurements started on 26 March 2008 (day 1) at stage R2. On day 32, all plants were at R5 stage, but the leaves of two plants of cultivar BRS 154 fertilized with 350 mg N had already senesced. On day 39, all plants were at stage R6/R7, except for those of BRS 154 + 350 mg N and two plants of BRS 262 fertilized with 175 mg N, which had already lost some leaves.

duration of our experiment. The adjusted R^2 was consistently higher in nodulated than in N-fertilized plants (Table 3). The estimated maximum rates of photosynthesis were higher in nodulated plants than in N-fertilized plants, and these maximum rates occurred 2 days later. The estimated maximum protein content was also higher in nodulated plants (65.9 mg·g^{-1} DW) compared with N-fertilized plants (49.2 mg·g^{-1} DW). The regressions predicted that the maximum protein content in nodulated plants would be achieved 6 days later than in N-fertilized plants, but R^2 of N-fertilized plants was very low. A more realistic comparison, considering nodulated plants on the one hand and fertilized plants with 350 mg of N on the other hand, gives a delay of just 3 days.

Starch and soluble sugars in leaves

In Experiment 2, starch concentration in the leaves at the R2 stage ranged from 6.0 to 9.9 mg g^{-1} FW in nodulated plants and 10.7–15.9 in N-fertilized plants (Table 4). There were no differences between the two soybean cultivars but there was a large difference between nodulated and N-fertilized soybean plants (Table 4). The average soluble sugar content in leaves was 8.8 mg·g^{-1} FW in both nodulated and N-fertilized plants, but the ratio starch:soluble sugars was significantly higher in N-fertilized than in nodulated plants.

Chlorophyll content

Statistical analysis based on repeated measurements revealed that there was no effect of cultivar on chlorophyll concentration, but there was a strong effect of the N source. The effects of cultivar were only significant at $P = 0.055$, whereas the effects of N source were significant at $P < 0.001$, and the interaction of cultivar × N source was significant at $P = 0.017$. At the R2 stage (day 1), average chlorophyll concentration was 140 mg·m^{-2} in the nodulated plants, and 155 mg·m^{-2} in the N-fertilized plants (Experiment 3; Fig. 1). On the 16th day (R4/R5 stage), chlorophyll concentration averaged 226 mg·m^{-2} in nodulated, and 172 mg·m^{-2} in N-fertilized plants. From stage R4/R5 onwards, the plants started to degrade chlorophyll and showed symptoms of leaf senescence (lower chlorophyll concentrations), but N-fertilized plants senesced more rapidly than nodulated plants (Fig. 1).

DISCUSSION

Our study demonstrated that nodulated soybean, with very effective N_2-fixing rhizobia, have higher rates of photosynthesis than N-fertilized plants, regardless of the leaf N concentration (Table 2). These results confirm that N_2 fixation is a more efficient method for legumes to stimulate photosynthesis than N fertilizer application (Brown & Bethlenfalvay 1988; de Veau *et al.* 1990; Zhou *et al.* 2006). Although leaf N concentration of N-fertilized plants declined in later stages, photosynthesis was not limited by leaf N. The threshold for N limitation of photosynthesis with an adequate light supply is assumed to be between 15 and 20 mg N g^{-1} leaf (Robertson *et al.* 2002), and in our study, even plants with smaller N concentrations accumulated more N than these suggested thresholds. Recently, Kaschuk *et al.* (2009) found an N-independent effect of rhizobia on photosynthesis, where rates of photosynthesis were stimulated by the photosynthate (C) sink strength of the symbioses. Assuming that C costs of N_2 fixation are higher than those of NO_3^- uptake (Minchin & Witty

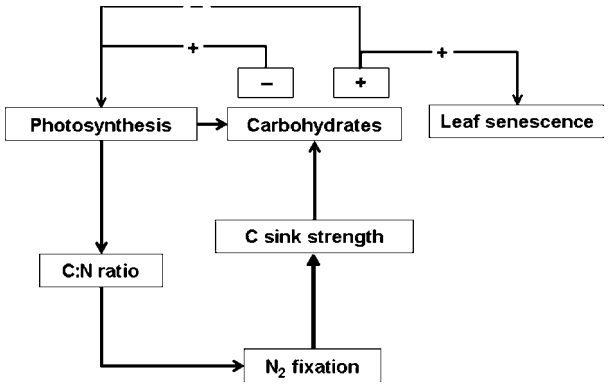

Fig. 2. Conceptual diagram of delayed leaf senescence in soybean plants with efficient nodulation. The C sink strength of N_2 fixation changes the sink:source ratio of soybean, and lowers the accumulation of carbohydrates in leaves. High accumulation of carbohydrates lowers rates of photosynthesis and triggers the process of leaf senescence. Low accumulation of carbohydrates stimulates rates of photosynthesis, which, in turn, allows high rates of N_2 fixation to be maintained. Arrows indicate the feedbacks between processes.

2005), our data support the hypothesis that higher C sink strength of N_2 fixation increases the rate of photosynthesis. Noteworthy is that this phenomenon can be exacerbated in a cultivar with lower shoots (Table 1). With the present experimental set-up, we could not estimate the rates of N_2 fixation, but we utilized N and ureide-N in the leaves as indicators for effective N_2 fixation. However, ureide-N is only a good indicator for N_2 fixation in the reproductive stages of nodulated soybean (Matsumoto *et al.* 1977; Herridge 1982). Ureide-N is strongly related to rates of N_2 fixation, as demonstrated by experiments with [^{15}N]-nitrogen gas (Ohyama & Kumazawa 1978) and acetylene reduction assays (McClure & Israel 1979; Herridge 1982; van Berkum *et al*. 1985), but may also be high prior to reproductive stages in plants receiving high rates of N fertilization (Polayes & Schubert 1984).

An important evidence for increased C sink strength due to N_2 fixation was that N-fertilized plants accumulated more starch in the leaves, despite lower rates of photosynthesis, than nodulated plants (Table 4). This suggests that nodulated plants achieved higher rates of photosynthesis because they had a larger demand for photosynthate. Previous studies have demonstrated that increased photosynthate demand prevents accumulation of carbohydrates in the leaves, and triggers the enzymatic machinery of the Calvin Cycle (Azcón-Bieto 1983; Goldschmidt & Huber 1992; Paul & Foyer 2001). The higher starch content in N-fertilized soybean plants suggests that photosynthesis is not limited by substrate (either ribulose bisphosphate or CO_2), enzymes (N and proteins), light or water availability. Furthermore, as both rhizobia-inoculated and N-fertilized soybean plants received the same amount of P in the nutrient solution, photosynthate export is not limited by inorganic P availability (Foyer & Spencer 1986; Fredeen *et al.* 1989). Therefore, differences in photosynthesis between nodulated and N-fertilized soybeans are most likely caused by increased C sink strength of the symbioses.

We also assessed the effects of nodulation on leaf senescence. Ono *et al.* (1996, 2001) demonstrated that leaves with low demand for photosynthate accumulate sugars and accelerate the symptoms of leaf senescence. We found strong evidence for a delay of leaf senescence in nodulated soybean as they always had higher rates of photosynthesis (Table 2), and sustained high concentrations of chlorophyll for a longer time (Fig. 1). Two processes occur simultaneously: first, N_2 fixation delays N removal from the leaves to the seeds by supplying N at rates of current plant N demand (*e.g.* Minchin *et al.* 1980); and second, C sink strength stimulates leaf activity and increased photosynthesis (Table 3). The two processes together are consistent with the hypothesis of Paul & Peliny (2003), who suggested that higher rates of photosynthesis prior to the early phase of senescence lead to a longer photosynthetically active life.

We confirmed our second hypothesis by showing a 2- to 3-day delay in the peak of photosynthesis and leaf protein in nodulated plants in comparison to N-fertilized plants receiving 350 mg of N (Table 3). Also Abu-Shakra *et al.* (1978) observed that nodulated soybeans with higher nitrogenase activity had prolonged photosynthetic activity. They suggested that longer photosynthetic activity is related to higher N availability in the leaves as a consequence of high N_2 fixation. In fact, the view that N alone regulates leaf senescence is too simplistic. Wittenbach (1982, 1983) and Crafts-Brandner & Egli (1987) removed the pods of poorly-nodulated soybeans receiving high amounts of N fertilizer, and thus delayed N reallocation, but this did not prevent a decrease in rates of photosynthesis. Hence, under these conditions, leaf photosynthesis was not limited by N, but rather it was limited by C sink strength of the pods (Wittenbach 1982, 1983; Crafts-Brandner & Egli 1987).

In Fig. 2, we summarize feedbacks from N_2 fixation to photosynthesis and leaf senescence, based on our results and previous studies. The figure indicates that the C sink strength of N_2 fixation prevents accumulation of sugars in leaves and, probably by triggering the Calvin Cycle, stimulates rates of photosynthesis. Increased rates of photosynthesis and higher N demand during the reproductive stage stimulate rates of N_2 fixation. In turn, N_2 fixation compensates for the degradation of proteins and chlorophyll while lowering carbohydrate concentrations in the leaves, which finally delays leaf senescence.

In conclusion, we demonstrate that C costs of N_2 fixation are compensated by increased leaf photosynthesis. We also show that allowing soybeans to fully rely on N_2 fixation does not compromise yield. Furthermore, due to higher leaf activity and continued N supply, N_2 fixation potentially delays leaf senescence, which increases the period of pod filling.

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