

## Relationship between Leaf Chlorophyll Content and Carbon Isotope Discrimination in Soybean

Madhavan Soundararajan

Department of Biochemistry, University of Nebraska-Lincoln,  
Lincoln, Nebraska 68588-0664,USA

**Abstract.** A significant positive correlation between carbon isotope ratio ( $\delta^{13}\text{C}$ , ‰) and total chlorophyll concentration [Chl] was observed in field-grown soybean (*Glycine max* L.) leaves with varying Chl levels. Leaf  $\delta^{13}\text{C}$  values became less negative by up to 4.5 ‰ with increasing Chl levels from 40 to 500  $\mu\text{mol m}^{-2}$ . The trend towards decreased leaf carbon isotope fractionation with increased Chl levels suggests an enhanced carboxylation capacity in these leaves. Soybean leaves showed a linear increase in GAPDH activity with increase in leaf [Chl] suggesting an enhanced Ribulose -1,5- biphosphate (RuBP) pool size. Levels of Ribulose biphosphate carboxylase (Rubisco) also increased in concert with leaf Chl levels. Less negative  $\delta^{13}\text{C}$  values in these leaves with increasing [Chl] is thus due to an enhanced carboxylation capacity, resulting from a larger RuBP pool size and increased Rubisco levels. Gas exchange studies of these leaves also indicated a correlation between Chl content and net photosynthetic rate. Results from this study demonstrate that leaf Chl provides a major molecular basis influencing leaf carbon isotope discrimination by regulating carboxylation strength.

**Keywords:** *Glycine max* L.cv. Hobbit; carbon isotope discrimination, chlorophyll, photosynthesis, ribulose 1,5-biphosphate carboxylase/oxygenase.

### 1. Introduction

Carbon isotope composition of whole plants and plant organs provides an integrated, long-term view of carbon assimilation. In terrestrial C3 plants, isotope fractionation can be described by the equation

$$\Delta\delta = a + \left[ (b - a) P_{\text{int}} / P_{\text{atm}} \right] - d \quad (1)$$

where  $\Delta\delta$  is  $\delta^{13}\text{C}_{[\text{source}]} - \delta^{13}\text{C}_{[\text{leaf}]}$ ,  $a$  is the discrimination due to diffusion (4.4‰),  $b$  is the discrimination by Rubisco (30‰),  $P_{\text{int}}$  is the internal partial pressure of CO<sub>2</sub> inside the leaf airspace, and  $P_{\text{atm}}$  is the partial pressure of CO<sub>2</sub> in the atmosphere [1,2]. The term  $d$  is expected to include contributions from respiration, isotopic changes due to transport of photosynthates and a variety of other factors. Several physical factors have been shown to influence the integrated balance of stomatal conductance and carboxylation, and thus affect isotopic discrimination in plants [3,4].

A molecular basis for the variation in foliar  $\delta^{13}\text{C}$  values of groundnut genotypes due to variation in Rubisco levels has been reported [6]. Leaf Chl content has received very little attention as a significant molecular basis for variation in leaf carbon isotope discrimination in plants. Sweetclover mutants with temperature-sensitive expression of Chl content showed a decrease in leaf carbon isotope discrimination with increasing Chl levels [7]. Total leaf Chl content has also been reported to be positively correlated with foliar  $\delta^{13}\text{C}$  in *Juniperus*, [8].

A variety of controls regulate leaf photosynthesis[8]. Manipulations resulting in the reduction of leaf Chl content induced true limitations in the rate of photosynthesis [9]. Photosynthetic rates have been positively correlated with Chl content in leaves of fieldgrown soybean [10] and peas [11]. A negative correlation between Chl content and photosynthetic rate has also been reported for certain cowpea mutants [12], and in

certain Chldeficient soybean isolines [13]. In this study we present evidence supporting a strong positive relationship between leaf Chl content and carbon assimilation, as indicated by both leaf carbon isotope discrimination and other physiological processes in field-grown soybean plants.

## 2. Materials and Methods

### 2.1. Plants

Various lines of soybean (*Glycine max* L.cv. Hobbit) plants were grown in test plots at the UNL Field Research and Development Station, Institute of Agriculture and Natural Resources, Mead, NE. Soybean plants grown at the Mead facility received modest amounts of supplemental nitrogen.

### 2.2. Chl Determinations

Fully developed, non-senescing leaves from the third or fourth nodes of approximately 10 week old soybean plants, were selected by Chl content. Leaf Chl determinations were made using a Minolta SPAD-502 meter . Rather than attempt to replicate individual measurements, individual plants of various soybean lines were selected to provide a large number of SPAD-502 meter readings with a wide range of values. The meter readings were converted to total leaf Chl content ( $\mu\text{mol m}^{-2}$ ) using an equation obtained from regression analysis of the raw meter readings and conventional Chl assays [14].

$$\text{Chl Concentration} = 10^{(\text{Meter Reading})^{0.265}} \quad (2)$$

### 2.3. Carbon Isotope Fractionation

Carbon isotope ratio measurements were made following published procedures [4]. The  $^{13}\text{C}$  content of  $\text{CO}_2$  is given as an isotope ratio, R,

$$R = \left[ \frac{^{13}\text{CO}_2}{^{12}\text{CO}_2} \right] \quad (3)$$

and  $\delta^{13}\text{C}$  is given by,

$$\delta^{13}\text{C} = 1000 \left[ \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right] \quad (4)$$

### 2.4. Quantitative relationship between [Chl] and Carbon Isotope Discrimination

The concentration of Chl should be related to  $\delta^{13}\text{C}$  through the Beer-Lambert law:  $I_f = I_i e^{-\alpha b C}$  where  $I_f$  is the intensity of light transmitted through the sample,  $I_i$  is the initial intensity of light,  $\alpha$  is the absorption,  $b$  is the sample thickness, and  $C$  is the concentration of the light-absorbing compound. The fraction of light absorbed is then equal to the quantity  $1 - I_f / I_i = 1 - e^{-\alpha b C}$ . The fraction of light absorbed by Chl should be proportional to the metabolic energy produced. The relationship between  $\delta^{13}\text{C}$  and [Chl] can be quantitatively described by the following function:

$$\delta^{13}\text{C} = \Delta\delta^{13}\text{C} \left( 1 - e^{-A_L \frac{[\text{Chl}]}{\text{Chl}_{\text{max}} - [\text{Chl}]}} \right) + \delta^{13}\text{C}_0$$

In this equation  $\Delta\delta^{13}\text{C}$  is a constant describing the total change in  $\delta^{13}\text{C}$  and  $\delta^{13}\text{C}_0$  is a constant equal to the  $\delta^{13}\text{C}$  value extrapolated to the complete absence of Chl.  $A_L$ , the leaf absorption constant, includes at least two factors: an extinction constant for the relative amount of Chl in the leaf and a leaf thickness constant. The constant,  $\text{Chl}_{\text{max}}$  is the maximum observed Chl concentration for the plant.

### 2.5. Gas Exchange

A LI-COR LI-6200 portable photosynthesis system with a 1L leaf cuvette was used to make gas exchange measurements on a subset of the leaves used for carbon isotope analysis. All gas exchange measurements were made at a PPFD of 1800-2100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , an RH of 50-60%, and a temperature around 27-29 °C.

## 2.6. Chloroplast GAPDH Assay

GAPDH activity measurements were measured using the method of [15] GAP oxidation was monitored spectro-photometrically by following the rate of NADPH formation (340 nm, 25 ° C).

## 2.7. Rubisco levels determination

Leaf extracts used for GAPDH assays served as the source for the determination of Rubisco levels by an indirect, sandwich enzyme immunolinked immunosorbent assay (ELISA). following the procedure of [16].

## 3. Results and Discussion

### 3.1. Chl Levels and Carbon Isotope Discrimination

Isotope discrimination decreased with increasing levels of Chl (Fig. 1). A difference of about 600  $\mu\text{mol}$  of Chl  $\text{m}^{-2}$  in leaves resulted in about a 4‰ change in carbon isotope discrimination. Carbon isotopic composition of leaves represents a long-term integration of carbon isotope discrimination due to a complex series of physiological and biochemical events. Isotope discrimination, in essence, reflects the balance between  $\text{CO}_2$  diffusion through the stomata and the chemical uptake of  $\text{CO}_2$ . In general,  $\delta^{13}\text{C}$  values of  $\text{C}_3$  plants become more negative when carboxylation limits  $\text{CO}_2$  uptake and less negative under conditions that limit  $\text{CO}_2$  diffusion [2]. The relationship between  $\delta^{13}\text{C}$  values and increasing Chl concentration in this study, parallels results obtained from our earlier studies on growth-chamber grown sweetclover mutants [6]. Less negative leaf  $\delta^{13}\text{C}$  values at increased Chl levels have been reported qualitatively. At leaf [Chl] of 500  $\text{mol m}^{-2}$  or above, it appears that carboxylation ceases to be limiting carbon isotope discrimination.

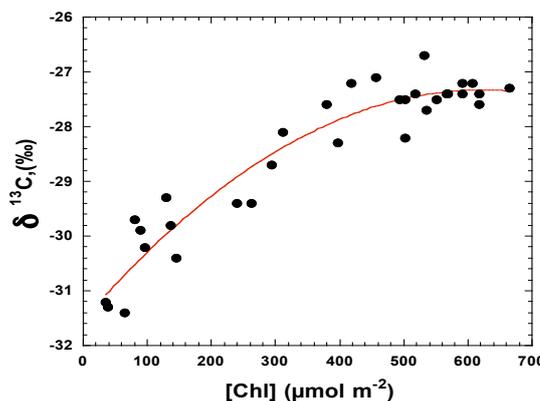


Fig. 1. Relationship between leaf chlorophyll concentration and  $\delta^{13}\text{C}$  (‰)

The observed relationship between  $\delta^{13}\text{C}$  values and Chl concentrations suggests an efficient photochemical process with increasing levels of Chl. This increased efficiency would result in an increased RuBP regeneration capacity by increasing the availability of ATP and NADPH.

Light energy availability within a leaf should be governed by the Beer-Lambert law. In this study, we have used this law to quantify the relationship between leaf Chl concentration and carbon isotope discrimination. It appears that even under reduced levels such 208  $\mu\text{mol m}^{-2}$  of Chl, carbon isotope discrimination in soybean plants is limited more by  $\text{CO}_2$  diffusion than by carboxylation.

### 3.2. Chl Levels and Stromal GAPDH Activity

Soybean leaves with varying levels of Chl when assayed for their chloroplast specific GAPDH activity, showed a linear relationship between GAPDH activity and Chl levels ranging from 100 to 500  $\mu\text{mol m}^{-2}$  (Fig. 2).

As indicated above,  $\delta^{13}\text{C}$  values of  $\text{C}_3$  plants become more negative under conditions that limit their carboxylation capacity. The term “carboxylation capacity” reflects not only the activity of the processes that synthesize RuBP but also the activation state and amount of Rubisco [17,18].  $\text{CO}_2$  assimilation rates in  $\text{C}_3$  plants can become limited by RuBP regeneration [19] which depends upon the availability of ATP and NADPH. NADPH-dependent GAPDH is a stromal enzyme that catalyzes the reversible conversion of 3-phosphoglycerate to GAP, using ATP and NADPH. Price [15] demonstrated that the RuBP pool size was

linearly related to GAPDH activity in transgenic tobacco engineered with an antisense RNA construct directed against chloroplast specific GAPDH. These antisense plants showed not only reduced activities of GAPDH but also had reduced Rubisco activity, leaf soluble protein and [Chl]. In the present study soybean leaves with varying levels of Chl showed a similar positive correlation between Chl concentration and GAPDH activity (Fig. 2) suggesting an increase in Chl levels tend to increase the RuBP pool size, effecting an enhanced carboxylation capacity.

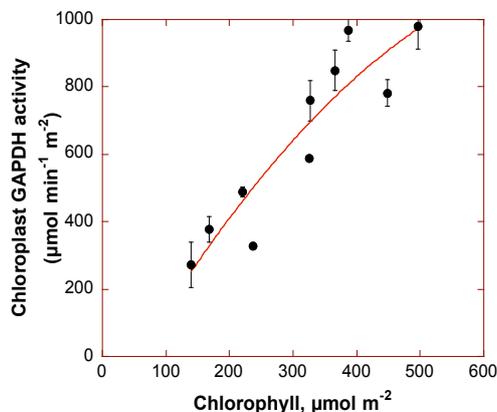


Fig. 2. Relationship between chloroplast GAPDH activity and total chlorophyll content in soybean leaves.

### 3.3. Relationship between Chl and Rubisco Levels

A linear relationship was observed between Rubisco amount and Chl levels ranging from 100 to 500  $\mu\text{mol m}^{-2}$  in soybean leaves with varying levels of Chl (Fig. 3). A decreased amount of Rubisco can also cause decreased carboxylation capacity. Transgenic tobacco plants with an antisense gene for the small subunit of Rubisco, not only showed a reduction in Rubisco levels and decreased Rubisco activity [20] but also had reduced Chl levels [21].

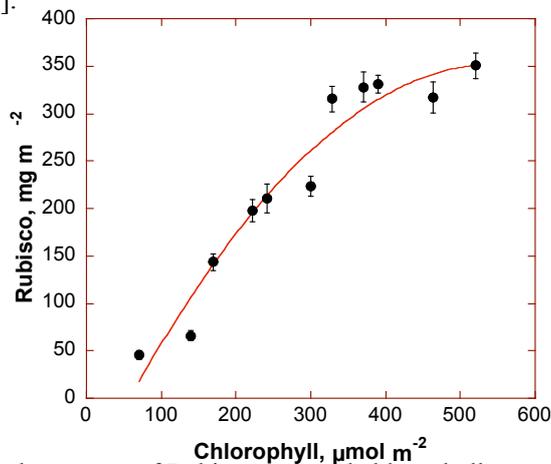


Fig. 3. Correlation between the amount of Rubisco and total chlorophyll concentration in soybean leaves.

Carbon isotope analyses of these transgenic plants showed more negative  $\delta^{13}\text{C}$  values (by about 3‰) than the control plants [20]. Rubisco content and carbon isotope discrimination were negatively correlated in groundnut genotypes [6]. A similar significant correlation between leaf [Chl] (from 100 to 350  $\mu\text{mol m}^{-2}$ ) and rubisco levels is apparent in soybean leaves (Fig.3). The observed, more negative  $\delta^{13}\text{C}$  values in these leaves may be due to a decreased carboxylation capacity resulting from decreased Rubisco levels.

### 3.4. Chl Levels and Leaf Photosynthesis

Increased foliar Chl concentration accompanied an increased rate of photosynthesis in soybean leaves (Fig. 4). A difference of about 25  $\mu\text{mol carbon fixed m}^{-2} \text{s}^{-1}$  in net photosynthetic rate was observed in leaves containing 100 to 450  $\mu\text{mol m}^{-2}$  of Chl (Fig. 4).

The observed positive relationship between net photosynthetic rate and Chl levels is consistent with previous reports [9,23]. Transgenic tobacco plants with antisense genes for GAPDH or the small subunit of Rubisco also showed a linear relationship between total Chl and net photosynthetic rate [22,15]. Photosynthetic rate is the product of light absorbed and quantum efficiency of photosynthesis. Results from

our study indicate that reduced photosynthetic rate at low Chl levels may be due to a limitation in the rate of supply of photochemical energy.

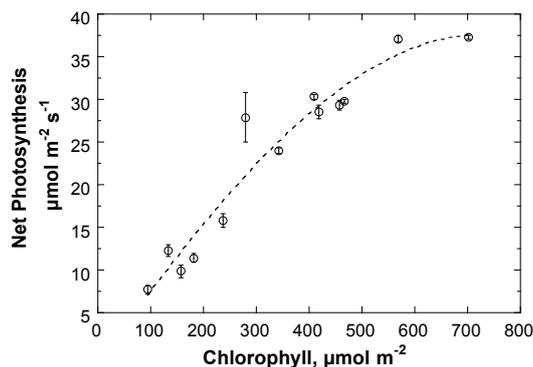


Fig. 4. Effect of varying chlorophyll concentration on net photosynthesis.

## 4. Conclusions

Results from this study suggest that chl regulates carbon isotope discrimination in soybean by regulating the carboxylation capacity. The changes that occur with increased levels of Chl must be significantly larger than the changes that occur in stomata. Foliar  $\delta^{13}\text{C}$  values thus appear to effectively communicate this relationship between Chl levels and processes associated with an integrated, long term carbon assimilation in  $\text{C}_3$  plants.

## 5. References

- [1] G.D. Farquhar, J.R. Ehleringer and K.T. Hubick, Carbon isotope discrimination and photosynthesis *Ann. Rev. Pl. Physiol. Pl. Mol. Biol.* 40, pp 503-537, 1989.
- [2] M.H. O'Leary, S. Madhavan, and P. Paneth, Physical and chemical basis of carbon isotope fractionation in plants *Plant, Cell and Environ.* 15, 1pp.1099-1104, 1992.
- [3] E.C. Meinzer, G. Goldstein, and D.A. Grantz, Carbon isotope discrimination in coffee genotypes grown under limited water supply *Plant Physiol.* 92, pp. 30-135, 1990.
- [4] S. Madhavan, I. Treichel, and M.H. O'Leary, "Effects of Relative Humidity on Carbon Isotope Fractionation in Plants". *Bot. Acta*, vol. 104, pp. 292-294. 1991.
- [5] N.R.C. Rao, M. Udayakumar, G.D. Farquhar, H.S. Talwar, and T.G. Prasad, Variation in carbon isotope discrimination and its relationship to specific leaf area and ribulose-1,5-bisphosphate carboxylase content in groundnut genotypes, *Aust. J. Pl. Physiol.*, 22, pp. 545-551, 1995.
- [6] M.A. Bevins, S. Madhavan, and J. Markwell, Two sweetclover (*Melilotus alba* Desr.) mutants temperature sensitive for chlorophyll expression, *Plant Physiology* 103, pp. 1123-1131, 1993.
- [7] P.W. Hill, L.L. Handley, and J.A. Raven, "*Juniperus communis* L. ssp. *communis* at Balnaguard, Scotland: Foliar carbon discrimination ( $\delta^{13}\text{C}$ ) and 15-N Natural abundance ( $\delta^{15}\text{N}$ ) suggests gender-linked differences in water and N use" *Bot. J. Scotl.* 48, pp. 209-224, 1996.
- [8] I.F. Wardlaw, "The control of carbon partitioning in plants". *New Phytol.* 116,, pp. 341-381, 1990.
- [9] S.E. Taylor and N. Terry, "Limiting factors in photosynthesis V. Photochemical energy supply colimits photosynthesis at low values of intercellular  $\text{CO}_2$  concentration", *Plant Physiol.* 75, pp. 82-86, 1984.
- [10] B.R. Buttery and R.L. Buzzell, "The relationship between chlorophyll content and rate of photosynthesis in soybeans", *Can. J. Pl. Sci.* 57, pp. 1-5, 1977.
- [11] S.L.A. Hobbs and J.D. Mahon, Inheritance of chlorophyll, ribulose-1,5 bisphosphate carboxylase activity and stomatal resistance in peas. *Crop Sci.* 25, pp. 1031-1034, 1985.

- [12] W.R. Kirchoff, A.E. Hall and M.L. Roose, "Inheritance of a mutation influencing chlorophyll content and composition in cowpea", *Crop Sci*, 29, pp. 105-108, 1989.
- [13] W.T. Pettigrew, J.D. Hesketh, D.B. Peters and J.T. Wooley, J.T. "Characterization of canopy photosynthesis of chlorophyll-deficient soybean isolines", *Crop Sci*. 29, pp. 1025-1029, 1989.
- [14] J. Markwell, J.C. Osterman and J.L. Mitchell, "Calibration of the Minolta SPAD-502 leaf chlorophyll meter", *Photosyn. Res.* 46, pp. 467-472, 1996.
- [15] G.D. Price, J.R. Evans, S. von Caemmerer, J.W. Yu, and M.R. Badger, "Specific reduction of chloroplast glyceraldehyde-3-phosphate dehydrogenase activity by antisense RNA reduces CO<sub>2</sub> assimilation via a reduction in ribulose biphosphate regeneration in transgenic tobacco plants" *Planta* 195, pp. 369-378, 1995.
- [16] E. Harlow and D. Lane, D, "*Antibodies: A Laboratory Manual*", Cold Springs Harbor Laboratory, Cold Springs Harbor, New York, 1988.
- [17] G.D. Farquhar and S. von Caemmerer, "Modeling of photosynthetic response to environmental conditions", In: *Physiological Plant Ecology II: Water relations and carbon assimilation*. (eds. Lange OL, Nobel PS, Osmond CB & Ziegler H) Vol. 12B, pp 549-587, Springer Verlag, Berlin, 1982.
- [18] G.D. Farquhar, "Models describing the kinetics of RuBP carboxylase-oxygenase", *Arch. Biochem. Biophys.* 193, pp. 456-468, 1979.
- [19] D.R. Geiger and J.C. Servaites, "Dynamics of self regulation of photosynthetic carbon metabolism", *Pl. Physiol. Biochem.* 32, pp. 173-183, 1994.
- [20] G.S. Hudson, J.R. Evans, J.R., S. von Caemmerer, Y.B.C. Arvidsson and T.J. Andrews, T.J. "Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants", *Pl. Physiol.* 98, pp. 294-302, 1992.
- [21] W.P. Quick, U. Schurr, R. Scheibe, E-D. Schulze, S.R. Rodermel, L. Bogorad, and M. Stitt, "Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" rbcS. *Planta* 183, pp. 542-554, 1991.
- [22] M. Stitt, W.P. Quick, U. Schurr, E-D. Schulze, S.R. Rodermel, and L. Bogorad "Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" rbcS. II Fluxcontrol coefficients for photosynthesis in varying light, CO<sub>2</sub> and air humidity", *Planta* 183, pp. 555-566, 1991.
- [23] B.L. Ma, M.J. Morrison and H.D. Voldeng,, "Leaf greenness and photosynthetic rates in soybean", *Crop Sci*, 35, pp. 1411-1414, 1995.