Photosynthesis and Photorespiration in Presenescent, Senescent, and Rejuvenated Soybean Cotyledons

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ABSTRACT
Various growth and physiological parameters were measured in germinating, presenescent, and senescing soybean (Glycine max [L.] Merr.) cotyledons and in cotyledons rejuvenated by epicotyl removal 18 days after planting. The maximal measured carbon dioxide exchange rates (CER) in the cotyledons were in the range of those reported for field-grown soybean leaves. Rejuvenated cotyledons accumulated total chlorophyll in excess of the maximum observed in presenescent cotyledons. When photosynthetic rates were expressed per cotyledon, the CER in rejuvenated tissue recovered to the maximal rates observed in presenescent cotyledons. Ribulose-1,5-bisphosphate carboxylase/oxygenase in rejuvenated cotyledons also recovered to the maximal amount seen in presenescent cotyledons so that CER appeared to be a function of ribulose-1,5-bisphosphate carboxylase/oxygenase content during most of the period studied. Observations of the postillumination outburst of CO₂ and ¹⁴C label in glycine indicated that photorespiration was occurring in the cotyledons and that photorespiration relative to photosynthesis was different in rejuvenated compared with presenescent cotyledons.

Soybean (Glycine max [L.] Merr.) is unusual among legumes because it possesses cotyledons that emerge and become green during germination. The cotyledons develop leaf-like characteristics, including photosynthetic capability, and function both as storage-mobilizing and photosynthetic organs (20). Data from Brown and Huber (4, 5) suggest that metabolism associated with the storage function disappears before there is loss of photosynthetic capacity. The cotyledons then begin to yellow and senesce as the seedling continues to develop. If the epicotyl is removed before the cotyledons become fully yellow and abscise, they remain attached to the stem and regreen. If axillary buds are removed as they grow out, the cotyledons persist for long periods.

Kruhl (12) showed that soybean cotyledons could lose up to 90% of their nucleic acids and up to 80% of their protein before senescence became irreversible. During a 15-d rejuvenation period, the cotyledons recovered a significant proportion of these lost components. Skadzen and Cherry (19) compared protein profiles from in vitro translated polyadenylated mRNA isolated from soybean cotyledons of different ages and found no translation products unique to presenescent, senescing, or rejuvenated cotyledons, although they did see quantitative differences in certain proteins. However, when proteins were extracted from cotyledons, specific proteins became prominent only in the rejuvenated cotyledons, suggesting that the rejuvenated state may be different from that of the presenescent cotyledon.

The rejuvenated cotyledon has not been well characterized physiologically. Photosynthesis in soybean cotyledons has been reported in only three studies, none of which included rejuvenated cotyledons (1, 4, 5, 9). The published data show that net photosynthesis in cotyledons is never much greater than dark respiration. Because soybean cotyledons can recover from senescence and survive for extended periods, they must be able to carry on net assimilation. The objective of this study was to characterize these cotyledons physiologically.

MATERIALS AND METHODS

Plant Material
Soybean (Glycine max [L.] Merr. cv Corsoy 79) was grown in flats of sterilized soil (1:1:1, soil:peat:perlite) in a greenhouse. The minimum temperature was always >18°C, and the maximum temperature was normally 22 to 24°C. Maximum daily irradiance was never less than 1200 μmol photons m⁻² s⁻¹. Seeds were screened to uniform size before planting, and cotyledon age was measured as number of days after planting. Plants were watered twice weekly with double-strength modified Hoagland solution (14) and on other days with tap water. Seedlings were thinned daily to eliminate plants not at the same developmental stage, as determined by visual morphological characteristics. For decapitation treatments, the stem, which included unifoliate leaves, trifoliolates, and apical bud (hereafter referred to as the epicotyl), was removed 1 or 2 cm above the cotyledonary node. After decapitation, axillary buds were carefully removed as they expanded.

As daylength, irradiance, and temperature varied throughout the year, there were variations between experiments in the exact timing of growth parameters; however, failure of the cotyledons to respond to epicotyl removal coincided with early expansion of the third trifoliolate, regardless of seedling age. The data reported are from one of a number of representative experiments with plants decapitated 18 d after planting.

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Gas Exchange

CERs\textsuperscript{2} were measured with an Anarad IRGA model AR500R (Anarad, Inc., Santa Barbara, CA) in the system described previously (14), modified to include condensation traps and bleed-out valves which reduced air flow into the gas analyzer (13). Experiments were conducted at 22 ± 1°C. Irradiance in the leaf chamber was 720 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\). Gas exchange measurements were always made between 10:30 AM and 4:30 PM. Approximately 30 min before the first series of measurements, flats were moved from the greenhouse to a growth chamber where irradiance at plant height was 340 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\). Cotyledons were removed from plants in the growth chamber.

Eight detached cotyledons were used for each set of light/dark CER measurements except for 2- and 4-d CER determinations when more cotyledons were used to compensate for lesser area per cotyledon. After the cotyledons reached steady-state photosynthesis in the sample chamber (10–15 min), the lights were turned off to allow for measurement of the PIB and dark respiration. The light/dark cycle was repeated. Five cotyledons were removed, frozen, and stored in liquid N\(_2\) until they were used for Rubisco determinations. The three cotyledons remaining in the sample chamber were used for \(^{14}\)C labeling. Transpiration was estimated from fresh weight losses.

\(^{14}\)CO\(_2\) Incorporation

The 20.2-L closed system described in ref. 13 was used for \(^{14}\)CO\(_2\) incorporation. \(^{14}\)C (1.11 \( \times \) 10\(^{12}\) Bq) was used for each set of four or fewer gas exchange measurements. After the cotyledons used for Rubisco determination were removed, the sample chamber was quickly connected to the closed system and photosynthesis of the remaining cotyledons monitored for 15 min. These labeled cotyledons were frozen and stored in liquid N\(_2\) until extracted for metabolite analysis.Disconnecting the closed system and freezing the cotyledons took <10 s.

Metabolite Extraction and Determination

The \(^{14}\)CO\(_2\)-labeled cotyledons were ground to a fine powder in liquid N\(_2\), and acid-stable metabolites were extracted with 2 N HCl. Insoluble material was removed by centrifugation, and the supernatant was filtered through a Millipore prefilter (Millipore AP20 13 mm) and extracted with chloroform. The aqueous phase was applied to a Dowex 50-H\(^{+}\) ion exchange column (5 \( \times \) 0.5 cm), and the acidic and neutral compounds were washed through with water. The basic fraction was eluted with 2 N NH\(_4\)OH, dried under air at room temperature, and resuspended in a minimal volume of water. Aliquots were spotted onto thin-layer cellulose plates (Kodak 13255 cellulose) and chromatographed in nonequilibrated glass tanks in butanol:acetone:water:diethylamine (20:20:10:3 [2]). The diethylamine was added just before starting the chromatography. Plates were air dried overnight before being used for autoradiography. The radioactive amino acid spots were located on the original TLC plates and quantified by liquid scintillation counting. Standard \(^{14}\)C amino acids were run on each chromatogram.

Quantification of Rubisco

Rubisco was quantified using the method of rocket immunoelectrophoresis. Antisera raised against soybean Rubisco were prepared by Diethelm and Shibbels (7). Agarose gels (BioRad Standard Low Electroendosmosis) containing 1.5% antisem were prepared (13) and electrophoresed for 16 h at 2 to 2.5 V cm\(^{-1}\) using Gelman high-resolution buffer (0.30 m) as the running buffer. The gels were processed as described before (7).

Samples and standards were each run twice on a gel. Samples were prepared by grinding the cotyledons to a fine powder in liquid N\(_2\). Protein was solubilized with 50 mM Bicine (pH 8), and insoluble material was removed by centrifugation. An aliquot of the supernatant was used for Rubisco quantification. Appropriate dilutions were made so that the amount of Rubisco added to a gel was between 0.3 and 0.6 \( \mu \)g. An aliquot of the supernatant was also used to measure total soluble protein (3) using fraction V BSA (Sigma) as a standard. Standards were prepared from Rubisco from the youngest, expanded trifoliolates of greenhouse-grown Corsoy 79 soybeans purified by the method of Paech and Dying (16).

Chl

Chl was extracted into cold N,N-dimethylformamide as described by Moran (15). The minimum fresh weight to solvent volume ratio was 1:20. If the cotyledon was sliced into four or five pieces, extraction with N,N-dimethylformamide gave Chl values comparable to acetone extraction, and Chl was much more stable in the N,N-dimethylformamide (98% of original values after 2 weeks, data not shown).

RESULTS

Growth Parameters

During this experiment, seedlings emerged by the fourth day after planting, at which time the cotyledons were greening and still enlarging (Fig. 1). By 18 d after planting, the third trifoliolate was beginning to unfold and the cotyledons were yellowing. By 20 to 21 d after planting, the cotyledons were fully yellow and/or abscised. Cotyledon fresh weight increased until day 8, remained relatively constant until day 12, decreased again until decapitation, and then remained relatively constant (data not shown). Maximal cotyledon area was attained by day 8 and remained relatively constant throughout senescence and rejuvenation (Fig. 1B). Total Chl per cotyledon increased until day 8 and then decreased steadily until decapitation or abscission (Fig. 1A). During rejuvenation, Chl increased to a maximum 1.25-fold greater than the maximum observed in presenescent cotyledons.

\textsuperscript{2} Abbreviations: CER, carbon dioxide exchange rate; PIB, postillumination outburst of CO\(_2\).
Rubisco

Rubisco could be detected immunochemically in 2-d-old, preemergent cotyledons, before there was detectable Chl. The amount of enzyme increased until day 6 and then decreased steadily until decapitation at day 18 (Fig. 2). After a 2-d lag, Rubisco increased during rejuvenation to the levels seen in 10- and 12-d-old cotyledons, about 75% of the presenescent maximum.

Total soluble protein was highest in 2-d-old cotyledons and decreased very rapidly until day 8, after which time the decrease was much slower (Fig. 2). After the epicotyl was removed, total soluble protein increased slowly, remaining fairly constant after 20 d rejuvenation at a level intermediate between that found in 10- and 12-d-old cotyledons. Rubisco represented about 50% of the total protein in both the 12-d-old cotyledons and in rejuvenated cotyledons beyond 6 d after epicotyl removal.

Gas Exchange

Because of the precipitous changes observed in Chl, CERs were expressed per cotyledon. Light CER was detectable 4 d

Figure 1. Total Chl (A) and area (B) of soybean cotyledons from germination until decapitation 18 d after planting (●) and after decapitation (○). The data are the means ± se from measurements of duplicate samples consisting of eight to 16 cotyledons that were determined individually. Error bars are missing from points where representation of the error was smaller than the data point character.

Figure 2. Rubisco (●, ○) and total soluble protein (■, □) in soybean cotyledons from germination until decapitation 18 d after planting (●, ■) and after decapitation (○, □). The data are the means ± se from measurements of duplicate samples consisting of five cotyledons except that six and 10 cotyledons were used from 4- and 2-d-old seedlings, respectively. Error bars are missing from points where representation of the error was smaller than the data point character.

Figure 3. Light CER (●, ○), dark respiration (■, □), and transpiration (▲, △) in soybean cotyledons from germination until decapitation 18 d after planting (●, ■, ▲) and after decapitation (○, □, △). The data are the means ± se from measurements of duplicate samples consisting of eight cotyledons except that 10 and 16 cotyledons were used from 4- and 2-d-old seedlings, respectively. Error bars are missing from points where representation of the error was smaller than the data point character.
after planting (Fig. 3). The exchange rate increased until day 8, remained relatively constant, and decreased after day 12 until it was not detectable in fully yellow cotyledons. There was little difference between presenescent and rejuvenated light CER maxima. Absolute light CER was only less than dark CER in the 4-d-old tissue (Fig. 3).

Light CER was correlated with Rubisco content during most of the experimental period (Fig. 4). Light CER increased by 60% by the first day after epicotyl removal without a corresponding change in dark CER or an increase in Rubisco content. The data represented by the open and solid squares in Figure 4 reflect this relationship. The transpiration rate also increased sharply during this period (Fig. 3). Thus, the initial rapid increase in CER corresponded to an apparent increase in stomatal aperture caused by epicotyl removal.

Two calculations were made from the dark gas exchange measurements. The first, an estimate of dark respiration, was calculated using the constant minimal CO₂ differential observed after 8 to 12 min of darkness. Dark respiration was highest in the youngest cotyledons and decreased to a relatively constant value by day 14, a level that varied little throughout the remainder of the experiment (Fig. 3). The second calculation was an estimate of the PIB and, hence, of photorespiration. It was made by subtracting the estimate of dark respiration from the maximum dark CO₂ differential, a nonsteady-state value that occurred approximately 30 s after darkening the tissue. The PIB was probably not different in the presenescent and rejuvenated cotyledons (data not shown). The shapes of the IRGA traces from light/dark transients in presenescent and rejuvenated cotyledons were very different, however (data not shown), probably because dark respiration was changing during reserve mobilization in the younger, presenescent tissue, making PIB calculations uncertain.

14CO₂ Incorporation

Calculations from acid-stable total incorporated 14C gave estimates of net photosynthesis that were mostly within 10% of the gas exchange measurements (data not shown). The labeling data should, therefore, relate well to calculations from gas exchange measurements (CER and PIB). Total label incorporated in glycine as a function of cotyledon age gave a pattern similar to that observed for light CER (data not shown).

The percentage of total incorporated label found in glycine was relatively constant from 4 through 12 to 14 d after planting (Fig. 5). Label in glycine then decreased and did not begin to increase above the value at decapitation (18 d) for 5 d. The percentage of label in glycine increased throughout rejuvenation, reaching a new, higher steady-state value 26 d after epicotyl removal. The percentage of label in glycine should be indicative of the rate of photorespiration as long as non-Rubisco carboxylation does not contribute significantly to the total 14C incorporated. Labeled glycine was detected during the period in which a PIB was not detectable, suggesting that photorespiration was occurring during that time.

DISCUSSION

Growth Parameters

Patterns of change in growth parameters observed in this study were qualitatively similar to those observed by others (5, 9, 11, 17), although differences in timing were seen. Seasonal variation was observed in the age of cotyledon when senescence was observed and, therefore, in the age after which the cotyledons no longer responded to epicotyl removal. Variation in timing of growth stages was probably related to

![Figure 5](https://www.example.com/f5.png)

**Figure 5.** The percentage of total 14C incorporated found in glycine in soybean cotyledons from germination until decapitation 18 d after planting (●) and after decapitation (○). Data are the means ± SE from measurements of duplicate samples consisting of three cotyledons except that four and six cotyledons used for 4- and 2-d-old seedlings, respectively. Error bars are missing from points where representation of the error was smaller than the data point character.
differences in the duration and intensity of light supporting different growth rates. In the studies reported here, the third trifoliolate was in its early stages of expansion when the cotyledons senesced and abscised, regardless of the season. Harris et al. (11) and Peterman and Siewod (17) reported similar timing between cotyledon abscission and third trifoliolate unfolding, although there may be varietal differences in this characteristic.

**Gas Exchange and \(^{14}\text{CO}_2\) Incorporation**

Maximum cotyledon light CERs measured in this study were in the low end of the range reported for field-grown soybean leaves (for example, see ref. 18) and were greater than those reported in other studies of soybean cotyledon photosynthesis (1, 4, 5, 9). Rates reported here were clearly in excess of dark respiration even in young cotyledons that showed high dark respiration rates. The very low rates reported by Abrahamsen and Mayer (1) were most likely a result of methodological limitations and of the use of low light intensities for growth and measurement.

In agreement with the data of Harris et al. (9), the CER of cotyledons increased to a maximum during early seedling development and then decreased to a minimal value during senescence. This pattern contrasted with the data reported by Brown and Huber (4, 5) which showed an early peak in light CER followed by a period of relatively constant CER, after which time CER increased to a maximum before rapidly declining to a very low level. Brown and Huber (4, 5) did not indicate at what age their cotyledons abscised, although the dry weight and Chl data that they reported suggest abscission at approximately 19 d, which is comparable with this study. They used lower irradiance for growth (450 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) and shorter daylength than we used in this study; however, Harris et al. (12, 13) used even lower irradiances (230 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) and obtained a CER pattern similar to the one we observed. The differences in irradiances probably account for the absolute differences in CER observed among the studies but not for the different patterns seen. The slower and lesser recovery of Rubisco activity and Chl in rejuvenated cotyledons observed by Harris et al. (10) compared with that found in this study is also likely due to the lower irradiances used by them for growth and measurements.

We saw a clear relationship between CER and total Chl in presenescent, senescing, and rejuvenating cotyledons (data not shown). The excess Chl that accumulated in the rejuvenated cotyledons, however, did not result in a corresponding increase in CER. To further characterize this differential response in rejuvenated cotyledons, CER was expressed as a function of the amount of cotyledon Rubisco and correlated with Rubisco concentration during most of the developmental sequence studied (Fig. 4). In the rejuvenated cotyledons in which excess Chl accumulation occurred, the lack of a corresponding increase in light CER may be attributed to a relatively constant Rubisco concentration.

There were two periods in this developmental sequence during which the relationship between light CER and Rubisco content was uncoupled or different. The first period included the most senescent cotyledons used during this study (18 d) and during the first 2 d of rejuvenation (Fig. 4). Equivalent amounts of Rubisco were measured in these cotyledons; yet, CER increased by 60% by the first day after epicotyl removal. This rapid increase may reflect the release of some stomatal limitation imposed by senescence (22), because transpiration also increased rapidly by the first day of rejuvenation. Friedrich and Huffaker (8) found a close correlation between CER and stomatal aperture and transpiration in their system, in addition to a high correlation between CER and Rubisco. The response time of the transpiration/stomatal aperture effect was more rapid than that of any other parameter measured.

Four- and 6-d-old cotyledons represented the second time period in which a different relationship between light CER and Rubisco concentration was observed: CER was less than expected based on Rubisco content. It is possible that CO\(_2\) diffusion was limiting in these fleshy organs, but this also represents a time during which these photosynthetic organs were still mobilizing stored reserves. If reserve mobilization occurs in the light, as is suggested by the data of Brown and Huber (4, 5), then light CER could be an underestimation of photosynthesis.

Presenescent and rejuvenated soybean cotyledons exhibited net photosynthetic rates in the range reported for true leaves. We also observed a typical \(\text{C}_3\) leaf PIB and, thus, photosynthesis rates comparable to those of leaves. However, a general relationship between the PIB and light CER was not observed, in contrast to the straightforward relationship observed by Bulley and Tregunna (6). In their study, CER was varied by changing light intensity so that PIB estimates were from the same soybean leaf. In this system, different light CERs came from plants in different developmental states. In addition to seeing differences in the relative magnitude of the burst, the shape of the PIB was different throughout this time frame, reflecting, perhaps, differences in pool sizes as well as differences in pool turnover rate. Dark respiration was changing during reserve mobilization in the younger, presenescent cotyledons, making PIB calculations uncertain.

The PIB was not detected in 4-d-old cotyledons or in late senescent and early rejuvenating cotyledons. Incorporation of \(^{14}\text{C}\) indicated, however, that photorespiration was occurring in these cotyledons. The percentage of the total label incorporated into glycine changed significantly during the time the developmental series was studied. In presenescent cotyledons, the percentage of label in glycine was fairly constant during a period of greatly changing light CER, suggesting a constant rate of photorespiration. In senescing and early rejuvenating cotyledons, the percentage of label in glycine was fairly constant during a period of greatly changing light CER, suggesting that the rate of photorespiration was changing, decreasing in the senescing cotyledons and increasing again in the rejuvenating organs. Senescent sunflower cotyledons produced less glycinate relative to CO\(_2\) fixed than nonsenescent cotyledons (2), suggesting a change in photorespiration relative to photosynthesis. In late rejuvenated cotyledons, the percentage of label in glycine was greater than it was in the presenescent cotyledons, suggesting that the relative rate of photorespiration had increased. Although CER in the rejuvenated cotyledons recovered to presenescent levels, if photorespiration is higher in the rejuvenated cotyledons, then, because CER is a net measurement, true photosynthesis may in fact be higher in the rejuvenated cotyledons than in the presenescent ones.
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LITERATURE CITED