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Photosynthesis and Other Traits in Relation to Chloroplast Number during Soybean Leaf Senescence[1]

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ABSTRACT

Soon after attaining full expansion, soybean (*Glycine max* [L.] Merr.) leaves enter a senescence phase marked by decline in photosynthetic rate and the progressive loss of chloroplast activity and composition. Our primary goal was to determine if this loss could be accounted for by sequential degradation of whole chloroplasts or by simultaneous degeneration of all chloroplasts. Total photosynthesis (TPs) measured as $^{14}CO_2$ uptake, chloroplast number, ribulose 1,5-bisphosphate carboxylase activity, uncoupled photosynthetic electron transport activity, soluble protein content, and chlorophyll content declined progressively during the 37 days after full leaf expansion. During this period, chloroplast number per unit leaf area was constant for all genotypes studied. We conclude that leaf senescence may be a two-stage process wherein the first stage chloroplast activity and composition declines, but chloroplast numbers do not change. During a brief terminal stage (11 days in our experiment), whole chloroplasts may be lost as well. As a second objective we wished to determine if variation in single-leaf total photosynthetic rate among soybean cultivars is related to corresponding variation in chloroplast number and/or chloroplast activity/composition. By comparing the means for three cultivars known to have rapid leaf TPs and for the three known to have slow TPs, we found the former group to be superior to the latter for all the previously mentioned leaf physiological traits. This superiority was related primarily to differences in chloroplast number and only secondarily to differences in activity and composition per chloroplast.

This paper is in part about genotypic differences in single-leaf photosynthetic rates, but mainly about the changes in single-leaf photosynthetic rates that occur during leaf senescence. Many experiments have shown the interrelatedness of leaf photosynthetic rate with other leaf traits among soybean (*Glycine max* [L.] Merr.) genotypes (3, 4, 6, 17). There are some inconsistencies, but the consensus that emerges from these experiments is that leaf photosynthetic rate, RuBP[3] carboxylase activity, photosynthetic electron transport activity, total or soluble protein content, Chl content, and specific leaf mass (all expressed per unit leaf area) are all more or less correlated. This suggests that genotypes that package more photosynthetic apparatus beneath each unit of leaf area will have more rapid photosynthetic rates than genotypes with less photosynthetic apparatus per unit leaf area. The question that we pose is how is the photosynthetic apparatus packaged: Do genotypes with rapid photosynthetic rates have more chloroplasts per unit leaf area, or does variation in chloroplast activity and composition explain differences in leaf photosynthetic rate?

The senescence phase of a leaf is characterized by a progressive decline in photosynthetic rate per unit leaf area. Most closely and consistently associated with this decline is the loss of RuBP carboxylase protein and activity (11, 14, 15, 20, 21). Uncoupled electron transport activity (5, 8) and Chl content (14, 15, 21) also diminish during senescence, but the onset and/or rate of decline may or may not be coupled with the decline in photosynthetic rate. Two hypotheses have been advanced to explain the loss of chloroplast composition and function. Either activity and composition per chloroplast remains constant with whole chloroplasts being sequentially destroyed (5, 12, 21, 22), or number of chloroplasts remain constant with a decline in activity and composition per chloroplast during senescence (10, 16). In this report, we present and discuss evidence that suggests that whole chloroplasts are lost only after a considerable decline in their composition and function.

MATERIALS AND METHODS

Culture. Six soybean cultivars ('Corsoy,' 'Amsoy,' 'Harosoy-63,' 'Ford,' 'Hawkeye,' and 'Richland') were field-grown under high fertility conditions and irrigation. The former three have rapid leaf photosynthetic rates, whereas the latter three have slower rates. Planting was on May 11. The experiment was in a split-plot design, with sampling dates assigned to whole plots and cultivars to the split-plots (hereinafter referred to simply as plots).

Sampling and Total Photosynthesis. Newly unfolding leaves were tagged on 10 plants in each plot on July 6. These leaves were the ones later sampled. Total photosynthesis per unit leaf area was measured on attached terminal leaflets of six tagged leaves in each sampled plot by $^{14}CO_2$-uptake (see Ref. 13 for details of procedure) on July 11, 18, and 25 and on August 1, 8, 15, and 24. Tagged leaves were about two-thirds expanded on July 11, 55 d after plant emergence, and were fully expanded 7 d later.

Two 1.27-cm^2 discs were removed from unexposed portions of each of the same six terminal leaflets and frozen in liquid N_2 for subsequent measurement of Chl, soluble protein, and the activity of RuBP carboxylase. Additionally, three (0.32 cm^2) discs were taken from each leaflet and preserved in FAA (10:50:5:35, 37% w/w formaldehyde:ethanol:glacial acetic acid:water) for chloroplast counting. The lateral leaflets were harvested the next day and taken to the laboratory on ice for measurement of uncoupled photosynthetic electron transport activity.

Photosynthetic Electron Transport. We found that leaflets could be stored on ice for up to 6 h with little loss of activity. Three to 6 of 12 lateral leaflets from each plot were pooled and coarsely chopped with a razor blade. The leaf pieces were placed

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[3] Abbreviations: RuBP, ribulose 1,5-bisphosphate; TPs, total photosynthetic rate.

on a brass window screen, which in turn, was over a 100-mesh, brass screen in a 55-mm plastic, 'break-apart' Buchner funnel. Nylon cloth netting (20 μm—Nitex, Tetko Inc., Elmsford, NY) was clamped between the two halves of the Buchner funnel. With the vacuum off, 20 ml of cold extraction buffer (4 mM Na_2HPO_4, 15 mM NaH_2PO_4 [pH 6.5], 0.3 M sucrose, 2% w/v PVP-40, and 12.5 mM K_2SO_4) was added, and the leaf pieces were vigorously mashed and worked over the screens with a small pestle. A slight vacuum was then applied, and liberated cells were collected on the nylon cloth. Free chloroplasts and other small debris passed through the cloth and were discarded. The mash was twice washed with 10-ml aliquots of extraction buffer. The Buchner funnel was broken apart, and the cells were washed off the netting into a 50-ml centrifuge tube. The extract was centrifuged by accelerating to 1500*g* and then decelerating. The supernatant was decanted, and the cells were resuspended in sufficient assay buffer (50 mM Tricine [pH 7.5], 0.2 M sucrose, 2 mM Ca $[NO_3]_2$, 5 mM KNO_3, 1 mM $MgCl_2$) to give about 0.2 to 0.4 mg Chl/ml buffer. This procedure is rapid and yields an abundance of intact cells—as judged by their ability to exclude Evan's Blue dye. Two, 0.25-ml aliquots were removed for Chl determination.

The method for measuring electron transport in whole cells was generously suggested by Dr. Charles Arntzen (personal communication). In an O_2-electrode (Hansatech Ltd., King's Lynn, Norfolk, England) a 0.1-ml aliquot of the cell suspension was combined with 0.9 ml of assay buffer. Aliquots of concentrated NaN_3 and methyl viologen were added, their final concentrations being 0.5 mM. After 60 s, the O_2-electrode was illuminated with a 300-W, film-strip projector, and O_2 consumption was monitored. After 120 to 180 s, uncoupling agents were added—gramicidin (final concentration of 5 μM) in 50% ethanol (0.5% v/v) and NH_4Cl (5 mM)—and the uncoupled rate was determined. Coupled and uncoupled rates were linearly related to Chl up to 50 g/ml. Photosynthetic electron transport activity per unit leaf area was calculated as the uncoupled rate of O_2 consumption per unit Chl multiplied by the amount of Chl per unit leaf area.

Chl and Soluble Protein. On d 2 after TPs sampling, six of the discs that had been harvested from each plot and frozen in liquid N_2 were homogenized in 100% ethanol by use of a Polytron (Brinkman). The total extract was filtered through No. 4 Whatman filter paper in a 11.5-mm Hirsch funnel. Chl content of the filtrate was determined spectrophotometrically according to Wintermans and de Mots (19).

The filter paper and cell residue was quantitatively transferred from the Hirsch funnel to a 50-ml centrifuge tube. Soluble proteins were extracted in 0.3 N NaOH by overnight incubation at 35 to 40°C. The alkaline protein solution was filtered (No. 1 Whatman). The filtrate was acidified with HCl and assayed for protein by Coomassie blue dye binding (2) by using BSA as the protein standard.

Ribulose 1,5-Bisphosphate Carboxylase. Also, on d 2 after TPs sampling, the remaining discs that had been frozen in liquid N_2 were ground in 5 ml of ice-cold, grinding buffer (14) with a small mortar and pestle. The extract was centrifuged at 4200*g* for 20 min. Aliquots were removed from the supernatant for determination of protein (2) and RuBP carboxylase activity (14). RuBP carboxylase activity per unit leaf area was calculated as activity per unit protein multiplied by the amount of soluble protein per unit leaf area.

Chloroplasts. Six of the discs that had been preserved in FAA were removed, dehydrated in an ethanol/xylene series, and embedded in paraffin. Sections 2 to 3 μm thick were cut from these discs with a microtome and stained with Safranin 0. Chloroplast number per unit leaf area was estimated as the number of chloroplasts in a cross-sectional area 17.5 μm long and bounded by the epidermal layers, divided by the leaf surface area associated with the cross-section (*i.e.* 17.5 μm times the thickness of the section). A preliminary study indicated that sufficient accuracy could be attained by counting two areas per section, two sections per leaf disc, and two leaf discs—a total of eight determinations per replicate. Chloroplast number could not be determined for the July 11 sample because the cells had not reached full expansion, and it was too difficult to discern individual chloroplasts.

RESULTS AND DISCUSSION

All the sampled leaves reached full expansion coincidentally at about 62 d after plant emergence from the soil. Therefore, differences among leaves on a given date should be as nearly independent of leaf age and environment as possible.

We counted an average of 9.4×10^7 chloroplasts/cm^2 or about twice what Watanabe (17) found in soybean unifoliolates. However, unifoliolate leaves on plants grown in conditions similar to those of Watanabe had about half the specific leaf mass of our field-grown, midcanopy leaves (18). The coefficient of variability was relatively high for chloroplast number/cm^2: 24% *versus* 19, 18, and 9% for RuBP carboxylase activity, TPs, and Chl content, respectively. Nevertheless, we could statistically detect differences of 8% between groups (*i.e.* between the mean of the three cultivars with rapid TPs and the mean of the three cultivars with slow TPs) and among dates. The magnitudes and variabilities associated with the rest of the data resemble what others have found for similar leaves (14, 15, 21, 22).

Group Effects. There were significant differences between group means for all six leaf traits expressed on a leaf area basis (Fig. 1). The group of cultivars with rapid TPs also averaged more chloroplasts, more soluble protein, more Chl, more rapid RuBP carboxylase activity, and more rapid photosynthetic electron transport activity than the group with slower TPs (Fig. 1).

These findings corroborate earlier work (3, 4, 6, 17) and suggest that genotypic differences in single-leaf photosynthesis of soybean are caused by associated variation in the amount of photosynthetic material per unit leaf area. On a chloroplast basis (Fig. 2), only RuBP carboxylase activity varied significantly among groups.

Therefore, variation in TPs among groups seems to be primarily associated with variation in chloroplast number per unit leaf area. This finding agrees with earlier work by Watanabe (17), who found that variation in chloroplast numbers explained 77% ($r = 0.88$) of the variation in photosynthesis among five cultivars.

Senescence Effects. TPs, RuBP carboxylase activity, and uncoupled electron transport activity all reached maximum on d 62, when leaves had just attained full expansion. Not only the onset but also the magnitude of declines in these activities are closely associated (Fig. 1). The amounts of Chl and soluble protein both peaked about 1 or 2 weeks later and remained near the peak content for a longer period before declining. The magnitude of the decline was generally less for these two traits than for TPs, RuBP carboxylase activity, or electron transport activity.

Chloroplast numbers per unit leaf area within groups, though variable, did not differ significantly throughout the sampling period. Therefore, we conclude, in agreement with Mae *et al.* (10) and Wardley *et al.* (16), that the declines in chloroplast traits observed during the first part of senescence resulted from aging of the entire chloroplast population rather than from sequential degradation of whole intact chloroplasts. Interestingly, the rates of decline in chloroplast activity and composition (Fig. 2) did not vary among genotypes.

Others have found that chloroplast number per cell declines during senescence, suggesting that the loss of whole chloroplasts may cause the decline in photosynthesis and chloroplast properties (5, 9, 12, 21, 22). However, we believe that these data can be reconciled with our findings. In nearly all these latter experi-

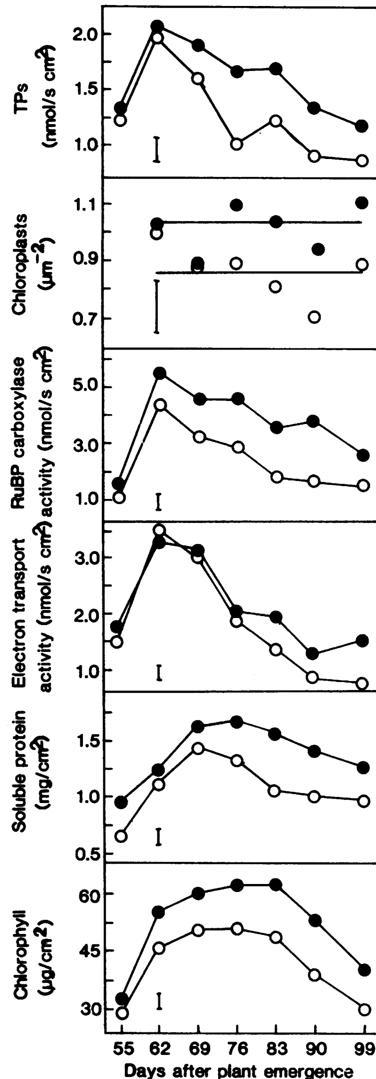

FIG. 1. Means for six leaf traits, expressed on a unit leaf area basis, for the rapid leaf TPs group: (●), Corsoy, Amsoy, and Harosoy-63, and for the slow leaf TPs group; (○), Ford, Hawkeye, and Richland. Each point represents 12 independent measurements, three cultivars each replicated four times (see "Materials and Methods"). $LSD_{.05}$ bars are for differences between group means within a date.

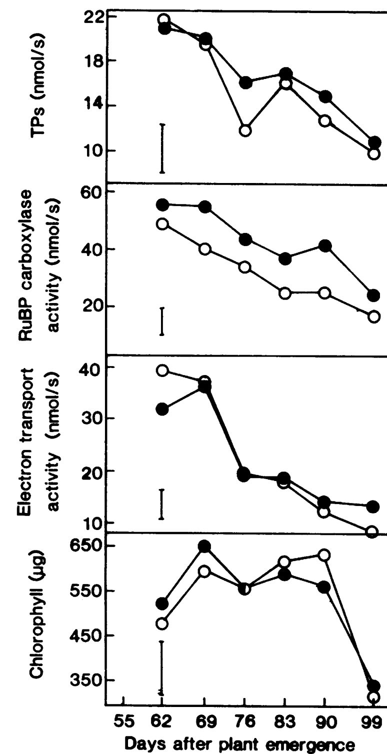

FIG. 2. Means for four measured leaf traits expressed on a chloroplast basis. Values are per 10^9 chloroplasts. Symbols and bars as described in the legend of Figure 1.

ments, considerable deterioration in activity of the entire chloroplast population had occurred before the onset of declines in chloroplast number. This suggests that leaf senescence is a two-phase process; in the first phase, chloroplasts deteriorate but do not disappear (our measurements were made during this phase), then late in the process, chloroplasts not only deteriorate but also disappear.

There is support for senescense being a two-phase process. Wittenbach *et al.* (21) using field-grown, upper-canopy soybean leaves found that photosynthetic rate declined by 20 to 60% before chloroplasts per cell began to decline. When Wittenbach *et al.* (22) induced senescence in attached wheat (*Triticum aestivum* L.) leaves by darkening them, RuBP carboxylase activity and photosynthetic rate per protoplast declined to nearly zero in 5 d, although 32% of the chloroplast population remained. In naturally senescing wheat leaves, Peoples *et al.* (12) found that, by 20 and 35 d after anthesis, RuBP carboxylase content had declined by 50 and 100%, respectively. Chloroplasts remained intact until 20 d after anthesis. Camp *et al.* (5) suggested that senescence in wheat leaves is caused by the sequential degradation of whole chloroplasts, but 25% of the maximum photosynthetic rate and 50% of the RuBP carboxylase and NADP-triose dehydrogenase activities were lost before chloroplast number likely began to decline. In primary wheat leaves, Mae *et al.* (10) observed that RuBP carboxylase and Chl contents declined by 98 and 55%, respectively, during the same period that chloroplast number declined by 13%. Thereafter, chloroplast number declined rapidly. Wardley *et al.* (16) could detect declines in chloroplast number per cell only in the oldest tissues of senescing primary wheat leaves. In these tissues, chloroplast number declined by about 22%, but not until RuBP carboxylase content had decreased by 80% and the Chl was gone. Younger portions of the leaf showed no decline in chloroplast number.

Electron microscopy reveals that considerable degeneration occurs preceding the loss of whole chloroplasts (7, 12, 21). Early in senescence, osmiophilic globuli appear. These progressively increase in size and number concurrent with, first, a disruption of thylakoid structure and, then thylakoid degeneration. Only after these changes have occurred do chloroplasts rupture and decline in number.

So the bulk of the physiological and anatomical data seems to be in agreement. Near the time of full leaf expansion photosynthetic rates are at a maximum. Soon thereafter, they begin to decline (Fig. 1; 5, 11, 14, 20–22). Coincidentally, osmiophilic globuli appear in the chloroplasts and begin to increase in size and number as the thylakoids degenerate (1, 7, 12, 21). Chl content begins to decline progressively at the same time as photosynthetic rate begins to decline or later. At some time, but not until extensive degeneration of the stroma and thylakoids has occurred, chloroplast number begins to decline (1, 7, 10, 12, 21). We probably did not observe declines in chloroplast number because we followed the course of senescence only until about 50% of the peak photosynthetic capacity was lost. It took 37 d for our leaves to lose the first 50% of the maximum photosynthetic activity and only 11 d or less to lose the rest.

CONCLUSIONS

Single-leaf photosynthetic rate differed among six soybean genotypes apparently because of differences in the amount of photosynthetic machinery per unit leaf area. The data supported the hypothesis that variation in amount of photosynthetic machinery was related primarily to differences in the number of chloroplasts per unit leaf area and only secondarily to differences in chloroplast activity and composition.

On the other hand, over the lifetime of individual leaves, differences in photosynthetic material and related differences in photosynthetic rate per unit leaf area are more closely related to chloroplast activity/composition than to number per unit leaf area. The leaves of all six cultivars exhibited identical ontogenetic trends with respect to the measured leaf traits; differences were only observed among genotypes in the magnitude of the traits at any given point in ontogeny.

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