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# Sink Removal and Leaf Senescence in Soybean[1]

## CULTIVAR EFFECTS

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### ABSTRACT

**Three cultivars of soybean (*Glycine max* [L.] Merr. cvs Harper, McCall, and Maple Amber) were grown in the field and kept continuously deflowered throughout the normal seedfill period. For all cultivars, deflowering led to delayed leaf abscission and a slower rate of chlorophyll loss. Compared to control plants, photosynthesis and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) level declined slightly faster for deflowered Harper, but for both McCall and Maple Amber, leaves of deflowered plants maintained approximately 20% of maximum photosynthesis and Rubisco level 1 month after control plants had senesced. Deflowering led to decreased leaf N remobilization and increased starch accumulation for all cultivars, but cultivars differed in that for McCall and Maple Amber, N and starch concentrations slowly but steadily declined over time whereas for Harper, N and starch concentrations remained essentially constant over time. SDS-PAGE of leaf proteins indicated that for all cultivars, deflowering led to accumulation of four polypeptides (80, 54, 29, and 27 kilodaltons). Western analysis using antisera prepared against the 29 and 27 kilodalton polypeptides verified that these polypeptides were the glycoproteins previously reported to accumulate in vacuoles of paraveinal mesophyll cells of depodded soybean plants. The results indicated that depending on the cultivar, sink removal can lead to either slightly faster or markedly slower loss of photosynthesis and Rubisco. This difference, however, was not associated with the ability to synthesize leaf storage proteins. For any particular cultivar, declines in chlorophyll, photosynthesis, and Rubisco were initiated at approximately the same time for control and deflowered plants. Thus, even though cultivars differed in rate of decay of photosynthetic rate and Rubisco level in response to sink removal, the initiation of leaf senescence was not influenced by presence or absence of developing fruits.**

The observation that fruit removal from soybean (*Glycine max* [L.] Merr.) plants leads to retention of green leaves long after leaves of normal plants have completely abscised has led to the contention that fruits have a regulatory function in the initiation and progression of leaf senescence (10, 12). Several studies, however, have demonstrated that pod removal or male sterility had little effect on total plant dry weight and nitrogen accumulation, nitrogenase activity, the initiation of Chl declines, and loss of photosynthetic activity and Rubisco[2] from leaves (1, 2, 4, 11, 16, 18, 19). Thus, while absence of fruits does decrease the rate of Chl degradation and delays leaf abscission, there is no apparent effect on the initiation of leaf senescence. Indeed, work of Wittenbach (18, 19) has indicated that, based on loss of photosynthetic activity and Rubisco, fruit removal leads to a more rapid functional senescence.

Recently, Heitholt and Egli (9) reported that continuous flower removal from the soybean cultivar McCall resulted in continued plant dry weight accumulation well after control plants had senesced, a result which is contrary to several previous studies. Apparently, soybean cultivars may differ in response to absence of developing reproductive structures. This would be consistent with results for maize (*Zea mays* L.) where, depending on the cultivar, ear removal leads to either markedly accelerated or slightly delayed leaf senescence compared to control plants with ears (2, 5).

This study was undertaken to examine the effect of continuous deflowering on several soybean cultivars, including McCall (9), to reconcile the differing reports concerning the relationship between developing fruit and leaf senescence.

## MATERIALS AND METHODS

**Plant Culture.** Seed of three indeterminant soybean cultivars (Harper [maturity group III], McCall and Maple Amber [both maturity group 00]) were planted on Spindletop Research Farm near Lexington, KY on May 31, 1985 and May 21, 1986. The soil type was a Maury silt loam (Typic Paleadalf). The experimental design was a randomized complete block design with four replicates. Cultivars were planted at 26 seeds $m^{-1}$ in three row plots which were 6.1 m long with 0.7 m row spacing. Within the middle row of each plot, four 0.5 m sections were thinned to 20 seeds $m^{-1}$ at growth stage V2 (7). Plants were sampled sequentially (three plants per sample) from the middle 0.3 m of the thinned sections. All plots were irrigated with a sprinkler irrigation system to minimize moisture stress. For the deflowering treatment, flowers were removed from plants every 3 d. Flower removal did not stimulate node production on the main stem and therefore, the leaf position sampled was identical for both deflowered and control plants.

**Sampling.** For all measurements, the second leaf below the uppermost leaf that was unrolled (edges were not touching [7]) was sampled. Photosynthesis measurements were made on two center leaflets for each plot. For all other measurements, three leaves $plot^{-1}$ were sampled. The middle leaflets were combined for Rubisco and electrophoresis analysis. The side leaflets were combined for nitrogen and starch analysis. Leaflets were transported from the field to the laboratory on ice. For Rubisco and electrophoresis analysis, three leaf punches (113 $mm^2$) were stored at −60°C until analysis. For nitrogen and starch analysis, the side leaflets were freeze-dried prior to grinding (20 mesh) and subsequent analysis. Similar results were obtained in both years of the experiment; all data presented will be for 1986 with the

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[2] Abbreviations: Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; DAP, days after planting.

exception of photosynthesis data.

**Photosynthesis.** Photosynthesis was determined with a LI-COR LI 6000 portable photosynthesis system. A 1073 ml leaf chamber was used and photosynthesis was determined on 1250 $mm^2$ of leaf area. Measurements were made on sunny days between 1030 and 1230 h EDST.

**Leaf Constituents.** Chl, total nitrogen, and starch were determined as previously described (4, 15).

**Electrophoresis.** SDS-PAGE was performed in 7.5 to 15% gradient gels as described by Salvucci and Ogren (13). Three leaf discs (113 $mm^2$ $disc^{-1}$) were homogenized in 0.5 ml buffer in a Ten Broeck homogenizer. The buffer contained 25 mM Hepes (pH 7.5), 1 mM EDTA, 5 mM isoascorbate, and 4 mM DTT. Samples were centrifuged in a microfuge for 10 min at 13000*g*. Protein was determined using Biorad (Biorad Laboratories)[3] protein reagent with BSA used as the standard. Each well was loaded with 60 $\mu$g of protein.

**Rocket Immunoelectrophoresis.** Rubisco quantity was determined by rocket immunoelectrophoresis as described by Wittenbach (17). Sample preparation was the same as for SDS-PAGE. Samples were diluted such that 1.5 $\mu$g of protein was loaded into each well. Soybean Rubisco purified according to Salvucci *et al.* (14) was used as the standard. Rubisco antisera (0.05% v/v) was kindly provided by V. A. Wittenbach (E. I. du Pont de Nemours and Co.) and was prepared as previously described (18).

**Western Blot Analysis.** Polypeptides separated by SDS-PAGE were electroblotted onto nitrocellulose as described by Salvucci and Ogren (13). Blots were probed with antisera prepared against the 29 and 27 kD polypeptides described by Wittenbach (20). Antisera was kindly provided by V. A. Wittenbach (E. I. du Pont de Nemours and Co.) and was prepared as previously described (20). Polypeptides were visualized using an alkaline phosphate linked anti-mouse immunoglobulin G system according to methods described by the manufacturer (Biorad Laboratories).

## RESULTS

The cultivar response to flower removal was similar in both years, thus only 1986 data is presented unless otherwise indicated. The typical visual response of soybeans to flower or fruit removal was reflected by Chl data in 1986 (Fig. 1). Although Chl levels began to decline at essentially the same time for deflowered and control plants of a particular cultivar, the decline of Chl was much slower for deflowered plants. Deflowered plants maintained green leaves long after complete leaf abscission for controls.

Although maturity group and thus flowering and maturity dates differed among cultivars, differences in the length of the seed filling period (days between growth stage R5 and R7) or the relative photosynthesis patterns were relatively small. For example, the number of days between growth stage R5 (7) and complete leaf abscission was 30 and 31 for Harper, and 24 and 26 for both McCall and Maple Amber, in 1985 and 1986, respectively. Also, rapid declines in photosynthesis began soon after growth stage R6 for controls of all cultivars in both years (Fig. 2), although photosynthesis declined more rapidly for McCall and Maple Amber, compared with Harper in both years. Thus, when viewed on a relative growth stage basis, photosynthesis patterns and seed filling periods were generally similar over years for control plants of all cultivars.

The differential effect of flower removal on the three cultivars was clearly indicated by seasonal patterns of photosynthesis (Fig. 2). Flower removal led to loss of photosynthesis at a similar, or perhaps slightly greater rate relative to controls for Harper. These data were very similar to results obtained by Wittenbach (18, 19)

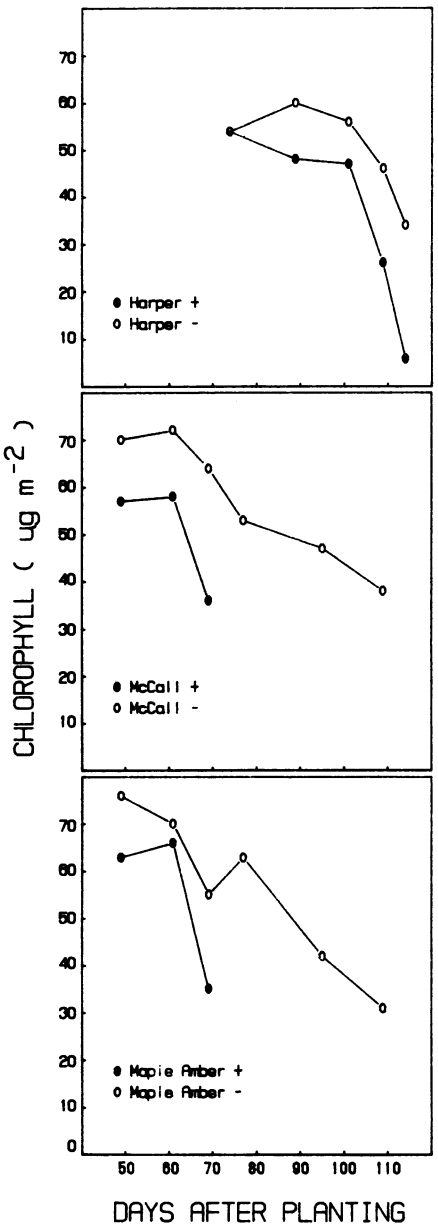


FIG. 1. Effect of flower removal on Chl concentration of leaves of soybean cultivars in 1986. Chl was determined on middle leaflets of the second leaf below the uppermost leaf that was unrolled. The average standard error of the mean for all data points was 3.2 for Harper, 3.3 for McCall, and 3.9 for Maple Amber. Control plants are indicated by closed symbols (●) and deflowered plants are indicated by open symbols (○). For McCall and Maple Amber controls, leaves had completely abscised by 77 DAP.

for two cultivars grown in either a field or growth room environment. Contrary to Wittenbach's results (18, 19) and our data for Harper, flower removal led to a slower rate of loss of photosynthesis for McCall and Maple Amber (Fig. 2). Declines in photosynthesis began at similar times for both control and deflowered plants, but deflowered plants maintained 15 to 20% of maximum photosynthesis approximately 1 month after control plants had senesced. These data were consistent with results of Heitholt and Egli (9) which indicated that dry weight accumulation of McCall continued at a much greater rate for deflowered compared to control plants at later stages of development.

The seasonal trends of Rubisco levels were similar to the trends

[3] Mentioning of a commercial product does not constitute endorsement by the United States Department of Agriculture.

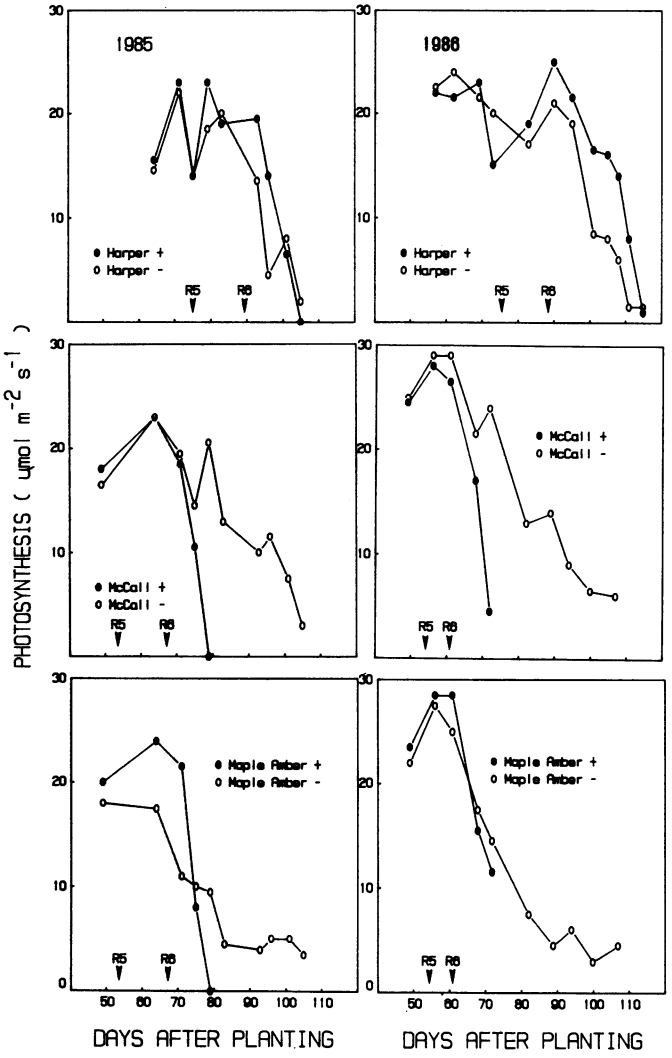


FIG. 2. Effect of flower removal on phytosynthesis of soybean cultivars in 1985 and 1986. Photosynthesis was determined on the middle leaflet of the second leaf below the uppermost leaf that was unrolled on clear days between 1030 and 1230 EDST. The average standard error of the mean for all data points for a particular cultivar was 1.9 and 1.6 for Harper, 1.4 and 1.6 for McCall, and 1.3 and 1.6 for Maple Amber in 1985 and 1986, respectively. Arrows denote the date when growth stages R5 and R6 (7) were reached for control plants. In 1986, leaves of McCall and Maple Amber controls had completely abscised by 77 DAP.

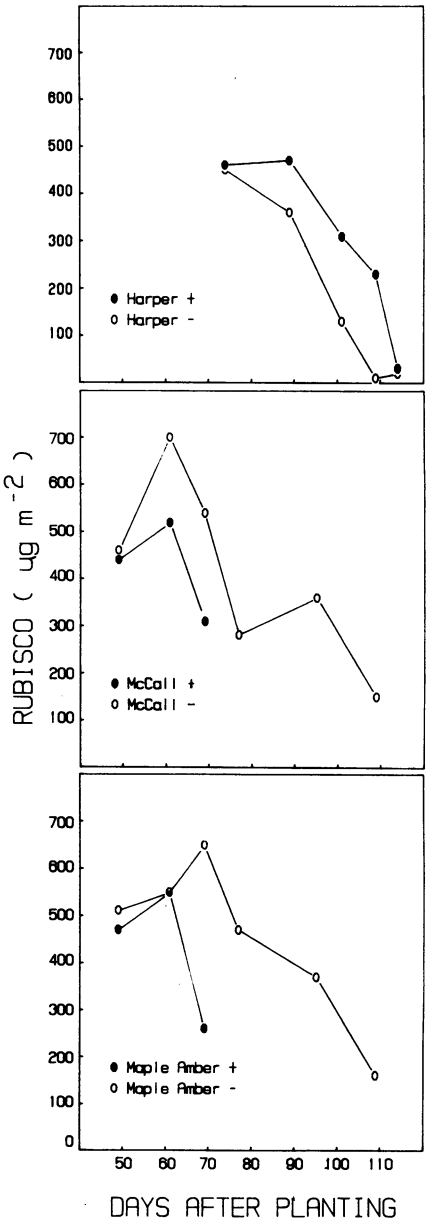


FIG. 3. Effect of flower removal on Rubisco levels of soybean cultivars in 1986. Sampling was as described in Figure 1. The average standard error of the mean for all data points for a particular cultivar was 24 for Harper, 42 for McCall, and 50 for Maple Amber.

of photosynthetic rate observed for leaves of control and deflowered plants of the three cultivars (Figs. 2 and 3). For Harper, deflowering led to a more rapid decline in Rubisco relative to controls (Fig. 3), a result nearly identical to previous work of Wittenbach (18, 19). In contrast to Harper, deflowered McCall and Maple Amber plants lost Rubisco much more slowly than controls. As with photosynthesis, leaves of deflowered McCall and Maple Amber had approximately 20% of the maximum Rubisco level 1 month after controls had senesced. Thus, Rubisco began to decline at approximately the same time for control and deflowered plants for all cultivars, but depending on the cultivar, deflowering resulted in either slightly faster or markedly delayed loss of Rubisco compared to controls.

For all cultivars, deflowering led to inhibition of N remobilization from leaves (Table I). There was little change in the N concentration in leaves of deflowered Harper. However, N concentrations for deflowered McCall and Maple Amber declined slowly but steadily over time. Deflowering caused an expected increase in leaf starch concentration, but as with nitrogen, trends over time differed for Harper compared with McCall and Maple Amber (Table I). Starch concentrations in leaves of deflowered Harper did not change significantly over time, whereas starch concentrations steadily declined over time for deflowered McCall and Maple Amber.

SDS-PAGE was used to visualize potential cultivar differences in leaf soluble proteins caused by the deflowering treatment (Fig. 4). Consistent with rocket immunoelectrophoresis data (Fig. 3), deflowered Harper lost both subunits of Rubisco (53 and 13 kD) more rapidly than controls (Fig. 4, lanes 11 and 12). Correlated with the loss of Rubisco was an increase in four protein bands roughly corresponding to the 80, 54, 29, and 27 kD polypeptides reported by Wittenbach (19). SDS-PAGE of leaf soluble proteins of McCall clearly indicated that both subunits of Rubisco were maintained in leaves of deflowered plants long after controls had

Table I. *Nitrogen and Starch Concentrations of Leaves of Control and Deflowered Soybean Plants at Various Stages of Development*

| | Sampling Time[a] | Harper | | McCall | | Maple Amber | |
|---|---|---|---|---|---|---|---|
| | | Control | Deflowered | Control | Deflowered | Control | Deflowered |
| | | | | $g\ kg^{-1} \pm SEM$ | | | |
| Nitrogen | 1 | 43 ± 1 | 38 ± 1 | 49 ± 2 | 48 ± 2 | 45 ± 2 | 40 ± 2 |
| | 2 | 36 ± 2 | 38 ± 1 | 34 ± 1 | 40 ± 2 | 28 ± 2 | 34± 2 |
| | 3 | 29 ± 1 | 34 ± 1 | —[b] | 38 ± 1 | —[b] | 36 ± 2 |
| | 4 | 20 ± 2 | 34 ± 2 | — | 32 ± 1 | — | 30 ± 1 |
| | 5 | | | — | 33 ± 1 | — | 28 ± 2 |
| Starch | 1 | 112 ± 12 | 179 ± 7 | 101 ± 6 | 141 ± 9 | 76 ± 4 | 189 ± 24 |
| | 2 | 129 ± 22 | 181 ± 14 | 71 ± 15 | 135 ± 18 | 51 ± 9 | 198 ± 28 |
| | 3 | 70 ± 6 | 188 ± 16 | —[b] | 97 ± 6 | —[b] | 159 ± 6 |
| | 4 | 28 ± 4 | 178 ± 32 | — | 103 ± 9 | — | 113 ± 12 |
| | 5 | | | — | 84 ± 15 | — | 89 ± 22 |

[a] For Harper, sampling times correspond to 90, 102, 110, and 115 DAP; for McCall and Maple Amber, sampling times correspond to 62, 70, 78, 96, and 110 DAP. [b] Leaves were completely abscissed from control plants at this time.

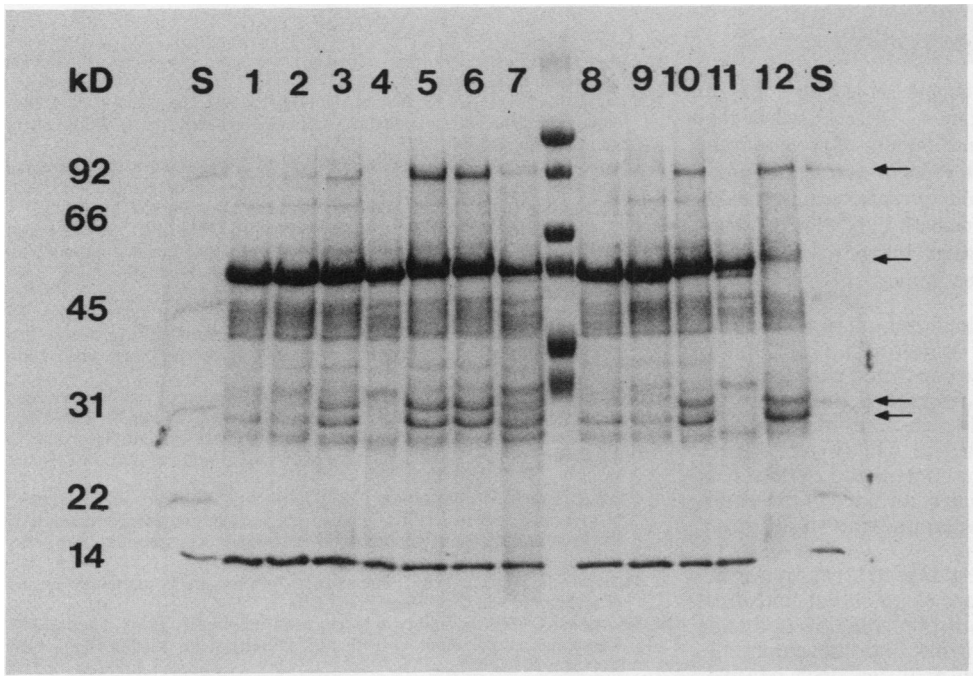


FIG. 4. Polypeptide profiles from SDS-polyacrylamide gel electrophoresis. Samples were from the 1986 experiment. Lanes 1 to 7 were loaded with 60 μg of soluble protein from leaves of McCall; treatments and sampling times were: control, 39 DAP (lane 1); control, 51 DAP (lane 2); deflowered, 51 DAP (lane 3); control, 59 DAP (lane 4); deflowered, 59 DAP (lane 5); deflowered, 67 DAP (lane 6); deflowered, 99 DAP (lane 7). Lanes 8 to 12 were loaded with 60 μg of soluble protein from leaves of Harper; treatments and sampling times were: control, 64 DAP (lane 8); control, 79 DAP (lane 9); deflowered, 79 DAP (lane 10); control, 99 DAP (lane 11); deflowered, 99 DAP (lane 12). Lanes marked by S were loaded with standards of the indicated mol wt. Arrows correspond to polypeptide bands which increased in intensity by the deflowering treatment.

senesced. Similar to Harper, however, leaves of deflowered McCall accumulated proteins in the 80, 29, and 27 kD region of the gel. Due to the presence of Rubisco, it was difficult to distinguish accumulation of the 54 kD polypeptide.

Western blots of an identical gel (same leaf extracts) confirmed that the two bands in the 29 and 27 kD region were indeed the same as reported by Wittenbach (20) (Fig. 5). Blots were probed with the same antisera used by Wittenbach which was prepared against the purified 29 and 27 kD polypeptides (20). Both polypeptides were present in leaves of control plants at beginning seed fill. Deflowering led to an increase in reaction intensity that paralleled the differing contents of Rubisco for Harper and McCall.

## DISCUSSION

Extensive research concerning the effect of fruits on leaf senescence in soybeans has established that, in general, fruit removal has no major effect on plant dry weight or nitrogen accumulation, although absence of fruits does delay leaf abscission, slow the rate of Chl loss, and essentially eliminate leaf nitrogen remobilization (1, 3, 4, 11, 16, 18, 19). Furthermore, Wittenbach (18, 19) has demonstrated that the absence of fruits did not appreciably affect the decline in photosynthesis compared to control plants with fruits, and that loss of photosynthesis was paralleled by loss of Rubisco. Wittenbach and colleagues (8, 20) have also demonstrated that Rubisco degradation in depodded plants was correlated with synthesis of at least four polypeptides, one of which is a glycoprotein composed of 29 and 27 kD subunits and localized in the vacuoles of leaf paraveinal mesophyll cells and associated bundle sheath cells.

The more recent study of Heitholt and Egli (9) was intriguing in that deflowered McCall soybeans continued to accumulate total plant dry weight after control plants had senesced. Our results have confirmed and extended the results of Heitholt and Egli (9). In the absence of fruits, both McCall and Maple Amber maintained photosynthetic competence long after leaves of control plants had abscised (Fig. 2). These data appear to be consistent with results obtained by Crafts-Brandner *et al.* (4) for the cultivar Harosoy, where depodded plants maintained some Rubisco activity after senescence of control plants. For the cultivar

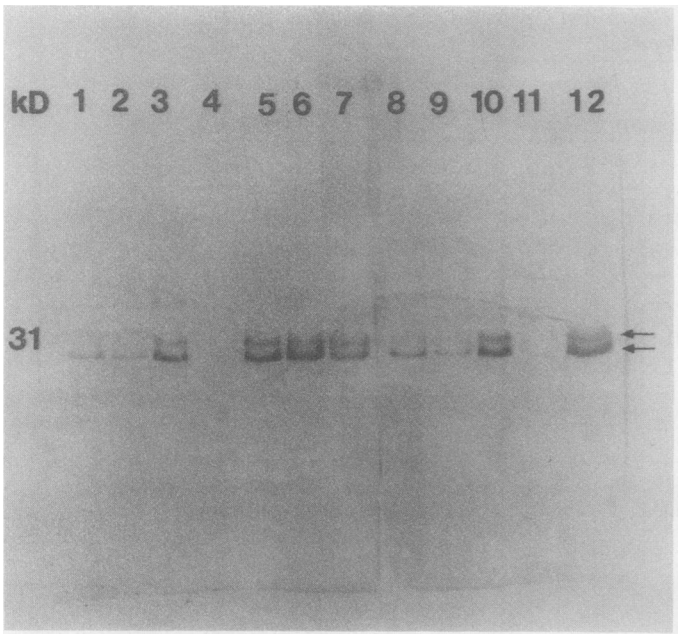


FIG. 5. Western blots of SDS-polyacrylamide gel. Lanes 1 to 12 correspond to treatments described in Figure 4. The gel used in the Western procedure was loaded with the same amount of protein from the same extracts used for the gel in Figure 4. Proteins were blotted onto nitrocellulose paper and probed with antibody prepared against the 29 and 27 kD polypeptides described by Wittenbach (20). Proteins were visualized with alkaline phosphatase-conjugated antibody. Location of the 31 kD mol wt standard is indicated in the figure.

Harper, however, absence of fruits resulted in more rapid decline in photosynthesis, similar to results of Wittenbach (18, 19). In all cases, declines in photosynthesis were correlated with Rubisco levels (Fig. 3). Even though cultivars responded differently to flower removal, it is important to note that declines in photosynthesis and Rubisco, as well as declines in Chl (Fig. 1) were initiated at essentially the same time for control and deflowered plants. Thus, leaf senescence was apparently initiated at the same time in the presence or absence of developing fruit in all three cultivars.

The cause of cultivar differences in response to flower removal is obscure. McCall and Maple Amber are of an earlier maturity group than Harper, but even though control plants flowered and senesced much earlier than Harper controls, plant development was similar for all three cultivars when analyzed on a relative growth stage basis. Furthermore, Heitholt and Egli's (9) experiments with McCall were conducted under natural daylengths in the greenhouse in both early summer and autumn. Thus, McCall apparently responds the same to flower removal under the differing daylengths of early summer and autumn.

SDS-PAGE of leaf soluble proteins (Fig. 4) indicated that, in the absence of fruits, leaves of McCall accumulated the same polypeptides previously reported by Wittenbach (19). In addition, Western analysis using antisera prepared against the 29 and 27 kD polypeptides (Fig. 5) confirmed that these polypeptides were indeed the same as previously observed by Wittenbach (20). Therefore, the differential response to flower removal between cultivars was not associated with any detectable differences in soluble protein composition of leaves. It has been suggested (19) that these newly synthesized proteins, which also were present in control plants at the time of beginning seed fill (19; Fig. 5), when leaf N levels are quite high, serve as a storage form of N somewhat analogous to starch, the predominant form of stored carbon in soybean leaves. Consistent with this suggestion, these polypeptides disappeared in control plants by the later stages of grainfill (Fig. 5), which is similar to commonly observed changes in leaf starch content during the grainfilling period (3). It can only be speculated that the ability to synthesize, store, or remobilize these polypeptides is somehow related to the differential loss of Rubisco between cultivars in the absence of fruits. It was observed that, for deflowered plants, both leaf nitrogen and starch concentrations did decline over time for McCall and Maple Amber whereas for Harper, leaf nitrogen and starch concentrations remained essentially constant over time (Table I).

Degradation of Rubisco in soybean can clearly be manipulated by flower or fruit removal. Based on the rate of nitrogen remobilization from leaves, it appears that cultivars may differ markedly in the rate of Rubisco degradation (6). Recent work with maize has also indicated that cultivars respond differently in response to ear removal and that control plants of different cultivars decline in photosynthesis and leaf nitrogen during grainfill at different rates (2, 5). Whole plant factors such as sink strength, nutrient and growth regulator supply from roots, and Rubisco level may interact to ultimately regulate the mechanism which controls Rubisco degradation. The large difference in Rubisco degradation created by deflowering Harper and McCall or Maple Amber may be useful for investigating the mechanism of Rubisco degradation.

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