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QUANTITATIVE CHANGES OF FATTY ACIDS IN SOYBEAN COTYLEDONS DURING SENESCENCE AND REGREENING

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Key Word Index—*Glycine max*; Leguminosae; soybean; cotyledon; senescence; regreening; chloroplasts; fatty acids.

Abstract— The quantity of total fatty acids in soybean cotyledons during aging, senescence and regreening has been studied. The greatest change in the fatty acid profile during the initial greening of the cotyledons (4–7 days after germination) was a 130% increase in the content of linolenate. Linoleate, as in the case of the other fatty acids, declined in the first 4 days and then increased by 7 days. Following the 10th day after germination, the quantity of palmitate, linoleate, and linolenate decreased continuously through senescence to 20–28% of the maximum quantity of each. When the cotyledons were regreened by removal of the epicotyl 15 or 16 days after germination, linolenate was present in quantities substantially higher than in the senescing cotyledon. On the 22nd day after germination, the quantity of linolenate in regreened tissue was 140% greater than that in senescing tissue of the same age. By contrast, the quantity of linoleate was only 30–40% greater in regreening tissue and the quantity of most of the other fatty acids was similar in both tissues. Similar changes in the quantity of chloroplast fatty acids were observed during this period. Removal of the epicotyl resulted in a higher level of chloroplast linolenate. During aging, the total chlorophyll and the number of chloroplasts reached a maximum on the 10th day and decreased rapidly during senescence. The amount of chlorophyll per chloroplast remained relatively constant during this period whereas the quantity of linolenate per chloroplast decreased during senescence. It is suggested that major structural changes observed in chloroplast membranes may be related to changes in fatty acid composition, but are not dependent on changes in chlorophyll concentration.

INTRODUCTION

Senescence of green tissue has been investigated in a number of plant systems. It has been suggested that the events which lead to the yellowing of a leaf are initiated in the cytoplasm [1, 2] and involve the appearance of proteolytic enzymes. Subsequently, chlorophyll and protein concentrations decrease in the senescing tissue. Isolated chloroplasts show very little decrease in chlorophyll over a 7 day period, indicating that chlorophyll breakdown is regulated in some way by cytoplasmic constituents.

Cotyledons from soybean and cucumber may be particularly useful for studying the mechanism of senescence since the tissues green rapidly and then senesce in a short period [3–7]. In cucumber, the chloroplasts undergo extensive changes in their appearance during senescence with a concomitant decrease in chlorophyll concentration [5, 7]. Draper also noted a large decrease in galactolipids over this same period but did not see a major change in fatty acid composition until the senescence was essentially finished, even though linolenate is a major component of the galactolipids.

Soybean cotyledons begin senescing *ca* 10 days after planting [3, 4]. During the next 6–8 days there is a large decrease in concentration of nucleic acids, protein and chlorophyll [3]. As in the cucumber cotyledon, major changes in the appearance of the chloroplasts occur over this period [4]. Isotope studies using UDP-glucose [$U^{14}C$] indicate that synthesis of galactolipids decreases [4] and there is a general decrease in the lipid content of the cotyledon over this period [8]. In addition to senescing in a short period, another interesting aspect of soybean cotyledons is that the senescence process can be interrupted by removing the epicotyl before the 15th or 16th day after planting [3].

After removing the epicotyl, the cotyledons regreen and there is a concomitant increase in the chlorophyll, nucleic acid and protein concentrations. In addition, the appearance of the chloroplasts reverts back to that of chloroplasts from 10-day-old plants [4]. Thus, it appears that the mechanisms occurring during the senescence process are stopped when the epicotyl is removed. This offers a useful tool to study events closely related to senescence and distinguishes them from events which are coincident with, but not necessarily directly related to senescence.

In the following study, the relationship between fatty acid composition and senescence of soybean cotyledons is studied. To date, previous studies have related to the percent composition of individual fatty acids and changes in lipid composition [7]. In this study, the actual amount of each fatty acid in cotyledons was determined during senescence and after epicotyl removal. Alterations in fatty acid concentrations might be expected since membrane alterations have been noted in the chloroplast [4]. Since the lipid and fatty acid composition of membranes varies, the observed membrane alterations could be related to specific changes in fatty acid composition.

RESULTS AND DISCUSSION

Soybean cotyledons emerge from the vermiculite *ca* 4 days after planting and begin to green. During this 4 day period there is a generally small but significant decrease in the concentration of most of the fatty acids (Table 1). The largest decrease occurs for the major fatty acid, linoleic acid, which represents 60% of the total fatty acids just after planting. Linolenic acid, however, does not decrease during this period. Over the next 3 day period, there is considerable greening of the cotyledons as well as an increase in the fr. wt of the cotyledons. During this period the quantity of linolenate increased 130% whereas the quantity of the other fatty acids only returned to levels which were present on the first day after planting. The concentration of fatty acid reaches a maximum 7–10 days after planting and then decreases continuously and rapidly after senescence begins until the cotyledon drops off the plant on the 20–25 day. The decrease in concentration is most apparent for palmitic, linoleic and linolenic acids, which are reduced to 20–28% of their maximum concentration.

If the epicotyl is removed on the 15th or 16th day there is a rapid reversal of senescence [3]. The yellowed cotyledons regreen with concomitant increases in chlorophyll, protein and nucleic acid concentrations. During this period, linolenate is present in concentration considerably higher than the concentration in plants of the same age but with the epicotyl intact and senescence uninterrupted. On the 22nd day, the concentration of linolenate is *ca* 140% higher in regreened tissue than in the senescing tissue, although still substantially below the concentration observed in 10-day-old cotyledons. The quantity of linoleate was only 30–40% higher in the regreening tissue and the quantity of the other fatty acids was similar in both tissues.

Huber and Newman have shown that major changes occur in the appearance of chloroplasts during senescence and that these changes are reversed during regreening [4]. The concentration changes of linolenate may be related to the observed structural changes of the chloroplast. Presumably the other fatty acids are not involved since their concentrations did not increase during regreening. It is possible, however, that there was a small response to regreening with respect to the quantity of the other fatty acids associated with the chloroplasts. This might not be detected because the concentrations of these fatty acids are relatively high outside the chloroplast whereas linolenic acid is principally located inside the chloroplast.

To determine if other fatty acids in the chloroplast were also changing, the concentration of chloroplast fatty acids in senescing and regreening tissue was determined. The yield of each chloroplast preparation was determined by relating the chlorophyll concentration in the chloroplast preparation to that of the whole cotyledon. The intactness of the chloroplast preparation was determined using phase-contrast microscopy and electron microscopy. Both techniques indicated that the isolation procedure yielded highly pure preparations of intact chloroplasts. In general, the changes in quantity of each fatty acid followed the same pattern observed for the whole cotyledon with the major changes occurring only in the quantity of linolenate during regreening (data not shown).

Since the changes in the concentration of linolenate approximated those observed by Krul for chlorophyll [3], it appeared that regulation of the concentration of these two compounds might be under similar control mechanisms. One explanation might be that the aging chloroplasts were simply degrading these compounds during senescence and resynthesizing them during regreening. A second alternative was that there was breakdown of whole chloroplasts or cells in the cotyledons during senescence. To determine the concentration of chloroplasts per cotyledon during senescence and regreening, the number of chloroplasts in a chloroplast suspension was determined with a hemacytometer and the chloroplast yield calculated by relating the chlorophyll concentrations of this suspension and the whole cotyledon. Knowing the chloroplast yield and number of chloroplasts in each preparation, the number of chloroplasts per cotyledon could be calculated. Comparing the

Table 1. Quantitative analysis of total fatty acids in aging and regreening soybean cotyledons

Age (days)	Fr. wt (g/cotyledon) (0.01)*	Fatty acids (μmol/cotyledon) 16:0 (0.04)*	18:0 (0.02)*	18:1 (0.08)*	18:2 (0.25)*	18:3 (0.05)*
1	0.19	0.88	0.20	0.67	3.5	0.60
4	0.18	0.68	0.15	0.45	2.4	0.56
7	0.26	0 89	0.19	0.63	3.3	1.38
10	0.36	0.63	0.17	0.68	3.0	1.39
13	0.32	0.49	0.14	0.54	2.1	1.03
16	0.30	0.40	0.14	0.46	1.5	0.71
19	0.27	0.37	0.11	0.41	1.4	0.55
22	0.22	0.26	0.09	0.31	0.90	0.34
25	0.15	0.25	0.08	0.35	0.93	0.27
19†	0.31	0.43	0.14	0.46	1.8	0.90
22†	0.30	0.35	0.13	0.36	1.2	0.81
25†	0.29	0.32	0.12	0.33	1.1	0.72

* Standard error of the mean ($n = 4$). † Epicotyl removed on 16th day.

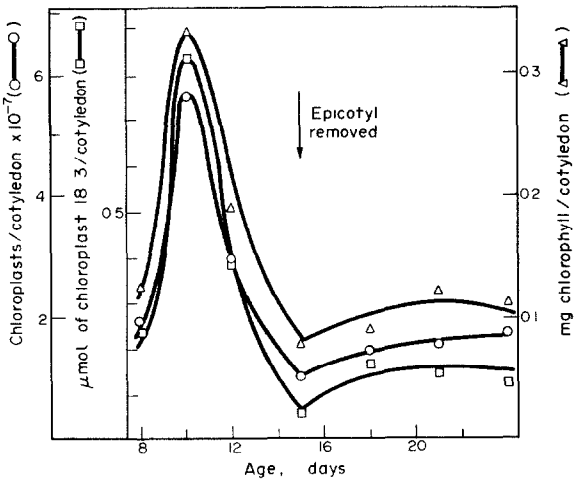

Fig. 1. Changes in number of chloroplasts and quantity of linolenate and chlorophyll per cotyledon in aging and regreening soybean cotyledons Epicotyls were removed on the 15th day to cause regreening

relative number of chloroplasts, chlorophyll concentration and linolenate concentration in cotyledons at various ages indicated that the large changes in the quantity in chlorophyll and linolenate are primarily related to the changing number of chloroplasts in the cotyledon (Fig. 1). The data shown were obtained using chloroplasts isolated by differential centrifugation. The substantial number of starch granules which appear in this preparation, particularly in younger chloroplasts, made counting chloroplasts difficult. Thus chloroplasts were also isolated by the method of Miflin and Beevers [9] using a discontinuous sucrose gradient. This yielded chloroplast preparations which were free of starch granules and the results agreed completely with the data illustrated in Fig. 1.

We confirmed that the changes in chloroplast number were not due to the isolation of a particular type of chloroplast associated with cotyledons at a specific stage of development by determining the ratio of chlorophyll *a* to chlorophyll *b* using the method of Arnon [10]. The chlorophyll *a/b* ratio decreases during senescence and if the chloroplast preparation from 15-day-old plants has a chlorophyll *a/b* ratio comparable to the whole cotyledon, it would indicate a representative sample of the chloroplasts from these cotyledons was being isolated. If, however, the chlorophyll *a/b* ratio in the chloroplasts was similar to that of the 10-day-old plants, it would indicate that the isolated chloroplasts were from an earlier stage of development and decreasing number of chloroplasts would simply reflect the inability to isolate a representative sample of chloroplasts from these cotyledons as compared to cotyledons at the stage of development of 10-day-old plants. The chlorophyll *a/b* ratios of cotyledons from 10-day-old and 15-day-old plants were 2.87 and 2.17 respectively. The chlorophyll *a/b* ratio of chloroplasts from 15-day-old plants was 2.29, indicating clearly that the data reflect the actual number of chloroplast present in cotyledons at the indicated ages and do not suggest that the isolation procedure resulted in the collection of a non-random sample of chloroplasts.

To determine how closely the linolenate and chlorophyll concentrations are correlated to the number of chloroplasts, the concentration of each per chloroplast was calculated and illustrated in Fig. 2. In general, the chlorophyll concentration in the chloroplast remains relatively unchanged over this period while the concentration of linolenate in chloroplasts was decreased on day 15 compared to its concentration earlier and during regreening. This decreased concentration of linolenate in chloroplast was observed in duplicate experiments and occurs at the time when lowest linolenate concentrations would be expected. It was surprising that a comparable decrease in chlorophyll concentration was not observed. These data would indicate that linolenate is indeed linked to major structural changes observed for chloroplasts during senescence and regreening. Since the linolenate is a major constituent of galactolipids [7], which are found principally in chloroplasts, the loss of linolenate is probably related to the decline in amount of diacyl glycerols of the chloroplast during senescence.

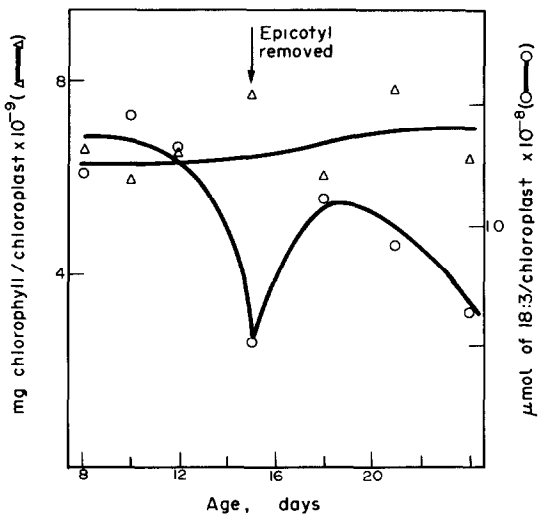

Fig. 2. Changes in quantity of linolenate and chlorophyll per chloroplast in aging and regreening soybean cotyledons. Epicotyls were removed on the 15th day to cause regreening.

The relatively constant concentration of chlorophyll per chloroplast in soybean cotyledons is in good agreement with the findings of Choe and Thimann who demonstrated that isolated chloroplasts from senescing oat leaves did not readily breakdown chlorophyll [2]. These and other experiments [1] have led to the hypothesis that senescence is controlled from outside the chloroplast. Data obtained here support that hypothesis and indicate that during the late stages of senescence of soybean cotyledons there is a major breakdown of chloroplasts which accounts for the disappearance of chlorophyll, as well as some of the fatty acids, proteins and nucleic acids.

EXPERIMENTAL

Soybeans (*Glycine max* L. cv Wayne) were grown under continuous light in vermiculite and supplied with a complete nutrient. Cotyledons were removed for lipid analysis at the designated ages. After 15 or 16 days of growth the cotyledons were faintly yellow and presumed to be in a state of senescence. At this time epicotyls were removed from some of the plants to induce the regreening process while other plants were left intact.

Isolation of fatty acids. Lipids were extracted by the method of ref. [11]. A 20 g sample of a known number of cotyledons was homogenized in 80 ml of $CHCl_3$–MeCH 1:1. The homogenate was filtered and the residue rinsed with additional solvent to give a final vol of 200 ml. Butylated hydroxytoluene was added to give a final concn of 0.005% (w/v) and a known amount of heptadecanoic acid was added as an internal standard. An aliquot of this soln was used to measure chlorophyll using the method of ref. [12] and the remainder was used for the lipid extraction. Isolated lipids were saponified and the free acids methylated with 14% BF_3–MeOH [13].

Chloroplasts were isolated by grinding a 30 *g* sample in 100 ml of isolation media [14] by 2, 3-sec pulses at low speed in an Oster blender. The homogenate was filtered through two layers of cheesecloth and two layers of Miracloth. The filtered homogenate was centrifuged at 1800 *g* for 1 min. The resuspended chloroplasts were counted using a hemacytometer. The chlorophyll per ml was determined as before and the fatty acids were isolated as described earlier.

Sucrose density gradient centrifugation. Resuspended chloroplasts were purified on a density gradient using the method of ref. [9]. The gradient consisted of 4 ml of 60% (w/w) sucrose, 6 ml of 60–42%, 5 ml of 42%, 10 ml of 42–30% and 3 ml of 30%. The samples were centrifuged for 5 min at 4100 *g* then for 10 min at 16300 *g*.

Fatty acids analysis. The fatty acid Me esters were analyzed by GLC using TC detector. All analyses were made isothermally at 185° and the Me esters were separated on a 2 m × 2 mm column of 17% Hi EFF 1BP on Gas Chrom P. Peak areas were

determined by triangulation and micromol of each fatty acid calculated by relating the peak areas to that of the internal standard. All calculations were corrected for the detector response to each fatty acid, which was determined using a standard Me ester soln.

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