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Nucleic Acid and Protein Metabolism of Senescing and Regenerating Soybean Cotyledons[1]

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ABSTRACT

An alternative to the leaf disk system for studies of the metabolism of senescence is described. The progress of senescence of soybean (*Glycine max* L.) cotyledons is arrested when the epicotyl is removed. Epicotyl removal at 16 or 17 days reversed the decline in nucleic acid, protein, and chlorophyll content in the cotyledon. Epicotyl removal at 18 days did not reverse the decline in the above components, and therefore the progress of cotyledon has passed the point of no return. Cotyledons lost 90% of their nucleic acid and 80% of their protein before senescence became irreversible. The rate of recovery in various macromolecular components after epicotyl removal did not occur in an equal manner. Nucleic acid was regenerated at a faster rate than chlorophyll, which was regenerated at a faster rate than soluble protein. The heavy nucleic acid components (ribosomal and heavy ribosomal messenger fractions) regenerated at greater rates than did the soluble RNA or DNA. No label from $^{14}CO_2$ was incorporated into DNA of the cotyledons when the epicotyl was present but label was incorporated into DNA after epicotyl removal.

The parallels between the mechanisms of cotyledon senescence and apical dominance are discussed.

The use of excised leaf disks to study the metabolism of senescence may be of value if the data obtained are relevant to the intact leaf. The metabolism of leaf disks is not the same as that of the intact leaf (14, 18, 21, 40). Intact leaf senescence is influenced by the other organs of the plant (20, 26, 27), whereas the leaf disk is free of this influence. Labeled metabolites added to the medium supporting excised leaf tissue (nucleotides, amino acids, orthophosphate, etc.) may replace endogenous metabolites which are diminishing or absent during leaf senescence. Enrichment of the medium with such metabolites may therefore change the direction of metabolism in excised disks. Products of macromolecular hydrolysis are rapidly exported from intact senescing leaves (33) but may accumulate in excised disks. Therefore, the pool size of a given metabolite might tend to decrease with time in intact leaves and increase with leaf disks. The contribution of wound metabolism (14, 18) and that of microorganisms (22) must be separated from the metabolism of senescence in isolated disks. Possibly the only advantage of using leaf disks for the study of the metabolism of senescence is convenience, but this may turn into a disadvantage if corrective measures for the above differences are not taken.

An alternative method for the study of senescence is described in this report. This method uses the intact leaf in which the metabolic rates can be estimated with simple precursors, and in which senescence can be deferred by endogenous substances. Senescence can be delayed and physiological vigor restored to leaves by removal of the apical portion of the plant before senescence has progressed too far (5, 8, 20, 27, 40). Removal of the epicotyl above a given pair of leaves at regular time intervals permits one to determine the time at which apex removal no longer defers senescence of the leaves in question. Before the time at which apex removal no longer defers senescence, the leaves contain a minimal amount of some substance(s) or structure(s) required for survival. This time shall be referred to as the point of no return. The period of recovery in which the progress of senescence is reversed can be referred to as regeneration. Metabolism to the point of no return and during the regeneration subsequent to apex removal can be studied by the incorporation of labeled CO_2.

MATERIALS AND METHODS

Soybean seeds (*Glycine max* L. var. Harosoy) were germinated in vermiculite in a growth chamber under 16-hr day length (29 C day, 25 C night) for 7 days. On the 7th day, seedlings were transplanted into 4-inch plastic pots containing garden soil and irrigated with 120 mg of 20-20-20 fertilizer per pot.

Cotyledons were harvested, frozen immediately, and stored at −20 C. Ribonuclease preparations were obtained by homogenizing the cotyledons in 0.3 M sucrose and filtering the homogenate through glass wool. The homogenates were then adjusted to a constant volume (2 or 2.5 ml/cotyledon) with 0.3 M sucrose and centrifuged at 1000*g* for 15 min at 5 C. Aliquots of the supernatant were assayed for RNase activity by the method described by Wilson (39). The pellet was not assayed for activity.

For isotope incorporation studies, intact soybean plants (17 days), or those from which epicotyls had been removed on the 16th or 17th day, were held for 24 hr in a Plexiglas chamber, in which 1.25 mc of $^{14}CO_2$ from barium carbonate were liberated with 50% lactic acid (16-hr day and 8-hr night, 30 C and 26 C, respectively). The initial concentration of CO_2 in the chamber was approximately 2.06%.

The soluble protein of cotyledons was extracted as described for RNase, but the centrifugation step was omitted. Aliquots (1 ml) of the extract were mixed with an equal volume of 10% (w/v) trichloroacetic acid and held overnight at 0 C. The

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mixture was centrifuged, the acid-soluble material was discarded, and the residue was dissolved in 0.1 N sodium hydroxide. Aliquots of the sodium hydroxide-soluble material were assayed by the procedures described by Lowry *et al.* (23).

Plastid protein was prepared and estimated by centrifugation of the crude sucrose homogenate at 5000*g* for 15 min at 5 C. The supernatant was decanted and assayed for protein by the above procedure (23). Chloroplast protein was expressed as the difference between the protein content of the crude preparation and that of the 5000*g* supernatant fraction.

The ^{14}C incorporated into each fraction was estimated by placing 0.4 ml of either the crude homogenate or the 5000*g* supernatant on a 10-cm disk of filter paper and by processing it by the procedures described by Mans and Novelli (25). Unprocessed disks containing an aliquot of the crude homogenate were used to estimate the total soluble ^{14}C of the cotyledon. The disks were placed in scintillation vials with POP (5.0 gm/l) and POPOP (0.3 gm/l) in toluene and were counted on a Packard Tri-Carb scintillation spectrometer.

Total nucleic acid was estimated by the methanol extraction procedure of Cherry (9) or by the phenol-Duponol method of Cherry *et al.* (10). Purified nucleic acid was chromatographed on MAK columns prepared according to the methods of Mandell and Hershey (24) with a linear gradient of NaCl (0.2 M–1.2 M) in 0.05 M NaH_2PO_4 (pH 6.7). Five-ml samples of the effluent were collected and the UV absorbance at 260 nm recorded for each. The amount of nucleic acid in each fraction was calculated by assuming that 1 mg of nucleic acid equaled 20 optical density units at 260 nm. Consecutive 1-ml portions from each tube were plated, dried, and counted on a gas-flow counter.

Dry weights were obtained by placing whole cotyledons in an oven at 80 C for 24 hr. Chl was extracted by boiling the cotyledons in methanol until no residual green color was observed. The methanol extracts were adjusted to volume, filtered through Whatman paper, and the absorbance determined at 600 nm. Chl content was estimated according to the procedures of Smith and Benites (34), using an absorption coefficient of 9.95 for both Chl *a* and *b*.

RESULTS

The point of no return for senescence of soybean cotyledons was determined by removing the epicotyl (a few mm above the cotyledons) on successive days after germination. In the plants which had the epicotyl removed on the 10th through the 16th day, 90 to 100% of the cotyledons remained green and nonsenescent. When the epicotyl was removed on the 17th day, 40 to 60% of the cotyledons remained green and, when it was removed on the 18th day, only 0 to 10% of the cotyledons remained green. Those cotyledons which did not abscise and begin to regreen following removal of the epicotyl were considered as nonsenescent. Usually, the cotyledonary leaves had become faintly yellow by the 17th day.

The changes in various cellular components relative to their content at the point of no return are shown in Figure 1. The slope of the decay curves (5–16 days) indicates that protein, nucleic acid, and Chl declined at (relatively) similar rates. The decline in RNase activity was slower than that of total soluble protein. The recovery rates for various macromolecular components were much slower than decay rates, and rates of recovery varied. Nucleic acid was regenerated most rapidly, then Chl, and finally protein. The dry weight of the cotyledon increased by 50% 15 days after epicotyl removal (12–20 mg). If the epicotyl were not removed, the contents of each component continued to decline during the 17th and 18th day.

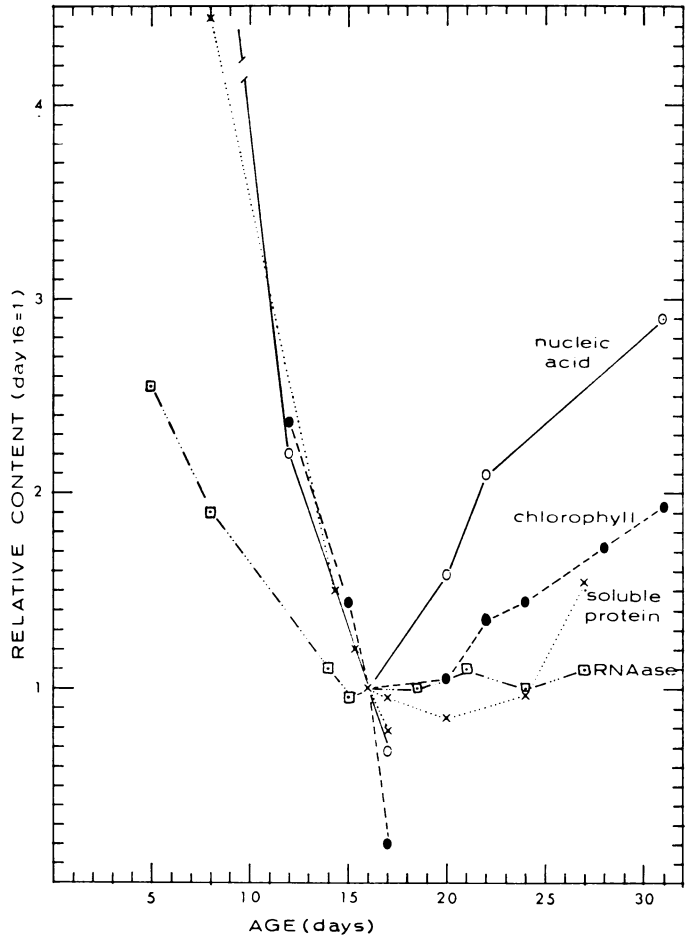

FIG. 1. Changes in nucleic acid, soluble protein, Chl and RNase in senescing and regenerating soybean cotyledons.

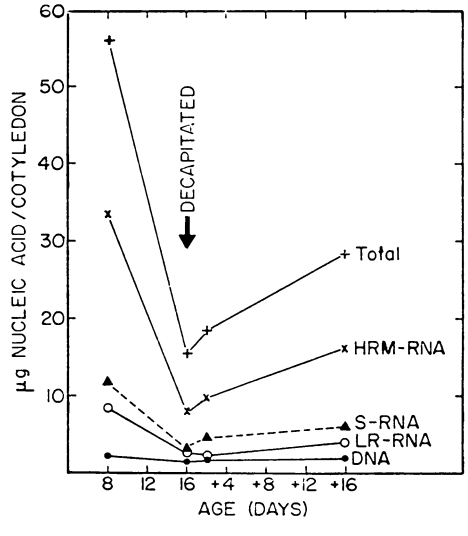

FIG. 2. Quantity of each nucleic acid fraction in senescing and regenerating soybean cotyledons. Each datum point was derived from a population of 10 cotyledons. HRM-RNA: heavy ribosomal-messenger RNA; LR-RNA: light ribosomal RNA.

The most rapidly increasing macromolecular component during regeneration was nucleic acid. Within the nucleic acid frac-

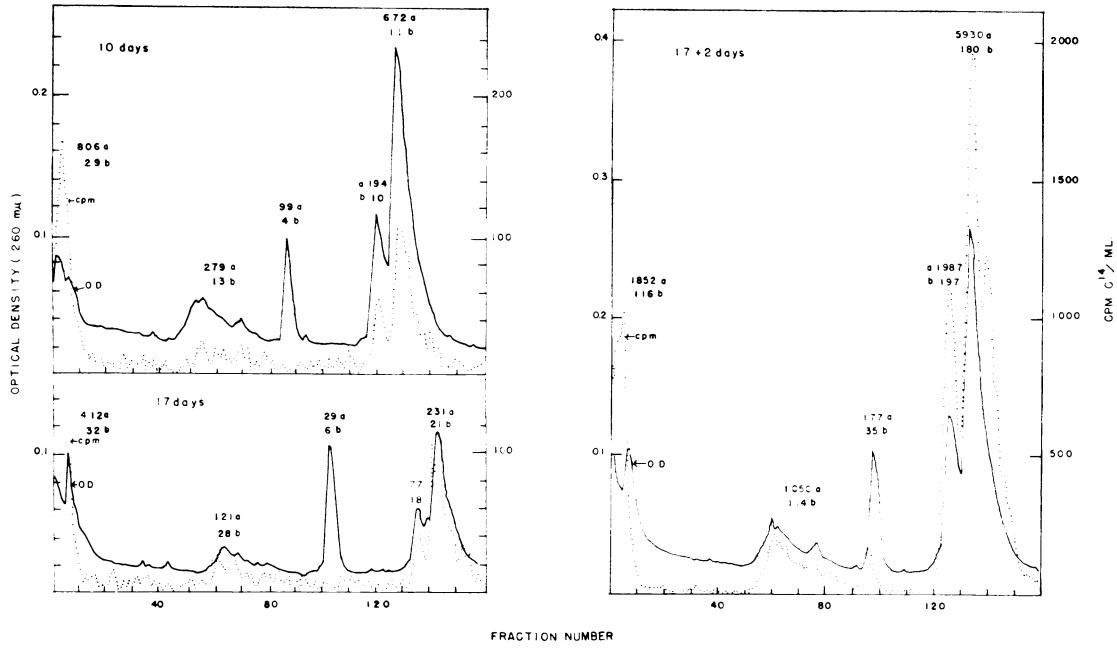

FIG. 3. Elution profiles of nucleic acid extracted from cotyledonary leaves at 10 days, 17 days, and 2 days after epicotyl removal. Cotyledons were fed 1.25 mc $^{14}CO_2$ 24 hr before extraction. Numbers followed by letter "a" represent total ^{14}C/fraction·cotyledon; numbers followed by letter "b" represent cpm/μg of nucleic acid. Peaks (from left to right) represent acid-soluble fraction, 4S and 5S (S-RNA), DNA, light ribosomal RNA (LR-RNA), and heavy ribosomal-messenger RNA (HRM-RNA). Each elution profile represents data derived from a population of 16 cotyledons.

Table I. *^{14}C Incorporation into Various Protein Fractions of Senescing and Regenerating Soybean Cotyledons*

Age	Total Soluble ^{14}C per Cotyledon	Total ^{14}C Protein[1]	Specific Activity	Plastid-rich Pellet	Specific Activity	Soluble Protein	Specific Activity
days	*cpm*	*cpm*	*cpm/μg soluble protein*	*cpm*	*cpm/μg soluble protein*	*cpm*	*cpm/μg soluble protein*
10	285,379	55,842	18	23,271	20	32,571	18
17	71,882	20,818	38	8,906	39	11,912	38
19[2]	1,341,431	382,368	239	115,972	179	266,329	280

[1] Total ^{14}C protein = plastid-rich protein + soluble protein.

[2] Epicotyl was removed on 17th day. The cotyledons were fed 1.25 mc of $^{14}CO_2$ for 24 hr before harvest. Each figure represents mean radioactivity derived from two replications of seven cotyledons each.

tion the most rapidly increasing component (during both senescent and regenerating phases) was the heavy ribosomal-messenger RNA fraction (Fig. 2). The tRNA and light rRNA fractions decayed and regenerated at similar rates. There was a barely perceptible change in the DNA component during senescence and regeneration.

The incorporation of label from photosynthetically fixed $^{14}CO_2$ into nucleic acid components during senescence, at the point of no return, and during regeneration, is shown in Figure 3. The absolute amount of label ("a" numbers) incorporated into nucleic acid declined between the 10th and 17th days but specific radioactivity ("b" numbers) increased during the same period. No incorporation of radioactivity into the DNA fraction was observed during this period. Two days after epicotyl removal the absolute and specific radioactivity in each nucleic acid component was greater than at the two earlier times and there was also an obvious incorporation of ^{14}C into the DNA component. The heavy fractions of nucleic acid displayed the greatest increases in absolute and specific radioactivity relative to the lighter fractions.

The total amount of label in cotyledons decreased with age but dramatically increased after epicotyl removal (Table I). After epicotyl removal, the amount of ^{14}C in the total soluble protein increased 15-fold, chloroplast protein 13-fold, and soluble protein 22-fold. This finding is consistent with the data of Callow and Woolhouse (5), who showed that cytoplasmic RNA recovered more rapidly than plastid RNA after epicotyl removal. The specific radioactivity of protein, as in the case of nucleic acid, increased between the 10th and 17th days, and even more dramatically after epicotyl removal.

DISCUSSION

Senescence may be considered to be the ultimate stage in the differentiation of an organ. The cotyledons on an intact plant begin this last phase of differentiation shortly after germination. When the epicotyl is removed before the point of no return, senescence is arrested. It is significant that the control of cotyledon senescence is a function of the whole plant and does not reside only in the organ. In some manner, epicotyl removal reverses the trend of depolymerization outpacing polymerization of such important macromolecules as nucleic acid, and hence, protein and Chl in cotyledons. The response to epicotyl removal is fairly rapid, occurring within 24 hr (as early as 12 hr according to Callow and Woolhouse [5]), and the time at which epicotyl removal no longer defers senescence is also somewhat limited (24 to 48 hr). This latter time limit, or the point of no return, suggests that the cotyledon has lost some substance(s) or structure(s) which cannot be replaced when the epicotyl is removed. The cotyledon can lose 90% of its nucleic acid and 80% of its protein before the process of senescence becomes irreversible. It would be of interest to obtain profiles such as those of Carr and Pate (7) comparing profiles just before and after the point of no return to determine which protein

species have been deleted and which new ones appear upon regeneration.

Senescence could result from a reduction in the genetic information potential (30). The loss of information potential may result from the physical loss of DNA or from the irreversible binding of histones to DNA (36). A slight amount of DNA was lost during senescence and no label was incorporated into this component until after the epicotyl was removed. It is difficult, therefore, to assess with this data whether either of the above mechanisms functions in cotyledon senescence.

The mechanism for correlational suppression of nucleic acid and protein synthesis in the cotyledon by the epicotyl may be similar to the suppression of axillary buds by the epicotyl. The presence of the intact epicotyl or a decapitated epicotyl treated with an auxin paste suppressed the synthesis of DNA in the apical cells of axillary buds. When the intact epicotyl or the auxin paste on the decapitated epicotyl was removed, DNA synthesis in the buds was initiated within 3 days (28). In the cotyledons, there was no incorporation into the DNA component while the epicotyl was in place, but after it was removed the DNA component became labeled. It is not clear whether such regulation is influenced by a local cytokinin-auxin balance (16, 37, 38), by hormone directed movement of metabolites from cotyledons to the apical regions of the plant (3, 31, 32), or both.

There are several differences between metabolism of senescing leaf disks and intact leaves as described by others (21), as well as those shown here. First, it is well known that significant amounts of label are incorporated into the DNA component of excised leaves (6, 10, 17, 29), whereas no significant label is incorporated into the DNA component of intact cotyledons. Labeling of DNA components of detached leaves may reflect wound healing reactions (14, 18) or bacterial contamination (22). Another contrast is that specific radioactivity of label incorporation declines with age in detached disks (29) but increases in intact cotyledons as shown here. An additional contrast between the intact and detached systems is that in the former, RNase activity declined with age (2, 8, 17), whereas in the latter, it tended to increase with age (21). It is not clear whether this discrepancy is a result of actual differences in enzyme activity or the means by which enzyme activity is expressed. If specific activity of the enzyme is expressed on a protein basis and relative protein content declines at a faster rate than the enzyme in question, specific activity will increase as decay of protein increases. Chin *et al.* (12) have reported striking differences in synthetic activities between intact and detached cotyledons. Finally, it is known that cytokinins defer the senescence of detached leaves (4, 15, 19, 29, 35) but seem to have little effect on intact leaves (unpublished data). It is possible that cytokinins have a role in senescence of intact cotyledons, since they may be synthesized in roots (15) and are translocated to other parts of the plant (19). However, the metabolism of senescence deferral of leaf disks by cytokinins is not the same, qualitatively, as deferral by epicotyl removal. In the excised leaves, all nucleic acid fractions are enhanced to the same degree (4, 6, 13, 35) in the presence of cytokinins. Senescence deferral by apex removal is also associated with an overall enhancement of nucleic acid synthesis, but the heavy fractions show much more activity than do the lighter ones. Therefore, factors other than or in addition to cytokinins may be associated with enhanced nucleic acid synthesis by epicotyl removal, or senescence deferral in intact organs may be qualitatively different from that of excised organs (21).

The sequence of regeneration of various macromolecular components of cotyledons is similar to that of regreening, glucose-bleached *Chlorella* cells (1). In both instances cytoplasmic protein appeared before plastid protein. In this respect, it is of interest that synthesis of cytoplasmic RNA precedes that of chloroplast RNA after epicotyl removal (5).

The dramatic increase in label fixed in cotyledons 2 days after epicotyl removal compared to those at the time of epicotyl removal raises some interesting questions. Does the presence of the epicotyl influence the amount of ^{14}C fixed by the cotyledon or does mobilization of photosynthate from cotyledons of intact plants lower the amount of ^{14}C in the organ? Does ^{14}C fixed in roots find its way to the cotyledons of plants if epicotyls are removed but not in intact plants? Finally, how might the presence of the epicotyl influence the relative activity of nucleic acid polymerases and depolymerases in the cotyledon?

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