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Studies on Genetic Male-Sterile Soybeans[1]

III. THE INITIATION OF MONOCARPIC SENESCENCE

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ABSTRACT

Soybean (*Glycine max* [L.] Merr.) germplasm, isogenic except for loci controlling male-sterility (ms_1) and nodulation (rj_1) was utilized to investigate the effects of reproductive tissue development and nitrogen source on the initiation of monocarpic senescence. The experimental genotypes (Ms_1Rj_1, Ms_1rj_1, ms_1Rj_1, and ms_1rj_1, were selected from a cross between N69-2774 and N59-5259, and were inbred to the F_5 generation. Greenhouse-grown plants were collected during the period of flowering (77 days after transplanting) until maturity (147 days after transplanting). Leaf tissues from the respective genotypes were analyzed at the various harvest dates for RNA, phenolic, and chlorophyll concentrations; acid protease activity; polypeptide banding patterns of chloroplast thylakoids; and chloroplastic ultrastructure.

Regardless of nitrogen source, total chlorophyll concentrations declined between 77 and 119 days after transplanting, resulting in a 40% loss of chlorophyll per square centimeter in all genotypes. Leaf chlorophyll levels continued to decline at a constant rate in male-fertile genotypes, but remained at a constant level (26 micrograms chlorophyll per square centimeter) in male-sterile genotypes, for the remainder of the study. With increased leaf age, a gradual disruption of thylakoid structures was observed, particularly in chloroplasts from the male-fertile genotypes. Chloroplasts from the male-sterile genotypes appeared to lose starch grains but increased their number of chloroplastic lipid bodies with leaf aging. These data suggest that monocarpic senescence in soybeans was initiated at or before flowering. Although reproductive tissue development probably augmented the process, the response attributed to seed formation was not apparent until the mid-pod fill stage (119 days after transplanting). All genotypes had similar changes in other cellular components that are recognized as indicators of plant senescence regardless of whether the plants produced seed.

Senescence is a biological process that ultimately kills the organism. Although a genetic basis for senescence in higher plants has not been established, certain biochemical changes attributed to that process, such as Chl degradation, appear to be under genetic control (24). To date, no direct relation is known between any genetic mechanism and the formation of biochemical agents responsible for initiating senescence in higher plants. Hence, the nature of the supposed senescence 'signals' remains unidentified (18) and there may be uncertainty about the stage of plant development which senescence begins (11, 12).

Soybeans have a monocarpic senescence pattern whereby the plant is limited to a single reproductive cycle (18). Monocarpic senescence may be monitored by observing declines in the concentration of cellular constituents such as Chl, protein, nucleic acids, and carbohydrates; or by the increase in the activities of various proteases, phosphatases, and nucleases (11, 13, 14, 19, 23). Although the mechanism regulating the biochemical aspects manifested during monocarpic plant senescence is unknown, the senescence process apparently can be delayed by removing reproductive tissues (13, 16, 17). Such experiments however, are tedious because of the plants' ability to initiate new reproductive tissues (6, 16). Furthermore, physical injury to the plant accrued through repeated manual attempts to prevent reproductive tissue development may cause metabolic aberrations that impair analytical studies of the senescence process. Therefore, it has been difficult to ascertain whether senescence is initiated at the time of flowering or as a result of fruit production.

Problems in such studies with soybeans have been alleviated by the use of genetic male-sterile germplasm (3, 25). When pollen vectors are excluded, male-sterile soybeans may flower but will not produce seed. Hence, the desired effect is achieved with minimum labor. We used soybean germplasm, isogenic except for loci controlling male-sterility (ms_1) and nodulation (rj_1), to investigate the effects of reproductive tissue development and nitrogen source upon the initiation of monocarpic senescence. Our findings present evidence that senescence in soybeans, irrespective of the mechanism for nitrogen nutrition, is not dependent upon seed production.

MATERIALS AND METHODS

Plant Material. Soybeans (*Glycine max* [L.] Merr. cv 'N69-2774' and cv 'N59-5259') were mated in 1978. The N69-2774 parent was homozygous recessive at the ms_1 locus governing male-sterility and homozygous dominant at the Rj_1 locus for nodulation. The N59-5259 parent was homozygous dominant for the Ms_1 locus and homozygous recessive for the rj_1 locus. From that mating, a fertile-nodulating (Ms_1 ms_1 Rj_1 Rj_1) line N80-96-1 and a fertile nonnodulating-type (Ms_1 ms_1 rj_1 rj_1) line N80-96-2 were selected and grown in this study. Detailed description of the genetic procedures and experimental conditions for plant

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culture have been reported previously (8).

Analytical Procedures. Leaf area was measured with a model LI-3000[3] area meter (Li-Cor, Inc.). Total Chl concentrations and Chl *a/b* ratios were determined by the procedure of Arnon (1). Total RNA was extracted from leaf homogenates in 0.1 M Tricine-NaOH (pH 7.8) with centrifugation at 25,000*g* for 10 min. RNA levels were measured with the procedure of Fish and Jagendorf (7). Total phenolic content was determined with the procedure of Pirie and Mullins (20). Acid protease (endopeptidase) activity was measured by the methods of Chrispeels and Boulter (5), with observation of the rate of azocoll hydrolysis at A_{525} from 1 to 3 h (pH 4.7, 37°C).

Electron Microscopy. Leaf segments were excised from male-sterile and male-fertile plants at 77, 92, 105, 119, 133, 140, and 147 DAT.[4] To insure uniformity, samples were collected from leaves of similar size and at a specific nodal position on the respective plants. Segments were cut into pieces (1 mm^2), suspended in 3% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4), and vacuum infiltrated to enhance penetration of the fixative. After a 1-h incubation period, the fixative was removed with five washes of 0.1 M Na-cacodylate buffer (pH 7.4). Tissue pieces were postfixed by a 1-h treatment with 2% (w/v) osmium tetroxide in 50 mM Na-cacodylate (pH 7.4). After two washes in deionized H_2O, the tissue was dehydrated in a series of graded acetone solutions and embedded in a mixture of low viscosity resins (21).

Chloroplast Thylakoid Isolation. Leaves collected from the respective male-sterile and male-fertile plants were homogenized in a grinding medium containing 0.4 M sorbitol and 0.1 M Tricine-NaOH (pH 7.8). The brei was filtered consecutively through 4 and then 12 layers of cheesecloth prior to centrifugation for 10 min at 1,000*g*. The resulting pellet was resuspended in a solution of 5 mM $MgCl_2$ and 10 mM Tricine-NaOH (pH 7.8), centrifuged for 5 min at 10,000*g*, and resuspended in the same medium.

SDS-PAGE. Chloroplast thylakoid proteins were separated by SDS-PAGE using the discontinuous buffer system of Laemmli (10). Electrophoresis was performed in a slab gel apparatus (22) with continuous 12% to 16% (w/v) polyacrylamide separating gel and a 5% (w/v) stacking gel. Thylakoid polypeptides were solubilized in a 65 mM Tris-Cl sample buffer (pH 6.8) that contained 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, and 2% (w/v) SDS. Samples with equal Chl concentrations were boiled for 2 min and applied to the gel sample wells. Electrophoresis was carried out at a constant current of 35 mamps. Gels were stained for 30 min for protein in a solution that contained 0.2% (w/v) Coomassie blue, 50% (v/v) methanol, and 7% (v/v) glacial acetic acid and were destained in a solution of 20% (v/v) methanol and 7% (v/v) glacial acetic acid. All data are presented as mean values for five replicate samples from each genotype at each date.

RESULTS AND DISCUSSION

Male-sterile soybeans, when grown under controlled greenhouse conditions, produced no seed and retained green leaves for an indefinite period beyond the life span of male-fertile siblings. The male-sterile plants exhibited an extended leaf longevity with either symbiotic nitrogen fixation or nitrate as the sole nitrogen source. Changes in the biological activity of male-sterile and male-fertile soybean leaves were determined at various intervals after the plants had flowered. Photosynthetic activities and starch accumulation patterns in the leaves of both genotypes have been reported previously (8). In that regard, net carbon exchange rates declined in both plant types with increased age, albeit higher levels of starch accumulated in the male-sterile tissues. Similar changes in photosynthetic activity have been observed with 'desinked' soybeans (17, 27). It also was noted (17) that total Chl levels (mg dm^{-2}) declined in control plants but remained constant with leaf maturation of desinked plants. Although the response of ribulose-1,5-bisphosphate carboxylase activity to pod removal differed between the reports (17, 27), leaf protein and starch levels remained high or increased in the treated plants. In addition, protease activities were not different among treatments before changes in net photosynthetic activities were found. No positive linear correlation, however, was shown between protease activity and the rate of decline in net photosynthetic rates in the respective treatments (27). Hence, a causal relation for the decline in photosynthetic rates could not be ascribed exclusively to senescence or a decline in leaf function.

The apparent prevention of plant death by avoidance of reproductive tissue development, however, stimulated our interest in determining whether the symptoms of monocarpic senescence could be detected in soybeans that did not produce seed. Thus, we conducted a corollary study with male-sterile and male-fertile soybeans differing in nodulation ability (8) in order to provide basic information on changes in cellular constituents that initiate the incidence of monocarpic senescence. However, it should be noted that the mode of nitrogen nutrition, symbiotic or nitrate, had no effect upon the individual responses observed in male-sterile or male-fertile genotypes.

Cellular RNA levels decreased by an average amount of 38.8% between 77 and 105 DAT in male-sterile and male-fertile genotypes (Fig. 1). Between 105 and 147 DAT, however, the cellular RNA levels tended to level off and averaged 33.5 ± 0.9 μg cm^{-2} in all genotypes. Generally, there were no statistically significant differences in RNA levels among genotypes at a given date. At 119 DAT, cellular phenolic levels increased linearly (by an average 68.5% above levels at 77 DAT) (Fig. 2). The phenolic concentration reached a plateau at an average level of 478.8 ± 10.6 nmol cm^{-2} in all genotypes between 133 and 147 DAT. Acid protease (endopeptidase) activity was not different statistically between male-sterile and male-fertile plants (77–105 DAT), but was significantly greater statistically in the male-sterile lines after 105 DAT (Fig. 3). Hence, these data indicated that mono-

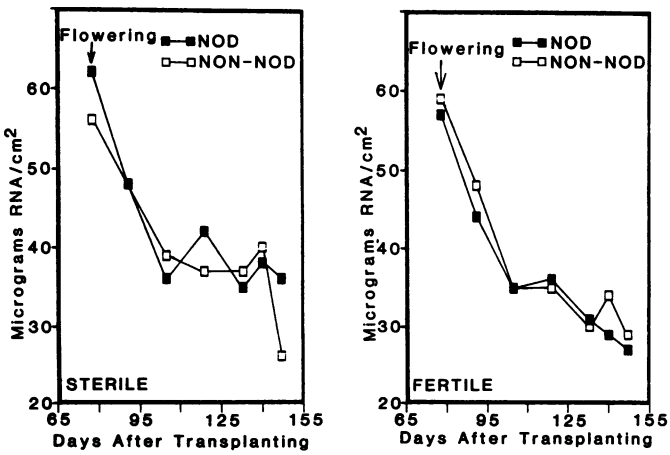

FIG. 1. Changes in cellular RNA levels during foliar senescence of nodulated (NOD) and nonnodulated (NON-NOD) genetic male-sterile and male-fertile soybeans. Leaf homogenates were clarified by centrifugation at 25,000*g* for 10 min, and RNA levels were determined by the procedure of Fish and Jagendorf (7).

[3] Mention of a trademark or a proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture or by the North Carolina Agricultural Research Service, and does not imply its approval to the exclusion of other products that may also be suitable.

[4] Abbreviation: DAT, days after transplanting.

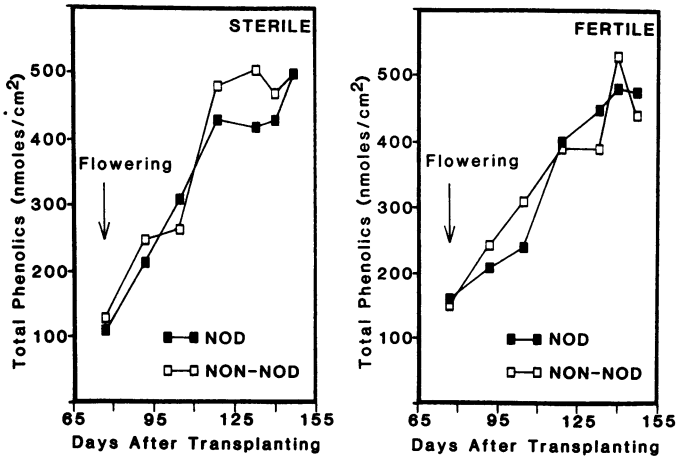

FIG. 2. Changes in the cellular phenolic content during foliar senescence of nodulated (NOD) and nonnodulated (NON-NOD) genetic male-sterile and male-fertile soybeans. Leaf samples were homogenized in aqueous methanolic HCl (50% methanol, 0.05% concentrated HCl), and the phenolic content was determined spectrophotometrically at 280 nm (20).

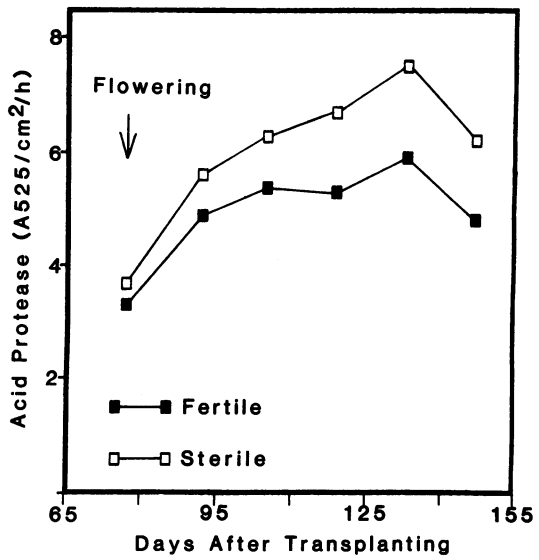

FIG. 3. Changes in the activity of acid proteases during foliar senescence of genetic male-sterile and male-fertile soybeans. Acid protease activity was determined according to the procedure of Chrispeels and Boulter (5).

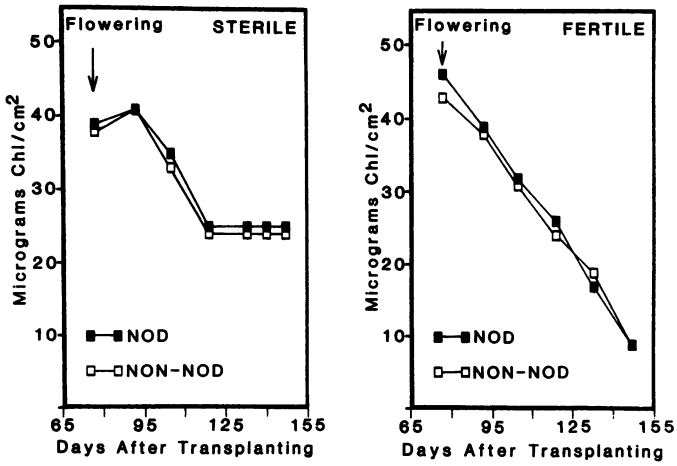

FIG. 4. Changes in the total Chl content during foliar senescence of genetic male-sterile and male-fertile soybeans. Leaf samples were homogenized in 80% acetone, and total Chl was determined spectrophotometrically according to the procedure of Arnon (1). NOD, nodulated; NON-NOD, non-nodulated.

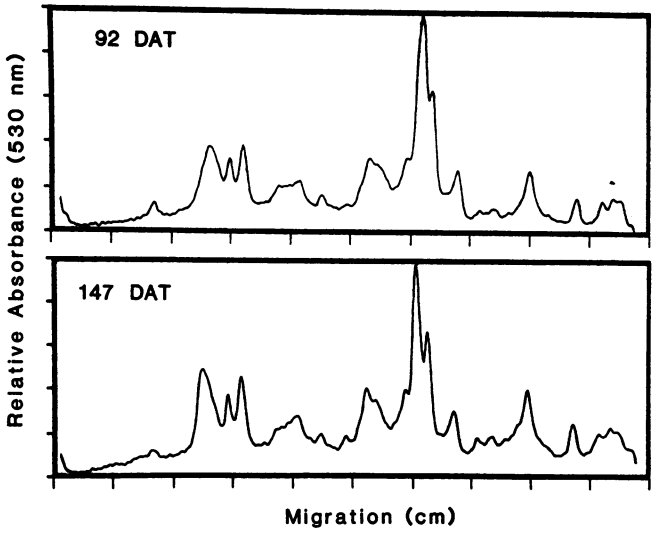

FIG. 5. A gel densitometric tracing of the polypeptide banding patterns for chloroplast thylakoids isolated from 92 DAT and 147 DAT male-fertile soybean leaves following SDS electrophoresis on a 12% to 16% polyacrylamide gel. Samples were loaded with equal Chl levels.

carpic senescence symptoms were apparent in soybean leaves at least from the time of flowering to maturity, and the associated changes in cellular constituents were not affected by reproductive tissue development until after 105 DAT (or 28 d after flowering).

Supporting evidence was evinced from the measurement of total Chl levels in the respective genotypes (Fig. 4). Total Chl levels in both male-sterile and male-fertile genotypes at 119 DAT were 40% lower than the levels of 77 DAT. Thereafter, male-fertile leaves continued to lose Chl at a constant linear rate, resulting in an 80% loss by 147 DAT. The male-sterile plants, however, maintained about 26 μg Chl cm^{-2} (119–147 DAT). Hence, the supposed biological process that 'signals' the onset of senescence occurred at least by the time of flowering. Apparently, the resulting effects were augmented in male-fertile plants by demands of seed development. In the absence of those demands in leaves of male-sterile plants, the end products of photosynthesis were apparently stored as starch, protein, and lipid, within a limited capacity, as suggested by Wittenbach (26).

Further analysis of chloroplast thylakoid polypeptides by SDS-PAGE showed similar banding patterns in the preparations from male-fertile leaves at 92 and 147 DAT (Fig. 5). Although not shown, all plants examined at all harvest dates had identical polypeptide patterns within the limitations of the technique used despite any accompanying difference in Chl levels. Because of these data, it was first assumed that only thylakoids from intact nonsenescent chloroplasts were pelleted in the isolation procedure. To test this assumption, the Chl *a*/*b* ratio was determined in whole leaf and purified thylakoid samples at the same tissue age. Because the Chl *a*/*b* ratio should decline in senescent leaves in proportion with the amount of chloroplast disruption (9), a representative sample of thylakoids should have the same Chl *a*/*b* ratio as the whole leaf. In that regard, the Chl *a*/*b* ratio for leaf tissues and corresponding thylakoids was not different at the respective dates. Thus, the polypeptide patterns were representative of tissue composition, and those proteins remained stable with leaf maturation. These data supported previous findings of

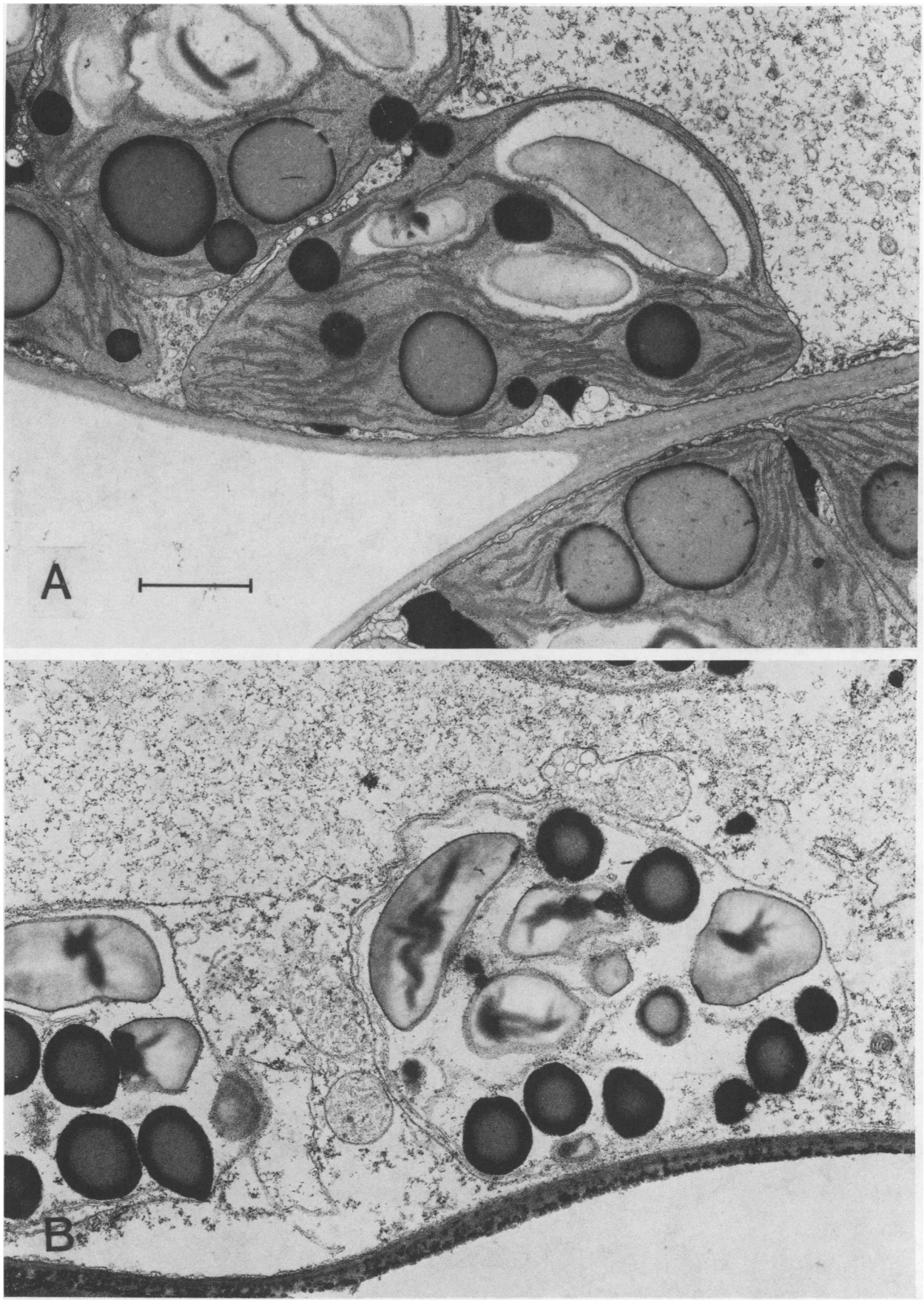

FIG. 6. Electron micrographs of chloroplasts from palisade cells of male-fertile soybeans at (A) 119 DAT and (B) 147 DAT. The magnification line represents 1.0 μm for both A and B.

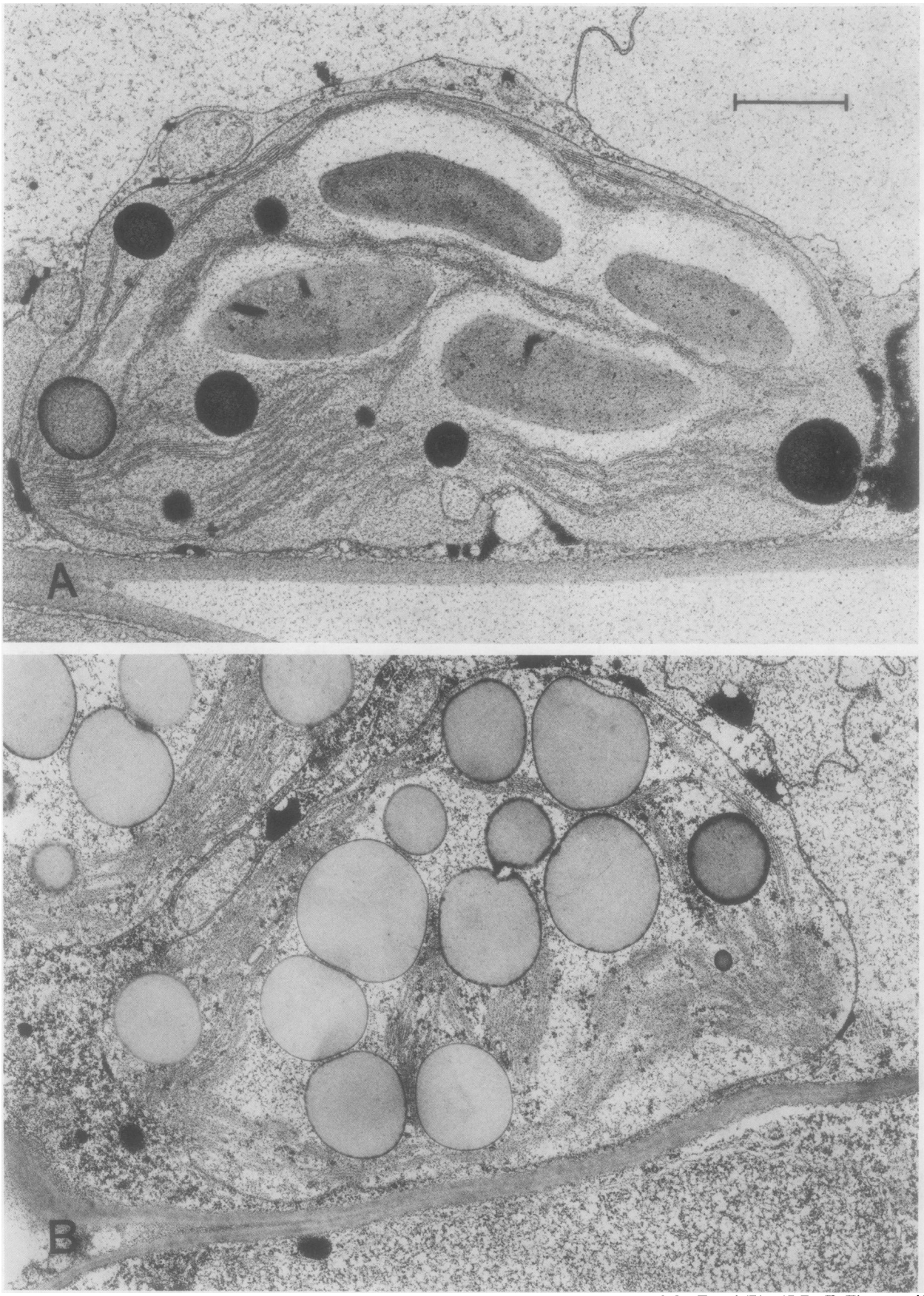

FIG. 7. Electron micrographs of chloroplasts from palisade cells of male-sterile soybeans at (A) 119 DAT and (B) 147 DAT. The magnification line represents 1.0 μm for both A and B.

senescent wheat leaves (4).

Additionally, the amount of Chl per chloroplast remained unchanged with leaf maturation. Therefore, we concluded that whole chloroplasts or possibly whole cells were lost sequentially in senescent leaves. A morphological study was conducted to visualize ultrastructural changes in leaf cells from male-sterile and male-fertile plants at 77, 119, and 147 DAT. At 77 DAT (data not shown) and 119 DAT, chloroplasts in male-fertile leaves (Fig. 6A) and male-sterile leaves (Fig. 7A) had well-defined thylakoids, several starch grains, and numerous osmiophilic bodies. In general, the osmiophilic bodies were smaller in size in the chloroplasts from male-sterile than from male-fertile leaves. Yellow leaves from male-fertile plants (147 DAT) had chloroplasts without a thylakoid network, decreased stromal density, numerous starch grains, and enlarged osmiophilic bodies (Fig. 6B). In contrast, chloroplasts from male-steriles leaves at 147 DAT had no starch grains, a dense stroma, a complete but somewhat distorted thylakoid network, and many large osmiophilic bodies (Fig. 7B).

The appearance of large osmiophilic bodies in chloroplasts from older leaves has been reported (26). The osmiophilic bodies have been suggested to contain various quinones (2). Recent evidence, however, has shown that the enzyme that catalyzes the synthesis of triacylglycerol, diacylglycerol acyltransferase (EC 2.3.1.20), was associated with chloroplast membrane fractions (15). Hence, it was suggested that storage lipids could be also contained within the osmiophilic bodies. This suggestion merits consideration, especially in the case of male-sterile plants where the leaf function has apparently shifted to photosynthate storage. In that regard, the triacylglycerol content of male-sterile leaves at 147 DAT was 21 times greater on a leaf area basis than the male-fertile counterpart.

In summary, we have demonstrated that the leaves of male-sterile soybean plants undergo numerous cellular changes generally attributed to senescence. Similar changes were found in the leaves of male-fertile siblings regardless of nitrogen source. Differences in total Chl content after 119 DAT (about midpod fill stage on the male-fertile siblings) was caused by the loss of whole chloroplasts. At that time, photosynthetic activity in male-sterile plant leaves fell to maintenance levels. Because of the demand for assimilates by developing seed, the end products of photosynthesis were sequestered and stored as starch and probably triacylglycerol. Hence, these data elicited our conclusion that the primary biological signal that initiated the senescence process in soybeans was received at or near the time of flowering. Any additional signal or response emanating from or as a result of seed development, therefore, must be considered as a secondary effect upon plant senescence.

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