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Differential Senescence of Maize Hybrids following Ear Removal[1]

II. SELECTED LEAF

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ABSTRACT

In conjunction with a study of the effects of ear removal on the senescence of whole maize (*Zea mays* L.) plants, visual symptoms and associated changes in constituent contents and activities of a selected leaf (first leaf above the ear) were determined. Leaves were sampled from field-grown eared and earless Pioneer brand 3382, B73 × Mo17, and Farm Services brand 854 maize hybrids at nine times during the grain-filling period.

Visual symptoms indicated the following sequence and rate of senescence: earless B73 × Mo17 > earless P3382 ≫ eared B73 × Mo17 ≫ eared P3382 ≥ earless FS854 > eared FS854. All earless hybrids showed increases in leaf dry weight and sugar content; however, the increases were transitory for P3382 and B73 × Mo17, but continuous throughout the grain-filling period for FS854, indicative of continued photosynthetic activity of the latter. All earless hybrids exhibited similar and transitory starch accumulation patterns. Thus, FS854 was an exception to the concept that carbohydrate accumulation accelerates leaf senescence. Ear removal resulted in accelerated losses of reduced N, phosphoenolpyruvate and ribulose bisphosphate carboxylases, phosphorus, chlorophyll, nitrate reductase activity, and moisture for P3382 and B73 × Mo17 plants. In contrast, the loss of all components (except phosphorus) was similar for the selected leaf of earless and eared FS854.

Although the loss of nitrate reductase activity, reduced N, and carboxylating enzymes accurately reflected the development of senescence of the selected leaf, the rate of net loss of reduced N and carboxylating enzymes appeared to be regulated. We deduced that the rate of flux of N into the leaf was a factor in regulating the differing rates of senescence observed for the six treatments; however, we cannot rule out the possibility of concurrent influence of growth regulators or other metabolites.

Pertinent background material has been reviewed in a companion paper (5). The complex nature of leaf senescence is clearly illustrated by the review of Thomas and Stoddart (14). Their views that leaf senescence is genetically controlled and programmed and that the causal factor(s) of senescence has not been conclusively identified remain valid. The cessation of numerous metabolic activities that are associated with the chloroplast and with the attainment of full leaf expansion, appears to predispose the leaf to senescence; however, the subsequent rate of senescence development can be affected by environment, growth regulators, ammonium nitrate (at least in *Nicotiana*), and genotype (14). With maize, another metabolic change associated with full leaf expansion was that the entry of nitrate into the leaf was greatly diminished or terminated (9). Although the changes in constituents and metabolic activities (loss of Chl, protein, enzyme activities, and accumulation of carbohydrates) have been intensively studied and associated with senescence, they are judged as symptoms rather than the cause of senescence.

The objectives of the current study concerning senescence of a selected leaf of maize during the grain-filling period were to (a) measure changes in selected metabolic parameters previously associated with senescence, (b) compare and contrast these patterns of change for eared and earless hybrids that exhibit different patterns of visual senescence, and (c) identify the trait that was most closely associated with effective leaf area duration of the three hybrids.

MATERIALS AND METHODS

Cultural Procedures. Cultural procedures, including ear removal and experimental design, were as described (5). Of the three maize hybrids used, Pioneer brand 3382 and Farm Services brand 854 are classed as 'stay-green' cultivars because their leaves remain green, while leaves of B73 × Mo17 are brownish yellow by the time of grain maturity.

Sampling. Plants were sampled between 0900 and 1100 h, nine times during the grain fill period; 4, 11, 19, 25, 30, 37, 44, 54, and 60 DAA.[2] Anthesis occurred at approximately July 12. At each sampling time, the selected leaf (leaf above the ear) from each of three representative plants per plot were combined for a sample to provide a total of 30 samples (five replications, six treatments). Leaves were placed on ice for transport to the laboratory. For each sample, the three leaves were stacked and folded once. A cork borer was used to cut discs (a total of 42 discs, 0.785 cm^2 $disc^{-1}$) from the midportion of the laminae. The disks were transferred to a preweighed vial, reweighed, frozen with liquid N_2, and stored at −20°C until extracted for gel electrophoresis. The midribs of the three leaves were then removed and discarded, and the laminae were chopped into 1 × 2 cm sections and thoroughly mixed, and subsamples were taken

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[2] Abbreviations: DAA, days after anthesis; NRA, nitrate reductase activity; RuBPCase, ribulose 1,5-bisphosphate carboxylase; PEPCase, phosphoenolpyruvate carboxylase.

for the various assays. Weight of discs was added back to obtain total laminae weight.

Assays. Subsamples of the fresh material were taken for NRA (4 g) and Chl (2 g). NRA was determined as described (11). Chl was extracted with 50 ml ethanol (95%, v/v) held in sealed glass bottles, and agitated for 20 h in the dark at ambient temperature. Aliquots (0.5 ml) were removed from the bottles and diluted with 5.5 ml ethanol, (95%) and A was determined at 649 and 665 nm. Chl concentrations were calculated according to Holden (6). Another weighed (4 g) subsample was dried to constant weight and reweighed for per cent moisture determination. The dried sample was then ground in a Wiley mill (20 mesh) and subsequently used for reduced N, phosphorus, sugars, and starch. The procedures used for these constituents have been described (5). For gel electrophoresis, 30 leaf discs (0.785 cm^2 $disc^{-1}$) from each sample were extracted in 3 ml of 20 mM Tris-HCl buffer (pH 7.3) containing 4 mM DTT and 1 mM EDTA using a Polytron homogenizer. The extracts were centrifuged for 15 min at 30,000g and aliquots from the supernatant fractions were assayed for protein by the Bio-Rad method (1). Based on the protein determinations, samples for SDS-PAGE were prepared containing equal amounts of protein in 20 mM Tris (pH 7.3), 1% (w/v) SDS, 5% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, and 0.005% (w/v) phenol red. The proteins were dissociated by immersing the samples for 2 min in boiling water. Aliquots containing 35 μg soluble protein were applied to each well. The polypeptides were separated on a 9 to 18% polyacrylamide gradient with a 5% acrylamide stacking gel in 0.1% SDS, using the buffer system described by Laemmli (7). Electrophoresis was carried out at a constant current of 40 mamp. Gels were stained overnight in a solution containing 0.1% (w/v) Coomassie blue, 40% (v/v) methanol, and 10% (v/v) glacial acetic acid and were

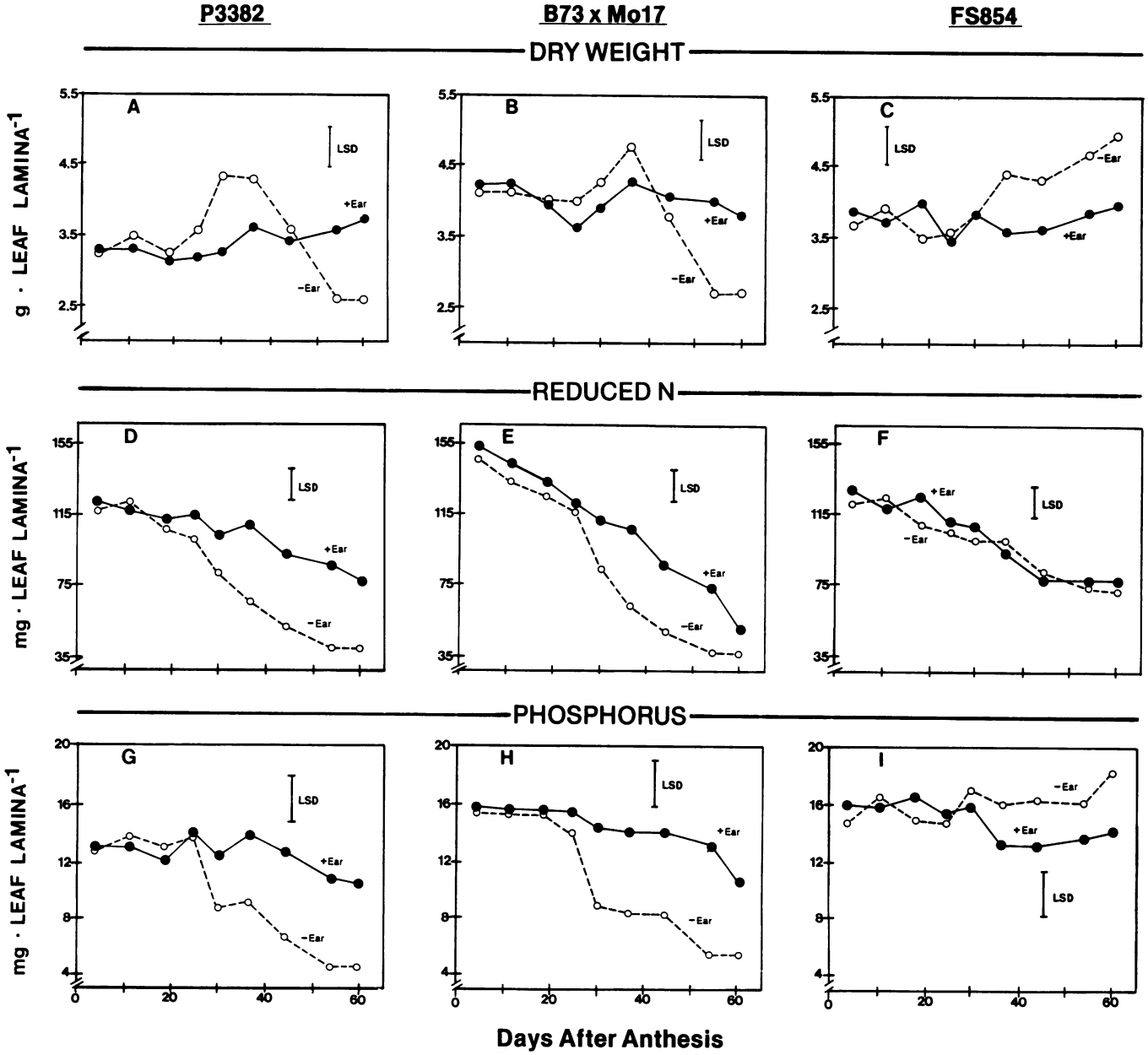

FIG. 1. Effect of ear removal on dry weight, reduced N, and phosphorus contents of the leaf above the ear of three maize hybrids. The primary ear shoot and any secondary ear shoots were bagged and excised (−ear treatment), compared with controls (+ear treatment). Leaf samples were collected and analyzed at nine times, specified as days after anthesis (July 12).

destained in 30% (v/v) methanol and 10% (v/v) acetic acid. Photographs were taken after the gels were dried.

Statistical Analyses. Statistical analyses were performed as previously described (5).

RESULTS

Visual Senescence. The marked differences in the visual senescence patterns of the selected leaf, among the hybrids, was similar to those described for the entire leaf canopy (5). However, in response to ear removal, the initial appearance of the red pigmentation occurred from 3 to 7 d later on the selected leaf than on the uppermost leaf. In response to ear removal, the selected leaf of P3382 and B73 × Mo17 plants senesced at a faster rate than the selected leaf of the eared plants. Although the selected leaf of the earless FS854 plant exhibited some red pigmentation of the midrib between 44 and 54 DAA, the visual symptoms on the selected leaf of eared and earless plants were essentially indistinguishable. For the eared plant, the selected leaf of B73 × Mo17 had a greenish yellow appearance while the selected leaf of P3382 and FS854 appeared green and viable at 60 DAA. The visual differences noted among hybrids and treatments were supported by significant hybrid treatment interactions (analyses not shown) for the various parameters measured and discussed in the following sections. These interactions were primarily due to the different response of FS854 relative to P3382 and B73 × Mo17 when ears were removed.

Dry Weight. For all hybrids, the general trend in dry weight accumulation of the selected leaf (Fig. 1, A–C) was similar to the trend exhibited by the composite leaf sample representative of the entire canopy as previously reported (5), except that the effects due to ear removal were delayed in the selected leaves. The selected leaf was one of the last leaves to senesce for both eared and earless plants.

In response to ear removal, leaf dry weight was unaffected until 25 DAA, increased between 25 and 37 DAA, and decreased rapidly between 37 and 54 DAA for both P3382 and B73 × Mo17, relative to respective controls. In contrast, leaf weight of earless FS854 plants was unaffected until 30 DAA and then increased until 60 DAA, relative to eared plants. The late season increase in leaf dry weight of the earless FS854 plants was attributed to a maintenance of photosynthetic activity and a decreased rate of translocation to the stalk. For all eared plants, the leaf dry weight remained relatively constant throughout grain fill.

Reduced N. In response to ear removal, the loss of reduced N content of the selected leaf was accelerated for P3382 and B73 × Mo17 (starting at 19 and 25 DAA, respectively) (Fig. 1, D–F). For FS854, the rate of loss from earless and eared plants was similar throughout. These patterns of N loss from the selected leaf were similar to the patterns of N loss from leaves representative of the total canopy (5). Regardless of treatment or hybrid, the selected leaves lost N throughout the grain-filling period and the rate of loss and content of N at 60 DAA showed a relationship with the visual symptoms of senescence. Leaves of eared P3382 and eared and earless FS854 plants that were green and viable appearing at 60 DAA retained about 60% of the N content present at 4 DAA, while leaves of earless P3382 and eared and earless B73 × Mo17 plants that senesced at or prior to 60 DAA retained only 24 to 32% of the N content at 4 DAA.

Phosphorus. Removal of ears had little initial effect on the phosphorus content of the selected leaf of P3382 and B73 × Mo17 plants; however, between 25 and 54 DAA, phosphorus was lost at an accelerated rate relative to controls (Fig. 1, G–I). In contrast, the phosphorus content of the selected leaf of earless FS854 plants increased between 25 and 60 DAA relative to control. The phosphorus content of the selected leaf of all eared hybrids remained relatively constant throughout grain fill. For

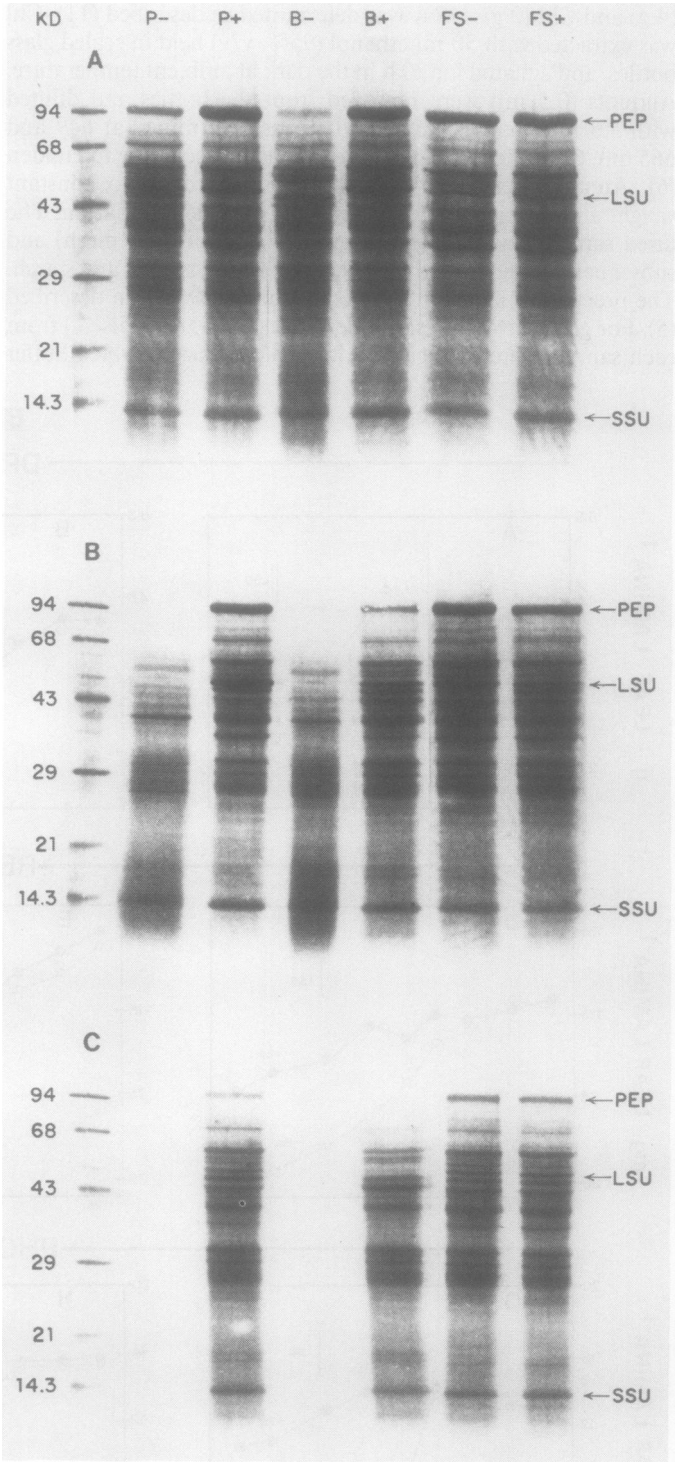

FIG. 2. SDS-gel electrophoretogram of polypeptides from the first leaf above the ear node for earless (−) and eared (+) plants of P3382 (P), B73 × Mo17 (B), and FS854 (FS) hybrids at 30 (A), 44 (B), and 60 (C) DAA. The protein standards and their respective mol wt in kD are shown in lane 1. The arrows indicate PEPCase (PEP) and the large (LSU) and small (SSU) subunits of RUBPCase. The low moisture content of the leaves of earless P3382 and B73 × Mo17 plants at 60 DAA precluded assay.

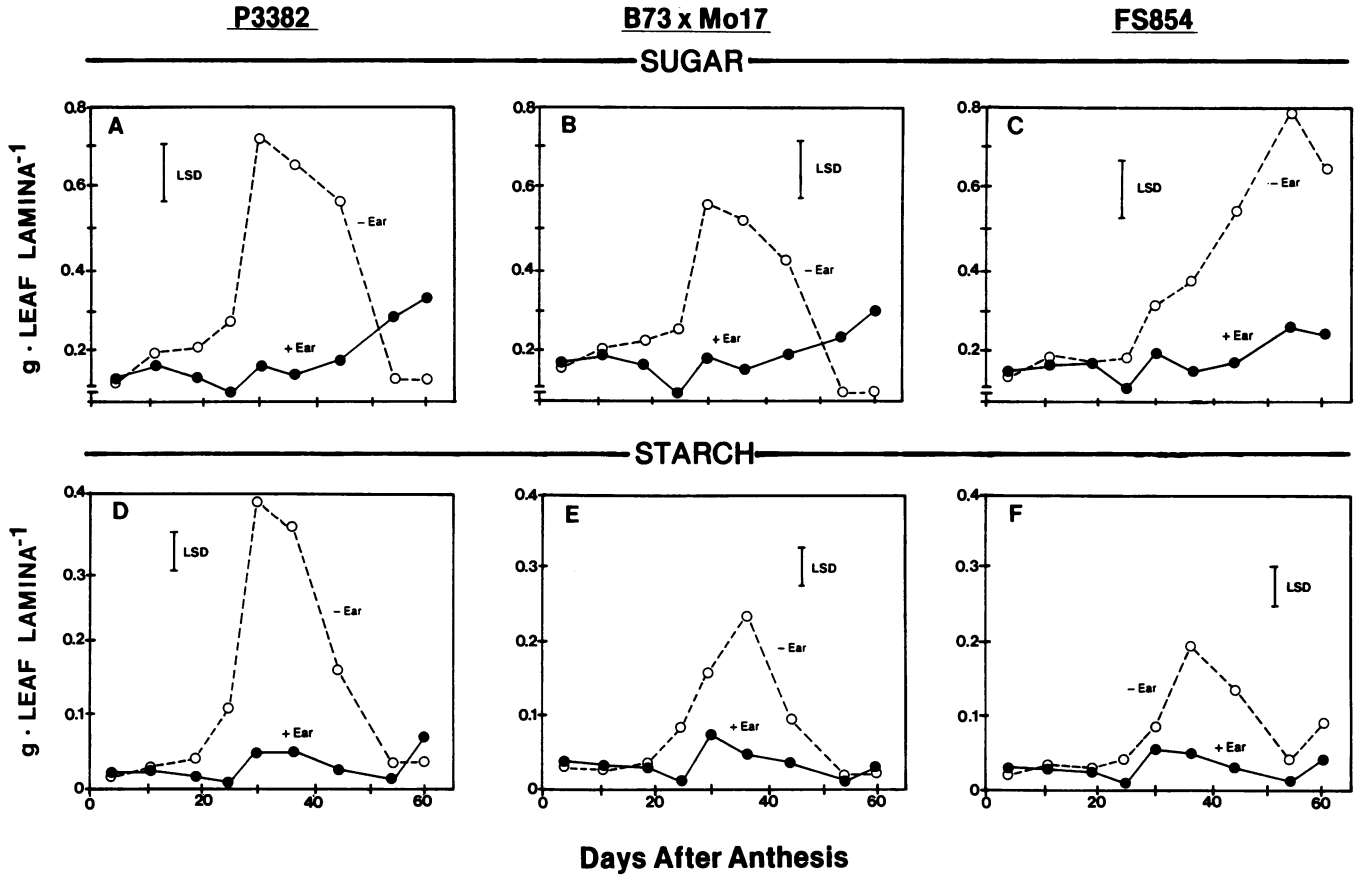

FIG. 3. Effect of ear removal on sugar and starch contents of the leaf above the ear of three maize hybrids. Treatments and sampling were as indicated in Figure 1 legend.

the earlier senescing B73 × Mo17 plants, the leaf at 54 DAA retained 80% of the phosphorus content present at 4 DAA. In contrast to reduced N (Fig. 1, D–F), it did not appear that phosphorus was remobilized from the leaves to support early ear development. For eared P3382 and B73 × Mo17, significant losses of leaf phosphorus were confined to the last 15 d of the grain-filling period. Estimates made with the whole plant data (5) showed only 15 to 18% of the phosphorus of the grain was remobilized from the vegetation. The loss of leaf phosphorus appeared to be a result of, rather than the cause of, leaf senescence.

Gel Electrophoresis. The soluble protein polypeptide profile (Fig. 2, A–C) showed that, relative to controls, ear removal resulted in an accelerated loss of PEPCase and the large subunit of RuBPCase extractable from leaves of P3382 and B73 × Mo17 plants. For the eared and earless FS854 plants, the changes in protein band densities were similar throughout the grain-filling period. Although not clearly evident in Figure 2 (60 DAA), the original gels for the earless FS854 plants showed a slight increase in density of the broad poorly defined bands around 14 and 29 kD compared to the eared FS854 plant. In contrast with senescing soybean leaves (15), the gel patterns provided no evidence for the formation of newly synthesized polypeptides (proposed as temporary storage proteins) during the course of leaf senescence in earless maize.

The loss of the photosynthetic enzymes from the selected leaf with time (Fig. 2) was concurrent with the loss of reduced N from the same leaf (Fig. 1, D–F). This association is consistent with the report of Morita (10) that 90 to 95% of the N lost from the leaves of a normally senescing rice plant was lost from the chloroplast. With corn 80 to 85% of the N lost from the leaves was from the chloroplast (unpublished).

Judged by the disappearance of the photosynthetic polypeptide bands, increased diffuse banding, and decreased band density over time (Fig. 2), the sequence and rate of senescence was: earless B73 × Mo17 > earless P3382 > eared B73 × Mo17 > eared P3382 > earless FS854 ≥ eared FS854. This sequence was consistent with visual and other metabolic symptoms of senescence.

Sugar. In response to ear removal, sugar content of the selected leaves increased slowly, reached maximum levels by 30 (P3382), 37 (B73 × Mo17), and 54 (FS854) DAA, and then declined thereafter (Fig. 3, A–C). For all eared hybrids, sugar content of the leaves remained relatively constant until 44 DAA and then increased, especially P3382 and B73 × Mo17. The differential late season accumulation of sugar among the eared hybrids was attributed to the balance between photosynthetic activity, transport, and ear demands. Swank *et al.* (13) found that the average rate of grain fill was 2.2, 1.6, and 3.9 g dry weight d^{-1} between 44 and 57 DAA for P3382, B73 × Mo17, and FS854, respectively.

Starch. The starch content of the selected leaf of the earless plants was unaffected until 20 to 25 DAA, increased to maximum levels by 30 to 37 DAA, and then declined (Fig. 3, D–F). Regardless of treatment or hybrid, the patterns of starch content were similar. The accumulation of sugar between 44 and 54 DAA and starch between 54 and 60 DAA implied that some photosynthetic activity was retained until 60 DAA by the leaf of the earless FS854 plant. This view was also supported by the whole plant data (5).

Although for all hybrids, ear removal led to carbohydrate accumulation in the leaf, the accumulation of carbohydrates was not closely associated with the rate of development of visual senescence symptoms or retention of the carboxylating enzymes.

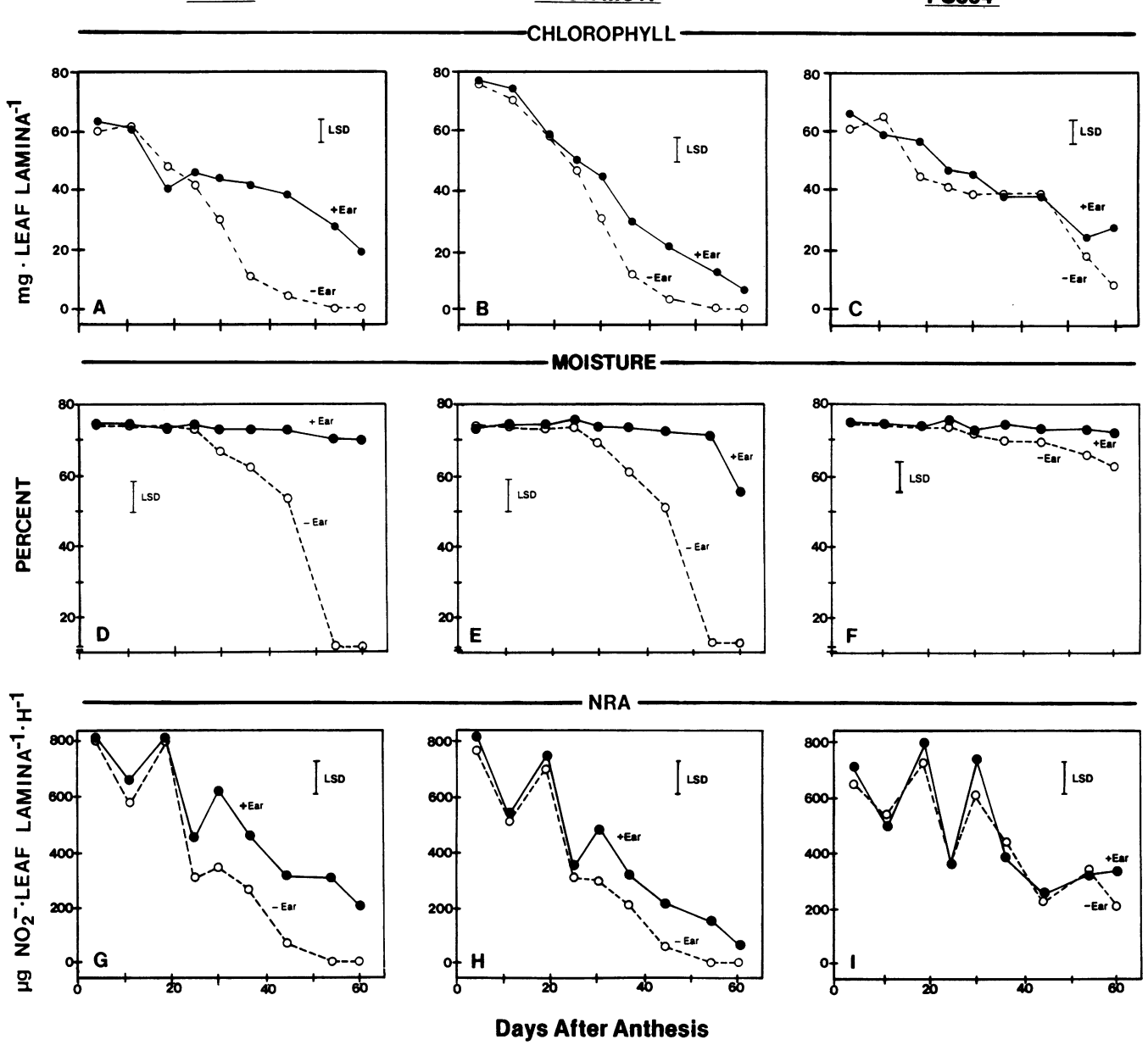

FIG. 4. Effect of ear removal on moisture and Chl contents, and on NRA of the leaf above the ear of three maize hybrids. Treatments and sampling were as indicated in Figure 1.

The differences among the eared hybrids in visual senescence patterns and the rate of loss of the carboxylating enzymes were not closely associated with the accumulation of carbohydrates by the selected leaf. These observations indicated that leaf carbohydrate accumulation was a symptom rather than a cause of senescence.

Chlorophyll. The patterns of Chl loss from the selected leaf were similar to the loss of reduced N for all hybrids and treatments (Fig. 4, A–C; Fig. 1, D–F). The concurrent loss of both components was consistent with the concept of continuous chloroplast degradation during the reproductive period.

Ear removal resulted in an accelerated loss of Chl starting at 25 DAA for P3382 and B73 × Mo17 while Chl loss was unaffected for FS854 until 54 DAA. The selected leaf of P3382 and B73 × Mo17 earless plants was devoid of Chl by 54 DAA. For all eared hybrids, the loss of Chl from the selected leaf was nearly linear; however, the rate of loss was greater for B73 × Mo17 than the other hybrids. Based on the average Chl contents between 44 and 60 DAA, the sequence and rate of senescence were: earless B73 × Mo17 > earless P3382 > eared B73 × Mo17 > earless FS854 > eared P3382 = eared FS854.

Moisture. Ear removal resulted in an accelerated loss of water from the selected leaf of P3382 and B73 × Mo17 plants beginning about 25 DAA (Fig. 4, D–F). The leaf of these two hybrids was air dry by 54 DAA. Although the general trends indicated that water loss from the leaf of earless FS854 was initiated by 30 DAA, the difference in moisture content between the eared and earless leaf was not significant until 54 DAA. This progressive differential in moisture percentages between eared and earless FS854 plants supported the view that leaf senescence of all hybrids was affected by ear removal (5), but that the rate of the accelerated senescence was extremely slow for FS854. For all

eared hybrids, the moisture values were similar except for the more rapid desiccation of the leaf of B73 × Mo17 plants between 54 and 60 DAA.

Because the retention of moisture probably reflected cell integrity, this parameter was judged as a reflection of the course of senescence rather than a cause of senescence. For all eared and earless hybrids, the moisture patterns were comparable to other metabolic parameters in providing a reasonably accurate reflection of the onset and development of leaf senescence. Based on the per cent moisture at 60 DAA, the sequence and rate of senescence was: earless B73 × Mo17 = earless P3382 > eared B73 × Mo17 > earless FS854 > eared P3382 > eared FS854.

NRA. An accelerated loss of NRA from the selected leaf of earless relative to eared P3382 and B73 × Mo17 plants was initiated by about 25 DAA (Fig. 4, G–I). In contrast, the patterns of NRA were similar for eared and earless FS854 plants until 54 DAA. Those plants that retained green, viable-appearing leaves throughout the grain-filling period (eared P3382, eared and earless FS854) had 200 or more units of NRA in the selected leaf at 60 DAA. By 60 DAA, NRA was not detectable in the selected leaf of earless P3382 and B73 × Mo17 plants but was present at 8, 21, 30, and 40% of the maximum amounts present earlier in the season for eared B73 × Mo17, eared P3382, earless FS854, and eared FS854, respectively. From these patterns, the sequence and rate of senescence was: earless B73 × Mo17 = earless P3382 > eared B73 × Mo17 > eared P3382 = earless FS854 > eared FS854.

The patterns of NRA (Fig. 4, G–I) and reduced N (Fig. 1, D–F) were similar for each of the six treatments, especially for the last half of the grain-filling period. In all six cases, the retention of NRA was associated with the retention of reduced N by the leaf. Nitrate was not measurable by our routine assay in maize leaf laminae after anthesis, and therefore NRA patterns could not be related to possible differential nitrate availability.

DISCUSSION

In the following paragraphs, we present our concepts on the role of nitrate and reduced N in the development (not the cause) of senescence of intact leaves. The patterns of NRA of the selected leaf (Fig. 4, G–I) as a function of hybrids and treatments were similar to the patterns of reduced N (Fig. 1, D–F), carboxylating enzymes (Fig. 2), and Chl (Fig. 4, A–C). All four parameters provided a reasonable reflection of the development of the visual symptoms of senescence. Based on the work of Shaner and Boyer (12), we believe that the variations in NRA levels were due to variations in the amount of nitrate fluxing into the leaves during the grain-filling period. This view is supported by the similarity of NRA (Fig. 4, G–I) and reduced N (Fig. 1, D–F) patterns of the selected leaf.

Recent work with rice has shown that the influx of N into the leaf and the synthesis of RuBPCase were closely associated throughout most of the life span of an intact selected leaf (8). They used immunochemical techniques and ^{15}N (as NH_4^+) to show that about 90% of the N influx into and RuBPCase synthesized during the life of the leaf occurred by about 1 week after full leaf expansion. New nitrogen (^{15}N) continued to flux into the leaf at a constant low rate until leaf death (63 d after full expansion) and RuBPCase synthesis continued at a constant low rate for at least 42 d after full leaf expansion. They were unable to determine whether the continued slow rate of synthesis of RuBPCase was due to slow turnover in the existing chloroplasts or the presence of chloroplasts still in the developmental stage (3). This work showed that some N could enter the rice leaf after full expansion, and our data (Fig. 4, G–I) and the N accumulation by the whole plant (5) implied that the amount of N fluxing into the leaf varied as a function of hybrid and treatment.

Previously, Meeker *et al.* (9) showed that nitrate flux into the leaves of young corn seedlings was essentially terminated by full leaf expansion. The patterns of NRA and concentration of nitrate in the leaves were similar throughout the life of the first four leaves of the seedling. Based on these various experiments, we conclude that although the loss of NRA, reduced N, and carboxylating enzymes were accurate reflections of the development of senescence of the selected leaf, the rate of net loss of reduced N and the carboxylating enzymes was regulated, at least in part, by the availability of reduced N (flux and assimilation of nitrate).

Our attempts to find a close relationship between the loss of N from the leaves and proteolytic activity have been unsuccessful (4). In addition to the repression of the chloroplast genome and suppression or cessation of chloroplast rRNA synthesis (2, 14), the depression or cessation of the flux of both nitrate and reduced N into the leaf of maize and rice (8, 9) were also associated with the completion of leaf expansion. Although the actual time of initiation of the net N loss from the intact rice leaf was obscured by the continued influx of N into the leaf, measurable net losses of N did not occur until 9 to 12 d after full leaf expansion (8). We view the rate of flux of nitrate and other metabolites into the leaf as a factor affecting the rate of senescence development in maize. Identification of the factor(s) that regulate the flux of N into the leaf throughout its life span may be helpful in identifying the factor(s) that cause senescence.

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