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Limiting Factors in Photosynthesis

II. IRON STRESS DIMINISHES PHOTOCHEMICAL CAPACITY BY REDUCING THE NUMBER OF PHOTOSYNTHETIC UNITS[1, 2]

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ABSTRACT

It has been proposed that Fe stress may be used in the study of limiting factors in photosynthesis as an experimental means of varying photochemical capacity *in vivo* (Plant Physiol 1980 65: 114–120). In this paper the effect of Fe stress on photosynthetic unit number, size, and composition was investigated by measuring P_{700}, cytochrome (Cyt) *f*, chlorophyll (Chl) *a*, and Chl *b* in sugar beet leaves. The results show that when Fe stress reduced Chl per unit area by 80% (from 60 to 12 micrograms per square centimeter), it decreased the number of P_{700} molecules per unit area by 88% and Cyt *f* per unit area by 86%; over the same range the Chl to P_{700} ratio increased by 37% but there was no significant change in the Chl to Cyt *f* ratio. These data suggest that Fe stress decreases photochemical capacity and Chl per unit area by diminishing the number of photosynthetic units per unit leaf area.

The ratio of Chl *a* to Chl *b* did not change with Fe stress. This suggests that the proportion of light-harvesting Chl *a*/*b*-protein complex within the photosynthetic unit remained constant. Electron microscopy of chloroplasts revealed that the decrease in the number of photosynthetic units which occurred during Fe stress was accompanied by a reduction in the number of granal and stromal lamellae per chloroplast and by a reduction in the number of thylakoids per granum.

In the first paper of this series (26), it was proposed that mineral nutrient stress might be used as an experimental tool in the study of limiting factors in photosynthesis. Data were presented which showed that Fe stress in sugar beet plants reduced the photochemical capacity of leaves. These data also showed that the maximum rate of photosynthesis per unit Chl was not decreased by Fe stress, suggesting that the size of the photosynthetic unit was unchanged. The hypothesis tested here was that the reduction in photochemical capacity which accompanied Fe chlorosis resulted from fewer photosynthetic units per leaf area rather than from a change in photosynthetic unit size.

According to current models of the photosynthetic unit there is 1 molecule of P_{700} and 1 molecule of Cyt *f* per photosynthetic unit (5) (except in some low-light-grown plants [4, 6]). On this assumption, we measured P_{700} and Cyt *f* as a means of estimating the number of photosynthetic units per unit leaf area. The ratio of Chl/P_{700} and Chl/Cyt *f* provided indices of photosynthetic unit size (4, 5). In addition, we measured the Chl *a*/Chl *b* ratio. Brown *et al.* (9) and Genge (12) concluded that Chl *b* is located only within the light-harvesting complex while Chl *a* is found throughout the photosynthetic unit. Based on their work we assumed that a change in the amount of light-harvesting Chl *a*/Chl *b*-protein complex per photosynthetic unit would be indicated by a change in the Chl *a*/Chl *b* ratio.

Since earlier work indicated that Fe stress may influence the formation of the entire light-harvesting and electron transport apparatus (25, 26), electron micrographs were prepared to show the progressive effects of Fe stress on chloroplast structure.

MATERIALS AND METHODS

Plant Culture and Harvest Procedure. Sugar beet plants (*Beta vulgaris* L. cv. F58-554H1) were cultured in growth chambers at 25 C with an illumination of 35,000 lux (7.0 mw cm^{-2}; 45 nE cm^{-2} s^{-1} [400–700 nm]) supplied over a 16-h daylength. The procedure for culturing and inducing Fe stress was the same as previously described (26). Leaves were harvested from two control plants and three Fe-stressed plants between 7:00 and 8:00 AM. After removal from the plant the harvested leaf was immediately enclosed in a plastic bag and stored at 2 C until it was used (usually on the same day).

P_{700} Extraction and Assay. The method employed for the extraction and assay of P_{700} was modified from that used by Shiozawa *et al.* (22). After removing the midrib the leaves were chopped and ground for 5 s in a Waring Blendor with a preparative solution containing 0.5 M sucrose, 0.1 M Tris-HCl (pH 8.0) and 30 mM sodium ascorbate. The resulting suspension was filtered through six layers of cheesecloth and centrifuged at 30,000*g* for 5 min. The pellet was resuspended and washed in 40 ml of a wash solution containing 0.10 M Tris-HCl (pH 8.0) and 30 mM sodium ascorbate and centrifuged at 30,000*g* for 10 min. This procedure gave a pellet which consisted largely of chloroplast lamellae. These were resuspended in a small volume and diluted to give a Chl concentration of approximately 800 μg/ml using the wash solution. A 0.5-ml aliquot of this suspension was diluted to 5 ml to give an assay solution with a final concentration of 0.5% (v/v) Triton X-100, 0.1 M Tris-HCl (pH 8.0), 30 mM sodium ascorbate and a Chl concentration of about 80 μg/ml. After 30 min at room temperature the assay solution was centrifuged at 2,500*g* for 10 min and the supernatant assayed immediately, or, frozen at −8 C prior to measurement.

The assay of P_{700} was carried out with an Aminco DW2 spectrophotometer which was modified by the insertion of an extension tube and a matt black baffle (with holes for the measuring beam) between the sample cuvette and the photomultiplier. This arrangement was made to minimize the amount of sample fluorescence reaching the photomultiplier. The concentration of P_{700} was determined using the dual wavelength mode by measuring the difference in *A* at 698 nm (slit width 3.5 nm) between the pho-

[1] This work was partly supported by the Beet Sugar Development Foundation. Taken from a thesis submitted by S. C. Spiller in partial fulfillment of requirements for the Ph.D., University of California, Berkeley.

[2] This work was presented at the Annual Meeting of the American Society of Plant Physiologists at Blacksburg, Virginia, June 29, 1978 (Plant Physiol. 61: S-87).

tooxidized and dark-reduced sample with 730 nm as the reference wavelength. An extinction coefficient of 64 mM^{-1} cm^{-1} (14) was used to calculate P_{700} concentration from the difference between ΔO.D. (698–730 nm) in light and in darkness. Blue actinic light was provided by means of an incandescent microscope lamp filtered through two Corning No. 4-96 filters. *A* was first measured in darkness and then at each of three increasing irradiances to ensure that the P_{700} was fully photooxidized. Actinic light was prevented from reaching the photomultiplier by two red Corning filters (No. 2-64) taped to the face of the photomultiplier.

Determination of Chlorophylls. To determine Chl *a* and Chl *b*, leaf plugs of known area were ground in a glass homogenizer with powdered sodium ascorbate and a solution containing 80% (v/v) acetone in water. The resulting suspension was made up to volume with 80% (v/v) acetone and centrifuged. The *A* of the supernatant was measured at 645 nm and 663 nm and the coefficients of Mackinney (16) used to determine the amounts of Chl *a* and Chl *b* per unit leaf area. The Chl content of the P_{700} assay solution was determined directly from the measurement of *A* at the 670 nm peak using an extinction coefficient of 60 mM^{-1} cm^{-1} (22).

Cyt *f* Extraction and Assay. The midrib was removed from harvested leaves and the leaf blade chopped and ground in a Waring Blendor in a preparative solution consisting of 0.4 M sucrose, 20 mM Tricine-KOH (pH 8.0), 10 mM NaCl and 30 mM sodium ascorbate. The suspension was filtered through six layers of cheesecloth and centrifuged at 30,000*g* for 5 min. The pellet was resuspended in 40 ml of a wash solution consisting of 20 mM Tricine-KOH (pH 8.0), 10 mM NaCl and 30 mM sodium ascorbate and centrifuged at 30,000*g* for 10 min. This pellet was resuspended in a second wash solution of 20 mM Tricine-KOH (pH 8.0) and 10 mM NaCl and centrifuged at 30,000*g* for 10 min to remove sodium ascorbate. The chloroplast pellet was resuspended in a minimal volume of a solution containing 50 mM Tricine-KOH (pH 8.0) and 5 mM $MgCl_2$. An appropriate aliquot was added to the assay solution containing 50 mM Tricine-KOH (pH 8.0), 5 mM $MgCl_2$, and 1% (v/v) Triton X-100 to give a final concentration of 65 to 110 μM Chl. The Triton was included to eliminate interference from absorption by the high potential form of Cyt b_{559} (3). The concentration of Cyt *f* was determined by measuring the ΔO.D. at the 554 nm peak obtained by ferricyanide oxidation and hydroquinone reduction of the sample using the split beam mode of the Aminco DW2 spectrophotometer (3). An extinction coefficient of 19.7 mM^{-1} cm^{-1} was used (based on Forti *et al.* [11] and personal communication from D. S. Bendall).

Electron Microscopy. Blocks of leaf tissue (0.5–1.0 mm^3) were fixed for 2 h in a solution consisting of 4% glutaraldehyde and 50 mM Sørensen's phosphate buffer (pH 7.2). The fixed tissue was rinsed using a solution of 50 mM Sørensen's phosphate buffer (pH 7.2) three times, 15 min per rinsing. Postfixation was carried out using 1% OsO_4 in 50 mM Sørensen's phosphate buffer (pH 7.2) for 2 h and rinsed in the buffer solution for 10 min. The tissue was dehydrated using a series of ethanol-water solutions, 30, 50, 70, 85, 90, and 95% (10 min per solution), followed by two changes in 100% ethanol (20 min each) and two changes in propylene oxide solution (20 min each). The leaf tissue was embedded in Araldite. Sections were stained with Reynold's lead citrate and saturated aqueous uranyl acetate. The electron microscopy was carried out using a Siemens 1A electron microscope.

RESULTS

Number and Size of Photosynthetic Units. The effect of Fe stress on the number of photosynthetic units was determined by withholding Fe hydroponically from the plant, and then measuring the number of P_{700} and Cyt *f* molecules in leaves with varying degrees of chlorosis. Chlorosis often occurred as early as 2 days from withholding. Leaves with different Chl contents were easily obtained by visual selection of plants which had been without Fe

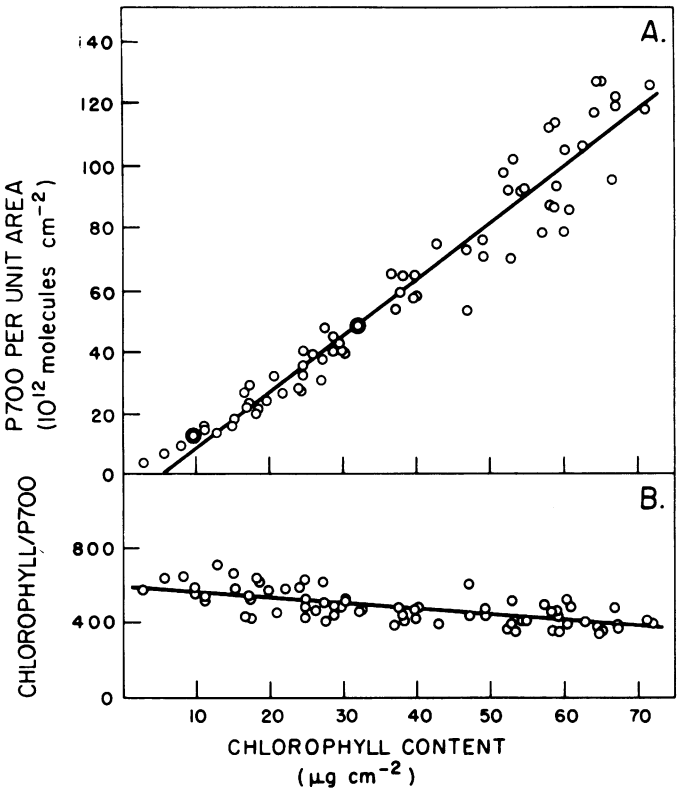

FIG. 1. Number of P_{700} molecules per unit leaf area (A) and the Chl/P_{700} molar ratio (B) for leaves with different Chl contents. Chl contents below 40 μg cm^{-2} were obtained by withholding Fe from plants for varying periods. (Regression lines are $y = 1.81\ x - 9.82$ [$r = 0.97$] for P_{700}/area and $y = -3.15\ x + 598$ [$r = -0.70$] for Chl/P_{700}).

for periods ranging from 2 to 10 days. On any given day, typically five leaves were harvested; two were removed from control (Fe-sufficient) plants, and three from Fe-stressed plants. The data, which were combined as shown in Figures 1 and 2, demonstrate that the number of P_{700} molecules/area and the number of Cyt *f* molecules/area were each linearly related to Chl/area. The regression equations for the two sets of measurements were surprisingly close (see legends of Figs. 1 and 2) and showed that with an 80% reduction in Chl/area (from 60 to 12 μg cm^{-2}) there was an 88% reduction in P_{700}/area and an 86% reduction in Cyt *f*/area.

Although these results clearly indicate a reduction in the number of photosynthetic units per unit leaf area[3], there may also have been an additional effect of Fe stress on photosynthetic unit size. The Chl/P_{700} ratio increased by 37% with decrease in Chl/area from 60 to 12 μg cm^{-2}, but there was no significant increase in Chl/Cyt *f*. The Chl/P_{700} ratio of Fe-sufficient plants was 423 compared to 411 for Chl/Cyt f (Table I). This suggests that sugar beets normally contain about 400 Chl molecules per photosynthetic unit with 1 molecule of P_{700} and 1 molecule of Cyt *f*. Under severe Fe stress the Chl/P_{700} ratio increased to 570 compared to 433 for Chl/Cyt *f*. Based on the means the Cyt *f*/P_{700} increased from about 1 to 1.32, implying that the composition of the photosynthetic unit with respect to P_{700} may have been altered by Fe stress.

Iron stress did not appear to alter the amount of light-harvesting Chl *a*/*b*-protein complex per photosynthetic unit since the ratio of Chl *a*/Chl *b* did not change significantly (Table I). The data show, however, that Chl *a*/Chl *b* ratios were more variable in the

[3] Comparison of these data on a per unit area basis is valid since Fe stress did not change leaf thickness (25).

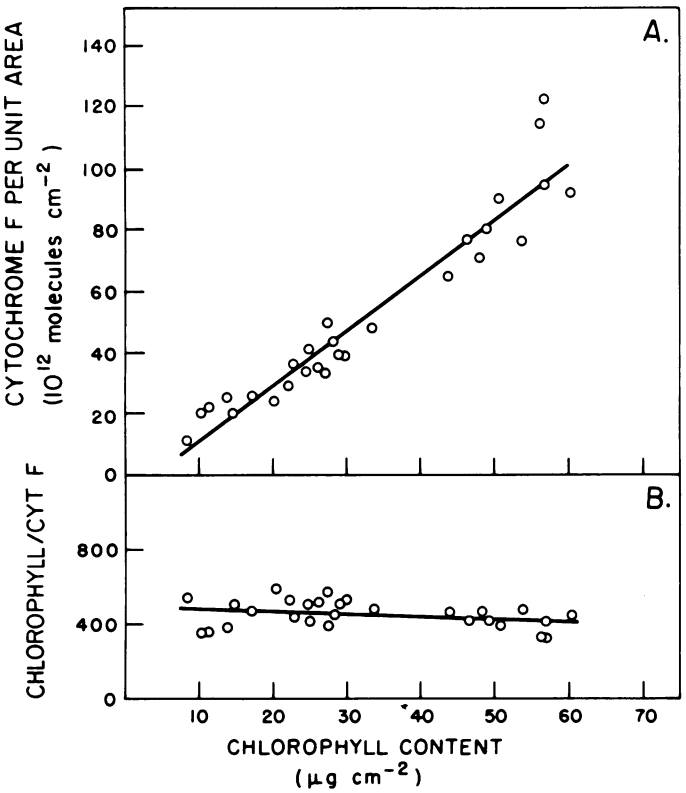

FIG. 2. Number of Cyt *f* molecules per unit leaf area (A) and the Chl/Cyt *f* molar ratio (B) for leaves with different Chl contents. (Regression lines are $y = 1.80\ x - 7.35$ [$r = 0.96$] for Cyt *f*/area and $y = -1.41\ x + 496$ [$r = -0.32$] for Chl/Cyt *f*).

severely Fe-stressed plants. The standard deviation in severely Fe-stressed plants was ±20% of the mean (3.83) compared to ±6% for the Chl *a*/Chl *b* ratio in control plants (3.29). This increased variability may have resulted from some methodological difficulties we experienced when working with Fe-stressed leaf tissue: first, the very low Chl concentrations in Fe-stressed leaves were difficult to measure accurately; and second, browning occurred when Fe-stressed leaves were macerated unless a high concentration of sodium ascorbate (30 mM) was used. The possibility remains, however, that some interference in the assay of Chl (or P_{700}) occurred.

Chloroplast Structure. Sugar beet chloroplasts typically exhibit a large number of granal and stromal lamellae with as many as seven or eight thylakoids per granum (Fig. 3A). As the Chl content of the leaf decreased with progressive Fe stress, the number of grana per chloroplast and the number of thylakoids per granum decreased. This is shown in Figure 3, B–D, which show chloroplasts at different stages of Fe stress. The severely Fe-stressed chloroplast (Fig. 3D) appears to be spherical or sausage-like in shape with very few lamellae present within a large stroma. There are almost no grana present in this cross-section although there are sites which appear to have two appressed thylakoids forming a rudimentary granum. The osmiophilic globules clustered in two regions at opposite ends of the lamellae are typical of severely Fe-stressed chloroplasts (when OsO_4 is used in fixation [15]) and may represent lipids and carotenoids accumulated in the absence of membrane formation (20). The reduction in chloroplast lamellae with iron stress seemed to occur uniformly in that all of the plastids within the cells of an Fe-stressed leaf had fewer lamellae and reduced grana (Fig. 4).

DISCUSSION

The results of the present work support the hypothesis that Fe stress decreased the number of photosynthetic units per unit area and that this resulted in both chlorosis and a reduction in photochemical capacity. We believe that photochemical capacity is decreased because Fe stress decreases the number of PSI reaction centers (P_{700}) and electron carriers (Cyt *f*). The linear relationship between maximum photosynthesis and Chl content observed in the earlier work with sugar beets (see ref. 26, Fig. 4), and by Emerson in his work with Fe-stressed *Chlorella* (10), seems to be a relationship between photosynthesis and the number of photosynthetic units per unit of photosynthetic tissue. A similar relationship may apply to the Fe stress data of Willstätter and Stoll (30), Bottrill *et al.* (8), and Spencer and Possingham (23).

Table I. *Effect of Fe Stress on Composition of Photosynthetic Unit*

Molar Ratios	Control Leaves >40 μg Chl cm^{-2}	Fe-stressed Leaves 20–40 μg Chl cm^{-2}	Severely Fe-stressed <20 μg Chl cm^{-2}
Chl *a*/Chl *b*	3.29 ± 0.20	3.38 ± 0.31	3.83 ± 0.75
	N[a] = 84	N = 63	N = 48
Chl/P_{700}	423 ± 62	486 ± 66	570 ± 76
	N = 30	N = 24	N = 17
Chl/Cyt *f*	411 ± 54	491 ± 60	433 ± 81
	N = 10	N = 12	N = 6
Cyt *f*/P_{700}	1.03	0.99	1.32

[a] N = No. samples/mean.

Because of the different effect of Fe stress on the Chl/P_{700} and on Chl/Cyt *f* ratios, it is not clear whether Fe stress increased photosynthetic unit size in sugar beets. In any case, the increase in Chl/P_{700} was no more than 50%. Öquist (19) found a much greater effect of Fe stress on the Chl/P_{700} ratio of the blue-green alga, *Anacystis nidulans* and that Fe stress depressed photosynthetic activity on a per unit Chl basis. Öquist's work differed from our own, however, not only in terms of plant species, but also in the procedure employed for initiating Fe stress. Öquist induced Fe stress by culturing *Anacystis* with a small concentration of Fe (0.1 mg $FeCl_3$/liter) in the culture solution. Such a procedure where Fe was continually supplied at insufficient levels may have resulted in secondary effects of Fe stress on the photosynthetic system. We tried to minimize secondary effects by withholding Fe from fairly mature sugar beets for short periods of not more than 10 days. The more mature green leaves supplied sufficient carbohydrate to maintain near normal rates of growth in young leaves as they developed chlorosis (25). Similarly, Emerson found that glucose was necessary to sustain *Chlorella* growth during his iron stress experiments (10).

Experimental evidence currently indicates that Chl *b* and xanthophyll are located in the light-harvesting Chl *a*/*b*-protein complex whereas Chl *a* and β-carotene are present in all parts of the unit (9, 12, 27). Since Fe stress did not significantly change the Chl *a*/Chl *b* ratio, there was no evidence that the amount of light-harvesting complex per photosynthetic unit was affected by Fe stress. Fe stress did not change the Chl *a*/*b* ratio in tomato (23) or spinach (8), although Stocking (24) found that the ratio was decreased in Fe-stressed maize. The composition of the light-harvesting complex may have changed, however, with respect to carotenoid content since there were significant increases in the molar ratio of β-carotene to Chl(*a*+*b*) and in the ratio of xanthophyll to Chl(*a*+*b*) with Fe stress (26). It is also possible that the increased amounts of xanthophyll (measured in leaves) were located external to the lamellae, perhaps in plastoglobuli, which are denser staining in the Fe-stressed chloroplasts (Figs. 3 and 4). Plastoglobuli have been shown to contain carotenoids in mutant barley (29). Also, Sager (20) has shown that carotenoids located in plastoglobuli may be formed in yellow mutants of *Chlamydomonas* in the absence of Chl and lamellae formation.

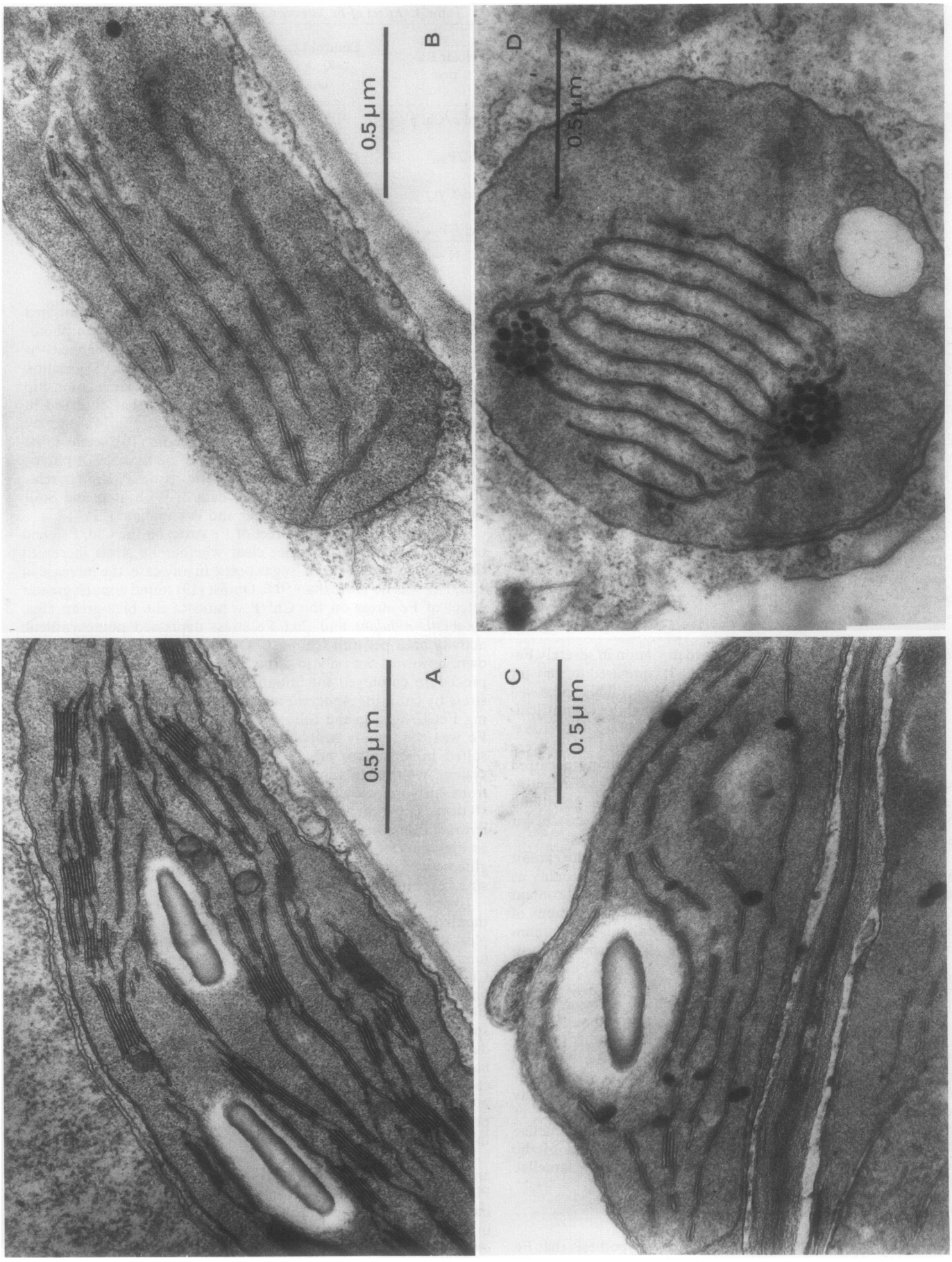

FIG. 3. Effect of Fe stress on chloroplast structure. A: chloroplast from an Fe-sufficient control leaf with a Chl content of >50 µg cm^{-2}; B: chloroplast from an Fe-stressed leaf with 15 µg Chl cm^{-2}; C: chloroplast from severely Fe-stressed leaf with 6.3 µg Chl cm^{-2}; D: chloroplast from leaf with less than 5 µg Chl cm^{-2}.

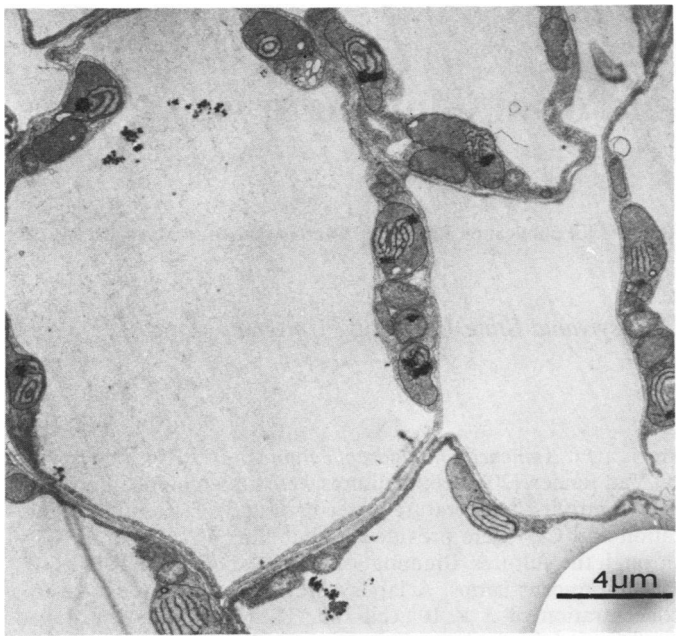

FIG. 4. Electron micrograph of cells from a severely Fe-stressed leaf with <5 μg Chl cm^{-2} showing that all of the chloroplasts have few lamellae.

The reduction in the number of photosynthetic units with Fe stress was accompanied by a decrease in the number of granal and stromal lamellae per chloroplast. Similar effects of Fe stress were observed in chloroplasts of maize (24, 28), tomato and spinach (28), tradescantia (15), and xanthium (7). Since Fe stress has a relatively small effect on chloroplast volume, chloroplast protein content, and RuBP carboxylase activity, and no effect on several leaf attributes including the number of cells or chloroplasts per unit area (25, 26), the chloroplast lamellae seem to be most affected by Fe stress. Another point of interest is the fact that Fe stress diminished the amount of grana stacking within the chloroplast while the Chl *a*/Chl *b* ratio remained low. These observations are in contrast to those of other investigators (17, 21) who have found that reduced grana stacking is associated with a high Chl a/b ratio.

Fe stress chlorosis exhibited some similarities to chlorosis in certain photosynthetic mutants of peanut (2), soybean, and cotton (1) in that there were fewer photosynthetic units per unit area and an increased Chl/P_{700} ratio. On the other hand, mutants of tobacco (18) and pea (13) had less Chl/area because of a reduction in Chl per photosynthetic unit. Fe stress differs from mutant-induced chloroses because it can be used to vary the number of photosynthetic units per unit leaf area progressively to less than 10% of the control. For this reason we believe it to be particularly suitable for use as an experimental tool for the control and study of photochemical capacity *in vivo*.

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