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THE RELATION BETWEEN IRON AND CHLOROPHYLL CONTENTS IN CHLOROTIC SUNFLOWER LEAVES[1]

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The advent of highly stable iron complexes which are useful in plant nutrition and agriculture (9, 16) has led to a renewed interest in the study of iron nutrition, particularly in lime-induced chlorosis. However, the investigator soon faces the problem of the comparative iron status of green and chlorotic leaves. Some investigators have found less iron in chlorotic than in green leaves and have demonstrated a good correlation between iron and chlorophyll contents (8, 15, 18). But also, it has been found that chlorotic leaves may contain as much or more iron than comparable green leaves (7, 11, 19). Brown and Hendricks (2) state that the concentration of iron in plant tissues has not been a satisfactory index of deficiency. Consequently chlorosis is frequently considered to involve more than a simple iron deficiency. Leeper (10) has summed up this view in the following statement with reference to lime-induced chlorosis. "Either the iron enters the plant in a less useful form than normal, or something goes wrong with the reactions of iron inside the plant growing on certain soils. The latter seems more likely."

Although Iljin (7) did not believe lime-induced chlorosis to be due simply to a deficiency of iron, he and others have pointed out that many if not all of the observed changes in the composition of chlorotic plants are consequences and not causes of chlorosis. De Kock and Hall (4) have examined the chlorosis occurring in variegated plants and that caused by virological or pathological causes and found the same high phosphorus-iron and low calcium-potassium ratios as is found in other forms of chlorosis. Probably the only major reason that simple iron deficiency has not been generally accepted as the cause of chlorosis is due to the continuing reports that as much or more iron may be present in chlorotic leaves as in comparable green leaves.

Some time ago, it was pointed out that surface contamination of leaves with iron might account for the reported lack of correlation between iron and chlorophyll contents (8) and it was suggested that adequate washing of leaves was of great importance in obtaining positive correlations. Since then, thorough washing has become more or less a general procedure but conflicting results are still being reported. Thus it would appear necessary to establish definitely whether iron chlorosis is caused directly by lack of iron or by more complex phenomena involving metabolic disarrangements.

In a study of the absorption and translocation of

[1] Received December 6, 1955.

ethylenediamine tetraacetic acid, Weinstein et al (19) reported that sunflowers grown at pH 7 with $FeSO_4$ as the source of iron gave rise to chlorotic leaves containing as much total iron as green leaves from plants grown under similar conditions at pH 5. This appeared to provide a means of readily obtaining chlorotic leaves with iron contents as high as in comparable green leaves. Experiments were therefore undertaken to see if a stock of material containing "non-utilizable" iron could be obtained.

Materials and Methods

Initial experiments were carried out closely following the procedure of Weinstein et al. In these experiments, sunflower (*Helianthus annuus* L.) was the test plant. The seeds were germinated in sand and seven days later the seedlings were transplanted into aerated nutrient solutions. The plants were harvested about 4 weeks after transplanting. The composition of the nutrient solutions used for the first two experiments was just as described by Weinstein et al. Hoagland nutrient solution (0.005 M KNO_3, 0.005 M $Ca(NO_3)_2$, 0.002 M $MgSO_4$, 0.001 M KH_2PO_4, 0.5 ppm Mn, 0.5 ppm B, 0.05 ppm Zn, 0.02 ppm Cu and 0.01 ppm Mo) was used in the later experiments. Reagent grade chemicals were used to prepare stock solutions for the first experiment. In subsequent experiments, chemicals especially purified by the method of Stout and Arnon (17) were used. Distilled water was used for all nutrient solutions. In addition to the usual micronutrients, 3 ppm Cl as KCl was added since the recent work of Broyer et al (3) indicates that this element is probably essential. Surfaces in contact with the nutrient solution were painted with asphaltum varnish or Amercoat paint (Amercoat Corporation).

After harvesting, the fresh material was separated into various plant parts and each fraction weighed. The leaf samples which consisted of several leaves of similar age and appearance were carefully washed in a 2 % solution of Dreft detergent and rinsed with distilled water. The washed samples were dried at 65° C in an oven with forced ventilation. After drying, the material was weighed and ground using a glass mortar and pestle. Contact with iron or iron containing substances was avoided at all times. In those cases where chlorophyll was to be determined, samples were removed prior to the washing and the chlorophyll determined within a short period of time.

Total iron was determined colorimetrically by the ortho-phenanthroline method after ashing a suitable amount of the ground material with redistilled concentrated H_2SO_4 and iron free 30 % H_2O_2 (8).

Samples (about 2 gm) for chlorophyll determination, obtained from the fresh unwashed material were weighed and ground with washed sand using a pinch of $CaCO_3$ and a few ml of acetone. The chlorophyll was extracted with 80 % (v/v) acetone-water and the absorption of the resulting solution measured at 663 and 645 mμ with a Beckman Model B spectrophotometer. Concentrations were calculated from this data and the values for the specific absorption coefficients

Table I

Iron Contents of Leaves from Sunflower Plants Grown at pH 5 and pH 7. Solutions Changed Every 2nd Day and 0.5 ppm Iron Supplied as $FeSO_4$

pH	Plant no.	ppm total Fe			Description
		Young	Recently matured	Old	
5	1	51.9	61.0	72.3	Very slightly chlorotic
	2	62.8	65.3	75.8	" " "
	3	68.8	66.3	70.4	" " "
5	1	70.4	78.6	78.4	Very slightly chlorotic
	2	57.8	59.5	68.1	Slight chlorosis
	3	81.0	74.2	73.1	Trace of chlorosis
5	1	40.3	44.7	60.3	Slight chlorosis
	2	49.8	46.8	58.9	Young leaves slightly chlorotic, old leaves green
	3	49.6	50.7	61.2	" " " " " " "
7	1	26.8	27.9	29.9	Moderate chlorosis, veins green
	2	26.3	26.0	33.7	Moderate chlorosis
	3	29.2	31.8	40.3	Moderate chlorosis, veins green
7	1	24.3	32.3	33.0	Moderate chlorosis
	2	29.4	32.7	33.5	" "
	3	24.5	33.6	36.3	" "
7	1	20.9	23.6	27.1	Severe chlorosis, some green veins
	2	27.1	28.4	37.2	" " " " "
	3	37.7	41.5	52.0	Slight to moderate chlorosis

Av. dry wt of entire plant = 14.5 gm for pH 5 set and 8.9 gm for pH 7 set.
Av. Fe content of stems = 15 ppm for pH 5 set and 7 ppm for pH 7 set.
Av. Fe content of petioles = 13 ppm for pH 5 set and 8 ppm for pH 7 set.
Av. Fe content of roots = 3000 ppm for pH 5 set and 1200 ppm for pH 7 set.

of chlorophyll a and b published by Mackinney (12). All data in the tables are expressed on a dry weight basis.

Results

Constant Iron Supply: For the first experiment, three 15-l crocks containing 3 sunflower plants each were maintained at pH 5 and 3 similar crocks were maintained at pH 7. The solutions were renewed every other day. 0.5 ppm Fe as $FeSO_4$ was added each time the solutions were renewed. An attempt was made to keep the pH constant by frequent adjustment between renewals. The seedlings were transplanted into the solutions on August 22 and the plants harvested 26 days later. The harvested leaves were classified according to age and degree of chlorosis. The data are given in table I.

Even the plants grown at pH 5 were not completely green although nearly so. Evidently climatic and cultural conditions were such that 0.5 ppm Fe was not quite sufficient. Nevertheless, the data of table I clearly show a correlation between iron content and degree of chlorosis. As a group, the leaves from plants grown at pH 5 were only slightly chlorotic and contained approximately 40 to 80 ppm Fe. The leaves of plants grown at pH 7 were moderately to severely chlorotic and contained about 20 to 40 ppm Fe. The only exception was the sample of old leaves from plant no. 3 listed at the bottom of the table. These leaves contained 52 ppm Fe but these plants were the least chlorotic of the pH 7 group. Analyses of other plant parts gave a similar picture. The iron values for the roots are probably not very meaningful because of the difficulty of removing precipitated iron from the root surface.

The results of this experiment differed so drastically from those reported by Weinstein et al (19) that a similar experiment was performed. This second experiment was carried out just as the first experiment except especially purified reagents were used in making up stock solutions. Rather than classify leaves according to appearance, the chlorophyll contents were determined. The seedlings were transplanted March 8 and the plants harvested 24 days later. The data of this experiment are summarized in table II. At this time of the year, better growth was obtained than in the previous experiment. The yields were considerably higher and not too dissimilar from those obtained by Weinstein et al (19).

As can be seen from the data of table II, there is a very good correlation between the amounts of total iron and chlorophyll with the exception of one sample of old leaves.

A comparison of the results of the above two experiments with those of Weinstein et al (19) emphasizes the problem previously stated; namely, some investigators find a correlation between iron and chlorophyll contents and others find no correlation whatever.

Fluctuating Iron Supply: In the following experiments, the iron-chlorophyll relationships were examined as a function of the variability of the rate of iron supply to the plant root. On April 8, sunflower seedlings were transplanted to complete Hoagland nutrient solution containing 0.1 ppm Fe as ferric sodium ethylenediamine tetraacetate (Fe EDTA). Subsequently on April 21, the plants were transferred to 15-l crocks, 3 plants to a crock. The Hoagland nutrient solution used in these crocks was prepared from especially purified stock solutions and no iron was added. These solutions were not changed during the remainder of the experiment. By May 6, chlorotic symptoms were well developed. On this date, leaf samples were taken for chlorophyll and iron analyses from one group of plants and 10 ppm Fe as Fe EDTA were added to the nutrient solution. One week later, leaf samples were again taken from this treated group of plants. Another group of plants was permitted to remain in the iron free solution until May 11, initial samples taken and 10 ppm Fe as Fe EDTA added. Samples were again taken one week later.

Table II

Iron and Chlorophyll Contents of Leaves from Sunflower Plants Grown at pH 5 and pH 7. Solutions Changed Every 2nd Day and 0.5 ppm Iron Supplied as $FeSO_4$

pH	Age of leaf	Ppm Fe	% Chlorophyll
5	R.m.*	88.2	0.827
	Old	101.0	0.678
5	R.m.	84.9	0.684
	Old	94.5	0.793
7	R.m.	55.5	0.538
	Old	59.0	0.570
7	R.m.	52.8	0.406
	Old	51.2	0.503
7	R.m.	40.6	0.288
	Old	38.7	0.377
7	R.m.	35.2	0.241
	Old	41.1	0.272

* R.m. = Recently matured.
Av. dry wt of entire plant = 24.0 gm for pH 5 set and 16.4 gm for pH 7 set.

The data for these experiments are presented in table III. The left side of the table gives the chlorophyll and iron data before treatment with iron and the right side gives the data one week after the addition of iron to the nutrient solution. The samples are arranged on the basis of chlorophyll content, i.e., the first sample on the right side of the table did not necessarily come from the same plant which furnished the first sample on the left side. In this experiment, both the initial and final samples consisted of matured and recently matured leaves.

An examination of the left side of the table shows that a reasonably good correlation between chlorophyll and iron contents was obtained by omitting iron from the nutrient solution. A comparison of this data with that from table II indicates that growth

TABLE III

THE EFFECT OF A FIFTEEN AND TWENTY DAY INTERRUPTION OF THE IRON SUPPLY ON THE IRON AND CHLOROPHYLL CONTENTS OF MATURED AND RECENTLY MATURED SUNFLOWER LEAVES. IRON SUPPLY RESUMED AT 10 PPM LEVEL WITH Fe EDTA

INTERRUPTION OF IRON SUPPLY	BEFORE ADDITION OF Fe		ONE WEEK AFTER ADDITION OF Fe	
	PPM Fe	% CHLOROPHYLL	PPM Fe	% CHLOROPHYLL
15-Day	14.8	0.0088	835	0.060
	19.5	0.0835	389	0.185
	21.4	0.165	490	0.192
	27.8	0.343	258	0.412
	27.8	0.432	442	0.458
	37.7	0.585	267	0.849
20-Day	18.1	0.0381	297	0.214
	22.6	0.152	543	0.450
	25.4	0.204	318	0.458
	24.2	0.252	525	0.651
	24.3	0.283	442	0.744
	34.4	0.486	359	0.722 *
	...	....	462	0.651 **
	...	....	462	0.286 †

* Green basal portions of leaf sample.
** Midsections of above sample.
† Chlorotic apical sections of above sample.

conditions in this experiment were such that iron was utilized more efficiently, the chlorophyll to iron ratio being considerably higher. It is quite apparent that the addition of iron to the previously iron free nutrient solution completely destroys the correlation between chlorophyll and iron. Furthermore, abnormally high iron contents are found in the leaves of plants so treated. When several leaves which were green at the base and chlorotic at the apex were examined, the same lack of correlation and extremely high iron content existed over the whole leaf. Although the entire leaf accumulated considerable iron, only a portion of the leaf responded by increased chlorophyll production.

In another experiment, the iron supply to the roots was interrupted for varying periods and the response observed. Sunflower seedlings were transplanted into Hoagland solution containing 1 ppm Fe as Fe EDTA on July 7. On July 18, the plants were transferred to nineteen 15-l crocks containing purified Hoagland solution but no iron. Each crock held 5 plants. One ppm Fe as Fe EDTA was added on the same day to the first crock. On the next day, 1 ppm Fe was added to the second crock. Addition of iron was made to individual crocks at subsequent intervals as listed in table IV. In this way the iron supply was interrupted for periods of time ranging from 0 to 30 days. Since the chlorotic plants made relatively little growth, it was neither necessary nor desirable to change the solutions. During this period a small amount of iron contamination kept the plants growing although at a reduced rate and they became progressively more chlorotic.

Initial samples for iron and chlorophyll analyses were taken from the plants in each crock at the conclusion of the minus iron treatments just before the addition of iron. Final samples were taken one week after iron had been added to the solution. In table IV, values for iron and chlorophyll contents are given for the initial and final samples. The initial samples consisted of recently matured leaves and the final samples consisted of recently matured and of old leaves.

In a general way the higher the iron content, the higher the chlorophyll content of the initial samples. A closer correlation was not obtained possibly because of the erratic nature of the small amount of iron contamination present and the long time interval between taking the first and last samples. Even in a most general way, there was no relation between the iron and chlorophyll contents in the final samples. The recently matured leaves were all high in chlorophyll content and contained widely differing amounts of iron. The older leaves which would correspond to the recently matured leaves of the initial samples also contained varying amounts of iron. These older leaf samples had lesser amounts of chlorophyll than the final recently matured leaves indicating that they had not completely recovered from chlorosis. The abnormally high iron values, previously observed, appeared possibly after a 2-day interruption and certainly after a 3-day interruption of the iron supply. The iron contents reached a peak in those plants subjected to a 12- to 14-day minus iron treatment.

TABLE IV

THE EFFECT OF VARYING THE DURATION OF THE INTERRUPTION OF THE IRON SUPPLY ON THE IRON AND CHLOROPHYLL CONTENTS OF SUNFLOWER LEAVES. IRON SUPPLY RESUMED AT 1.0 PPM LEVEL WITH Fe EDTA

PRETREATMENT—DAYS IN −Fe SOLUTION	BEFORE ADDITION OF Fe RECENTLY MATURED LEAVES		ONE WEEK AFTER ADDITION OF Fe			
			RECENTLY MATURED LEAVES		OLD LEAVES	
	PPM Fe	% CHLOROPHYLL	PPM Fe	% CHLOROPHYLL	PPM Fe	% CHLOROPHYLL
0	56.9	0.792	77.0	0.855	...	....
1	60.3	0.696	77.6	1.00	...	....
2	64.5	1.00	93.8	0.893	...	....
3	49.0	0.704	132	1.03	...	....
4	45.1	0.648	294	1.03	582	0.891
5	55.9	0.740	240	1.10	...	....
6	44.2	0.660	214	1.07	381	0.969
8	34.0	0.523	259	0.797	344	0.571
10	33.7	0.593	265	0.986	247	0.670
12	42.0	0.761	544	1.01	639	0.507
14	35.8	0.587	620	1.13	482	0.757
16	27.8	0.355	227	1.00	234	0.702
18	30.9	0.486	198	1.16	431	0.516
20	45.0	0.573	140	1.12	136	0.642
22	32.5	0.475	332	0.975	344	0.580
24	23.6	0.357	265	1.13	227	0.389
26	25.3	0.260	299	0.858	223	0.678
28	38.0	0.661	288	0.812	201	0.576
30	31.3	0.438	250	1.05	151	0.604

Four weeks after the end of the minus iron treatment, the iron and chlorophyll contents of recently matured leaves from plants whose iron supply was interrupted for 2, 3 and 4 days were examined. The chlorophyll content of these leaves which had developed some time after the iron supply was resumed varied from 0.799 to 1.28 % and the iron content varied from 44.7 to 73.5 ppm. By this time, an essentially normal relationship between iron and chlorophyll contents had been reestablished.

In the above experiments, the accumulation of iron and the irreversibility of chlorosis occurred in plants growing in solutions in which Fe EDTA was used as the source of iron. A check experiment using $FeSO_4$ gave very similar results, indicating that these effects are related to iron per se and are not peculiar to the complex.

Discussion

A lack of relationship between iron and chlorophyll contents was demonstrated by Weinstein et al (19) using greenhouse grown sunflowers. However, in our hands, similar experiments gave rise to plants showing a good correlation between iron and chlorophyll contents. Evidently some factor of which neither we nor they were cognizant was involved.

Even though nutrient solutions were changed every other day in the first two experiments described here, quite large and rapid changes in the pH of the nutrient solutions were observed. The changes were caused by selective absorption of ions by the roots and were related to general growth factors. Changes in pH affect the availability of iron when supplied as $FeSO_4$. At high pH, rapid oxidation and precipitation occurs. At low pH, iron is brought into solution. This behavior would be particularly prominent in the pH 7 cultures. In our experiments, an attempt was made to minimize the pH changes by frequent adjustment between renewals of the nutrient solution. Even with adjustment, pH decreases of a unit or more were occasionally observed in the pH 7 cultures. Since Weinstein et al (19) apparently did not control pH between renewals, their pH 7 plants might well have been exposed to a fluctuating iron supply. This could produce results comparable to those obtained by deliberately interrupting the iron supply.

The data of tables I, II and the left side of table III are typical of the results consistently found in this laboratory in the study of iron nutrition. For any given set of plants, a reasonably smooth curve is obtained by plotting the chlorophyll content against the iron content (8). The efficiency of the iron, i.e., the slope of the curve, varies from one set to another and is influenced by climatic and other factors.

The above relationship has been observed when the rate of iron supply was uniform or nearly uniform. If the rate is interrupted drastically, then very different results are obtained. After the iron supply is resumed, it is found that the correlation between iron and chlorophyll no longer exists and abnormally large amounts of iron are present in the leaves. The large increase in iron content occurs in old leaves and in leaves developed immediately after the resumption of the iron supply. Later growth contains considerably smaller amounts of iron.

The increased iron content resulting from a temporary interruption of the iron supply is not confined to chlorotic tissue only. It is found in the green leaves and green parts of semi-chlorotic leaves. Analytical data and autoradiographs with radioiron show no detectable differences in the amount of accumulation in green and chlorotic tissues. Presumably, the roots temporarily acquire a high capacity for the absorption of iron after being subjected to a minus iron treatment. This behavior is somewhat comparable to the enhanced uptake of salts exhibited by excised low salt barley roots. The high iron content of the leaf is similar to the "luxury consumption" of nutrients observed in foliar diagnosis.

In addition to the increased iron accumulation another equally important factor which may destroy the correlation between iron and chlorophyll contents is the observation that chlorosis tends to be irreversible except possibly in very young leaves. Slight or mild chlorosis shows good recovery on the addition of iron but more severely chlorotic leaves are incapable of turning green or at best do so only partially (13). When plants having chlorotic leaves were placed in solutions containing iron, the iron content of the leaves increased considerably but the yellow leaves never became completely green again except in cases of very mild chlorosis.

The inability of severely chlorotic leaves to recover completely may best be explained on the basis that iron is essential, directly or indirectly, for the production of chlorophyll and a deficiency of iron irreversibly damages the producing mechanism. Iron deficiency has been reported to change the relative activities of some plant enzymes (2). In green leaves, a considerably larger proportion of the nitrogen is present as protein nitrogen than in the case of chlorotic leaves (6), due perhaps to a direct relation between iron and protein synthesis or to a decrease in protein synthesis brought about by a reduced rate of photosynthesis in chlorotic leaves. Good correlations have been found between iron and protein contents as well as between iron and chlorophyll contents (1). Since one-third to one-half of the leaf protein is contained in the chloroplasts (14), large differences in protein content should involve the chloroplasts. When green and chlorotic sugar beet leaves were compared, fewer and smaller chloroplasts were present in the cells of the chlorotic leaf. If replacement of the disintegrated chloroplasts does not occur or occurs to a limited extent, then the leaf would be unable to recover completely from chlorosis. It is interesting that nearly a hundred years ago, Gris concluded that chlorosis was characterized by an imperfect development of the chloroplast caused by lack of iron salts (5).

A deficiency of iron may therefore progressively impair the chlorophyll producing mechanism to the point that chlorophyll synthesis is limited not by the

amount of iron in the leaf but by the inability of the leaf to produce chlorophyll. According to this view, it is not the iron which becomes inactive but rather the leaf becomes inactive because of the preceding period of iron shortage. In sunflower, this point is readily reached, a few days of minus iron treatment sufficing. The chlorotic leaves of other plants may retain some ability to turn green for periods as long as two or three months. An indication of this was observed with chlorotic pear and peach leaves in the field.

Basically, the type of chlorosis studied here is initially caused by a simple deficiency of iron. The deficiency and resulting chlorosis modify the behavior of the plant in such way as to destroy the correlation between iron and chlorophyll contents provided the appropriate conditions are present. In sunflowers, chlorosis is characterized by an increased ability to accumulate iron in the leaves and probably the entire plant and by incomplete reversibility of the chlorosis. When the rate of iron supply fluctuates, these factors appear to be instrumental in obscuring the relation between iron and chlorophyll contents. With a reasonably uniform rate of iron supply, a good correlation is maintained. Although this work does not deal with lime induced chlorosis, it seems highly probable that the above statements may apply equally well in that case.

Summary

1. If iron is supplied at a uniform rate, then good correlations are obtained between iron and chlorophyll contents in sunflower leaves.

2. If sunflower plants undergo a preliminary period of iron deficiency, then no correlation between iron and chlorophyll contents are found when the iron supply subsequently becomes adequate.

3. A deficiency of iron causes the sunflower leaf to accumulate excessive amounts of iron upon restoration of the iron supply, probably because of an enhanced iron uptake by the low iron plant.

4. Chlorosis tends to be irreversible so chlorotic leaves may not turn completely green even when large amounts of iron enter the leaf. It is suggested that iron is involved in chloroplast formation via protein synthesis, directly or indirectly.

5. Chlorosis is initially caused by a simple deficiency of iron but under some conditions, subsequent correlations between iron and chlorophyll contents may be obscured because of effects related to the deficiency.

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