NUTRIENT INTERRELATIONS IN LIME-INDUCED CHLOROSIS

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Introduction

Chlorosis, or a low chlorophyll concentration in plants, may be brought about by many factors. Light or temperature conditions, hereditary factors, pathogens, and nutritional derangements are all recognized as causing such a condition.

Disturbances in iron nutrition are probably the most commonly recognized nutritional disorders causing chlorosis. There are various ways in which the iron nutrition of a plant may be affected so as to bring about a chlorotic condition. Four of the more commonly recognized types are: (1), true iron deficiency; (2), an upset in the phosphate/iron balance; (3), an upset in the manganese/iron balance; and (4), lime-induced chlorosis. The latter is probably the most widespread disorder of this type, and it assumes serious proportions in the more arid regions of this country where high concentrations of carbonates of calcium and magnesium are present in the soil. In some localities, lime-induced chlorosis is considered to be the primary limiting factor in the production of certain tree fruits. Present control practices are generally temporary and far from satisfactory. If a better understanding of the physiology of this disorder could be attained, more efficient methods of control might be developed.

The other iron disorders mentioned above are not known to be directly associated with lime-induced chlorosis. True iron deficiency occurs experimentally in water and sand cultures when the iron level of the nutrient solution is kept low. The iron content of such chlorotic plants, when expressed on an appropriate basis, is naturally lower than that of normal green plants. Since iron is so widely distributed in most soils and since the amounts of iron necessary for normal plant growth are relatively small, this simple type of chlorosis probably occurs infrequently under field conditions.

The phosphate/iron balance in relation to chlorosis has been discussed at length by OLSEN (**19**). He has shown that plants grown in water cultures relatively high in phosphates, with iron supplied as the ferric ion, became chlorotic even though the total iron content of the leaves of the chlorotic plants was as much as, or more than, the iron content of green leaves. The chlorotic plants absorbed more phosphates, and OLSEN concluded that iron was precipitated as phosphate in the leaf tissue, particularly along the vascular bundles. This chlorotic condition was overcome either by reducing the phosphate supply or by increasing the calcium. OLSEN also showed that in water cultures the pH of the solution was effective in inducing chlorosis through its effect on the absorption of phosphates. Plants grown in solutions of pH 6 to 7 absorbed more phosphates and were chlorotic, while plants in solutions of either higher or lower pH were green. When iron was supplied in slightly ionized form (ferric citrate), the plants,

regardless of the pH, did not absorb sufficient phosphates to become chlorotic. CLARK (**5**) has reported somewhat similar effects. It is conceivable that this type of chlorosis may possibly occur in the field where heavy applications of phosphates are made, although so far as the writers are aware, no cases of chlorosis have been definitely correlated with this cause.

In Hawaii, where soils are high in manganese, a chlorotic condition of plants occurs that can be overcome by the addition of iron. RIPPEL (**25**) and MARTIN (**15**) have shown that a high manganese level in water cultures will induce chlorosis and that this condition could be overcome by increasing the iron level of the solution. Since the iron content of normal and chlorotic plants was found to be approximately the same, RIPPEL concluded that manganese affected the iron metabolism within the tissues of the plant. More recently, further studies of this manganese/iron balance in plants have been reported (**27, 28, 29**). These have shown that if the manganese concentration is too high, a typical iron chlorosis is produced which can be controlled either by increasing the iron level of the nutrient or by reducing the manganese level. The absolute amounts of these elements (over a fairly wide range) are immaterial as long as the proper balance exists between them. They concluded that "high concentrations of soluble manganese in the tissues are invariably associated with low concentrations of soluble iron and vice versa. This suggests the oxidation of ferrous to ferric ions by active manganese, in the inactivation and precipitation of iron in the form of ferric organic complexes."

Lime-induced chlorosis was thought at one time to have been due to a high calcium carbonate content of the soil raising the pH of the soil to such an extent that iron was made unavailable to the plant. When it was discovered that the iron content of normal and chlorotic plants was essentially the same (**1, 16, 17, 21, 32**), this hypothesis was no longer tenable. Since chlorotic leaves turned green when they were sprayed with iron compounds, the only possible conclusion seemed to be that iron in the chlorotic plant was present in an inactive form. OSERKOWSKY (**21**) first explored this avenue of approach. He found that during the early part of the growing season, the iron content of the 1 N HCl extract of pear leaves was in proportion to the chlorophyll content of the leaves. He concluded that a part of the iron in this extract was the "active" iron in chlorophyll formation. Since iron is not known to be part of the chlorophyll molecule, it is assumed that iron acts in a catalytic manner in chlorophyll formation, presumably as part of an enzyme. Thus the "active" iron of OSERKOWSKY might correspond to the iron in the enzyme that is responsible for chlorophyll formation.

It is the purpose of this paper to discuss some of the complex nutrient interrelations of lime-induced chlorosis and their effect on the iron metabolism of the pear and of certain other plants. While this work is only exploratory in nature, it is hoped that it may lead to a better understanding of the physiology of this disorder.

Methods

Analytical procedures

Surface contamination of leaf samples with dust is a major source of error in iron determinations and it was necessary to wash all samples. This was accomplished by swabbing each leaf with moist cotton followed by rinsing in distilled water. The samples were subsequently dried in a vacuum oven at 80° C.

One-gram samples were weighed out and ashed in a muffle furnace at about 450° C. After cooling, the ash was moistened with a small amount of water and then dissolved with 5 ml. of 6 N nitric acid. The mixture was brought to a boil to oxidize any ferrous iron to the ferric form. On cooling, the solution was filtered through #40 Whatman paper which had previously been washed with 6 N nitric acid and water. The filtrate was collected in a 50-ml. glass-stoppered volumetric flask and made to volume with water. When magnesium and phosphorus were to be determined on the same sample, it was necessary to add some alkaline material which did not contain magnesium, but which prevented the volatilization of phosphorus. A mixture of 4 grams of sodium nitrate and 1 gram of sodium hydroxide dissolved in 100 ml. of water was found to be satisfactory for this purpose. Five ml. of this solution was used per gram of material and the mixture was evaporated before ashing. If, after several hours' ashing, carbonaceous material was still present, the sample was cooled, moistened with a small amount of water, and 1 ml. of 6 N nitric acid added. After drying, the sample was re-ashed.

All colorimetric determinations were made with a photo-electric colorimeter of the test-tube type, using uniform tubes calibrated at 5 and 10 ml.

Iron was determined in the dissolved ash by a slight modification of the A.O.A.C. colorimetric method (**2**). A 5-ml. aliquot of the dissolved ash was transferred to a colorimeter tube and 0.5 ml. of 20 per cent. KCNS added. The tube was shaken and a reading taken immediately in the colorimeter using a green filter (Wratten #54). One division on the colorimeter scale was equivalent to about 0.1 microgram of iron per 5 ml. of test solution and Beer's law was followed to a reading of about 400. In all iron determinations special care was taken to prevent contamination of the sample with iron. The solutions were never allowed to come in contact with cork or rubber and all leaf samples were thoroughly washed before being dried or frozen. A frequent blank reading on all reagents was taken.

Calcium was determined by a modified volumetric micro-method (**12**). A 5-ml. aliquot of the dissolved ash was transferred to a wide-mouth 250-ml. Erlenmeyer flask. Five ml. of a saturated solution of ammonium oxalate was added, followed by 5 ml. of a saturated solution of sodium acetate. After the solution had stood overnight, the supernatant liquid was removed by means of a sintered-glass filter-stick with a layer of asbestos over the tip. The precipitate was thoroughly washed with a saturated solution of calcium oxalate made alkaline with a few drops of ammonium hydroxide. The pad and the precipitate were washed into a flask with distilled water. One ml.

of 6 N sulphuric acid was added and the solution was boiled to dissolve the calcium oxalate. After cooling, an excess of 0.01 N ceric sulphate was added and the solution was titrated with 0.01 N ferrous ammonium sulphate, using o-phenanthroline for an indicator.

Potassium was determined by a modified micro-volumetric method in the initial work, but this procedure was later discarded for a more rapid turbidimetric method, which has been described elsewhere (**11**). In the volumetric method a 5-ml. aliquot of the dissolved ash was transferred to a 250-ml. wide-mouth Erlenmeyer flask. One ml. of 6 N hydrochloric acid was added, followed by 2.5 ml. of glycerine and 8 ml. of KRAMER and TISDALL'S cobaltinitrite reagent (**10**). The mixture was allowed to stand overnight and the supernatant liquid was removed from the precipitate by means of a sintered-glass filter-stick with a layer of asbestos and celite over the tip. The precipitate and flask were washed at least 3 times with 70 per cent. alcohol and the wash liquid was continually removed with the filter-stick. The precipitate and pad of asbestos and celite were washed into the flask with distilled water and brought to a volume of about 30 ml. The mixture was boiled until the precipitate dissolved. After cooling, an excess of 0.01 N ceric sulphate was added and the solution titrated with 0.01 N ferrous ammonium sulphate using o-phenanthroline as an indicator.

Magnesium and phosphorus were determined by colorimetric methods that have been described elsewhere (**11**).

Manganese was determined by a slightly modified colorimetric A.O.A.C. method (**2**). A 5-ml. aliquot of the dissolved ash was transferred to a colorimeter tube. About 0.1 gm. of KIO_4 was added and the tube heated in a boiling water bath until maximum color had developed (at least 30 minutes). After the solution had been cooled and made back to the original volume with water, a reading was taken on the colorimeter using a green filter (Wratten #54). One division on the colorimeter scale was equivalent to about 0.25 microgram of manganese per 5 ml. of test solution. BEER'S law was followed up to a reading of about 400.

Chlorophyll was determined on fresh or frozen material by a slightly modified procedure of PETERING, WOLMAN, and HIBBARD (**23**). A red filter was used in the colorimeter (Wratten #29, with two pieces of ground glass to reduce light intensity). An ether solution of the chlorophyll was transferred to a colorimeter tube which was stoppered to prevent evaporation. The colorimeter was standardized against 5 X chlorophyll obtained from American Chlorophyll, Incorporated. Since BEER'S law was not followed exactly, a standard curve had to be prepared. In the lower ranges, 1 division on the colorimeter scale was equivalent to about 0.7 microgram of chlorophyll per 5 ml. of test solution.

All analyses reported in this paper are the average of duplicate or triplicate determinations.

FRACTIONATION PROCEDURES

Leaf samples of 100 to 200 sq. cm. in area (1 to 2 gm. dry wt.) were extracted with various solvents in the following manner:

The area of the leaves was obtained by tracing their outlines on paper and subsequently measuring the area with a planimeter. The leaf samples were then carefully washed to remove all adhering dust, placed in clean cellophane sacks, and frozen. Each sample, after it had been thawed, was ground in an appropriate-sized porcelain mortar. About 30 ml. of 50 per cent. acetone was added and the extract decanted through a piece of Whatman #41H filter paper on a 7-cm. Buchner funnel. The sample was further extracted several times with 50 per cent. acetone until a total of 100 ml. had been used. The grinding and extraction were continued, with pure acetone as a solvent, until the leaf tissue was reduced to a fine white or brownish powder. A total of 100 ml. of pure acetone was used in this later extraction. The filtrate was transferred to a separatory funnel and about 70 ml. of ether was added. The solution was mixed and allowed to stand several minutes to permit the ether layer to separate. The water layer was transferred to a 250-ml. beaker. The ether layer, containing the chlorophyll, was washed several times with water, and each of the washings was added to the water fraction in the beaker. The ether fraction was then transferred to a glass-stoppered graduated cylinder and made to 100-ml. volume with ether. Chlorophyll was determined on this fraction by the method previously indicated. In the text the ether extract of the water-acetone fraction is designated as the "ether" fraction.

The leaf residue from this extraction was treated with about 50 ml. of 0.1 N HCl and transferred to the Buchner funnel along with washings of 0.1 N HCl. The extraction was continued until 150 ml. of HCl had been used. The filtrate was transferred to a 250-ml. beaker and the residue was successively extracted in the same manner with 0.5 N HCl and then with 1 N HCl. The final residue, including the filter paper, was then transferred along with a little wash water to a 250-ml. beaker. One ml. of the sodium hydroxide–sodium nitrate ashing reagent was added to each extract and 5 ml. to the residue. All extracts were evaporated to dryness, and ashed at 450° C. in a muffle furnace as indicated previously.

In some cases (particularly with pear leaves) filtration was so extremely slow that it was necessary to separate the extract and residue by centrifuging. Both methods gave similar results.

By extracting first with 50 per cent. and then 100 per cent. acetone, it was found that a more rapid extraction was possible than by making a water extract, and the value of the water extract was still retained by separating the fat-soluble material with ether.

OSERKOWSKY'S extraction procedure (**21**) differed considerably from that given above. He used a larger initial sample (5 gm.) and extracted it with about 50 ml. of 1 N HCl by shaking for a definite period of time. After centrifuging, he washed the residue with an additional 50 ml. of acid.

In comparing OSERKOWSKY'S method with the one given here, it was found that fresh or frozen samples could be used as well as dry samples, but when cholorphyll was to be determined, it was necessary to use only fresh

or frozen material. Furthermore, repeated tests showed that practically all soluble material was removed with the first 50 ml. of solvent when a small (1 to 2 gm. dry wt.) sample was used. By using 150 ml. of solvent with this size of sample, a fairly complete extraction was assured.

Results

In preliminary work, an attempt was made to find by electrodialysis the iron fraction in leaves that was active in chlorophyll formation. OSERKOWSKY's results (**21**) indicated that only a small part of the iron in the leaf was active in chlorophyll formation, and that in leaves affected with lime-induced chlorosis, the iron was in an inactive form.

Fresh and frozen samples were suspended in distilled water between platinum electrodes 3 sq. cm. in area and about 3.5 cm. apart. Varying

TABLE I

IRON EXTRACTED FROM NORMAL AND CHLOROTIC BARTLETT PEAR LEAVES BY ELECTRODIALYSIS AT 180 VOLTS FOR 2 HOURS. ELECTRODES 3 SQ. CM. IN AREA AND 3.5 CM. APART. SAMPLES COLLECTED ON 7/16/40

SAMPLE	FRACTION	IRON PER 100 SQ. CM. OF LEAF AREA
		γ
Green leaves from chlorotic Bartlett tree	Dialysate	3
	Residue	84
	Total	87
Chlorotic leaves from same tree as above	Dialysate	5
	Residue	47
	Total	52
Leaves from chlorotic Bartlett pear tree that turned green after iron citrate was injected into the tree on 5/21/40	Dialysate	17
	Residue	156
	Total	173

voltages were tried for various lengths of time, but in no case could the amount of iron removed be correlated with the chlorophyll content of the leaves. Differences between fresh and frozen samples were usually not very great although more iron was commonly extracted from frozen samples. Table I shows some data typical of those obtained.

It was concluded from this work that the dialyzable iron was not correlated with lime-induced chlorosis, and that the iron active in chlorophyll formation was probably present in some complex insoluble form. An attempt was then made to extract the "active" iron from the leaf by some means other than that used by OSERKOWSKY (**21**). Green and chlorotic leaves were extracted with water, alcohol, acetone, 1 N acetic acid, and 1 N ammonium hydroxide, but the iron content of these extracts could not be correlated with chlorosis.

CALCIUM/POTASSIUM RELATIONS IN LIME-INDUCED CHLOROSIS

In order to determine whether some of the more common elements other than iron might be correlated with lime-induced chlorosis, green and chlo-

TABLE II

IRON, CALCIUM, AND POTASSIUM CONTENT* OF DELICIOUS APPLE LEAVES IN RELATION TO CHLOROSIS. SAMPLES TAKEN FROM PARTIALLY CHLOROTIC TREE GROWING IN CALCAREOUS SOIL ON 8/2/40

SAMPLE	Fe	Ca	K
	p.p.m.	%	%
Green leaves	188	1.90	1.13
Slightly chlorotic	205	2.36	2.71
Moderately chlorotic	173	1.78	4.49
Severely chlorotic	159	1.37	5.18

* Calculated on dry weight basis.

rotic leaves were analyzed for total nitrogen, phosphorus, calcium, potassium, and iron. There seemed to be no direct relation between the phosphorus and the nitrogen content and the degree of chlorosis. However, a striking degree of correlation between the amount of chlorosis and the potassium content was found (tables II, III).

A number of similar determinations were made on different kinds of plants and some typical results are presented in table IV. There was no consistent correlation between the total iron content and the degree of chlorosis but chlorotic leaves were always found to be considerably higher in potassium.

The higher calcium/potassium ratio in green than in chlorotic leaves raised the question whether the relationship between calcium and potassium in chlorotic leaves was modified when the leaves became green following iron citrate injection into the tree. Two severely chlorotic Bartlett pear trees growing on calcareous soil, one limb on each of which had been injected with iron citrate on May 21, 1940, furnished material for this purpose. The leaves on the treated limb on one tree became fully green, while on the other tree the leaves became only partially green. The results of the analyses are shown in table V. The injection of iron citrate did not reduce the potassium content of the initially chlorotic leaves to that of the normal green leaves, but the calcium content was greatly increased and, as a result, the calcium/potassium relation was restored to nearly that of normal green leaves.

TABLE III

IRON, CALCIUM, POTASSIUM, AND CHLOROPHYLL CONTENT OF BARTLETT PEAR LEAVES IN RELATION TO CHLOROSIS. SAMPLES TAKEN FROM PARTIALLY CHLOROTIC TREE GROWING IN CALCAREOUS SOIL ON 8/27/40

SAMPLE	Fe*	Ca*	K*	CHLOROPHYLL†
	p.p.m.	%	%	%
Green leaves	158	2.54	1.11	0.31
Slightly chlorotic	148	2.79	1.15	0.20
Moderately chlorotic	79	2.53	2.47	0.07
Severely chlorotic	43	2.97	3.55	0.03

* Calculated on dry weight basis.
† Calculated on fresh weight basis.

TABLE IV

IRON, CALCIUM, AND POTASSIUM CONTENT* OF PAIRED SAMPLES OF GREEN AND CHLOROTIC LEAVES FROM PARTIALLY CHLOROTIC PLANTS GROWING IN CALCAREOUS SOIL

PLANT	DATE	SAMPLE	Fe	Ca	K	Ca/K
			p.p.m.	%	%	
Bartlett pear	7/16/40	Green	87	2.43	0.54	4.50
		Severely chlorotic	52	1.13	1.90	0.59
Riland apricot	8/22/40	Green	86	1.70	3.20	0.53
		Severely chlorotic	326	1.86	5.05	0.37
Wild rose	8/27/40	Slightly chlorotic	155	1.53	1.54	0.99
		Severely chlorotic	318	1.87	2.29	0.82
Delicious apple	8/27/40	Green	183	1.22	1.51	0.81
		Severely chlorotic	83	0.89	2.89	0.31
Delicious apple	8/28/40	Green	40	1.02	1.98	0.52
		Moderately chlorotic	29	1.36	2.77	0.41
Slappy peach	8/31/40	Green	98	2.21	1.46	1.51
		Severely chlorotic	83	1.42	2.52	0.56
Bing cherry	9/27/40	Green	66	4.04	1.57	2.57
		Severely chlorotic	68	3.92	2.99	1.31

* Calculated on dry weight basis.

It is not known whether the calcium/potassium relations are the cause or the result of lime-induced chlorosis, but there seems to be a consistent correlation. The high level of potassium in chlorotic leaves is probably responsible for the burning that is often observed when chlorosis is severe.

LEAF FRACTIONATIONS IN LIME-INDUCED CHLOROSIS

In order to clarify these nutrient interrelations, an attempt was made to separate the various chemical elements into different fractions by successively extracting green and chlorotic leaves with a series of solvents. By means of this procedure it was hoped that some idea might be obtained as to the approximate types of compounds of the various elements that are present in both green and chlorotic leaves.

TABLE V

IRON, CALCIUM, AND POTASSIUM CONTENT* OF PAIRED SAMPLES OF BARTLETT PEAR LEAVES AS AFFECTED BY INJECTING IRON CITRATE INTO THE TREE. TREES INJECTED 5/21/40

SAMPLE	DATE	Fe	Ca	K	Ca/K
		p.p.m.	%	%	
Tree no. 1					
Chlorotic leaves from untreated limb	7/16/40	28	1.88	2.35	0.80
Leaves fully greened by injection	7/16/40	173	3.02	2.10	1.44
Tree no. 2					
Chlorotic leaves from untreated limb	7/25/40	31	1.51	1.85	0.82
Leaves partially greened by injection	7/25/40	72	3.22	2.03	1.59
Average normal pear leaves for comparison	7/25/40	95	2.64	.99	2.67

* Calculated on dry weight basis.

TABLE VI

IRON FRACTIONS IN PAIRED SAMPLES OF NORMAL AND CHLOROTIC BARTLETT PEAR LEAVES TAKEN FROM THE SAME TREE IN JUNE AND AUGUST COMPARED WITH CHLOROPHYLL CONTENT

FRACTION	Fe IN MICROGRAMS PER 100 SQ. CM. OF LEAF AREA			
	6/23/41		8/12/41	
	GREEN	CHLOROTIC	GREEN	CHLOROTIC
	γ	γ	γ	γ
Water	4	3	4	21
"Ether"	1	1	2	2
0.1 N HCl	14	15	20	9
0.5 N HCl	15	7	24	11
1.0 N HCl	7	7	15	4
Insoluble	28	44	68	80
Total	69	77	133	127
Chlorophyll in mg. per 100 sq. cm.	1.72	0.21	5.10	0.40

Using the previously described extraction procedure, samples of green and chlorotic Bartlett pear leaves, collected in June and August, were analyzed for iron, calcium, potassium, phosphorus, manganese, and magnesium. The data on iron are summarized in table VI. The chlorotic samples were from a very severely chlorotic tree while the green samples were from a normal appearing adjacent tree, both growing in calcareous soil. In June, only the 0.5 N acid extract showed lower in iron for chlorotic leaves than for green ones, while the water-soluble fraction was much higher.

OSERKOWSKY (**21**) obtained more iron in his 1 N acid fraction of chlorotic leaves later in the season than he did from green leaves, while early in the season he obtained more iron in this fraction in the green leaves. This might have been due to the presence of more readily soluble iron in his samples during the latter part of the growing season.

TABLE VII

CALCIUM, POTASSIUM, MAGNESIUM, PHOSPHORUS, AND MANGANESE FRACTIONS* IN GREEN AND CHLOROTIC BARTLETT PEAR LEAVES IN AUGUST

FRACTION	Ca		K		Mg		P		Mn	
	G†	C‡	G	C	G	C	G	C	G	C
	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ
Water	2100	3600	5500	14200	2700	3500	270	420	16	28
"Ether"	100	0	100	400	100	0	40	50	0	0
0.1 N HCl	11100	7700	300	3100	3200	1100	280	200	27	19
0.5 N HCl	9700	6300	0	100	100	200	60	40	2	0
1.0 N HCl	3000	1200	0	0	0	0	30	30	0	3
Insoluble	800	100	0	700	700	0	540	500	0	0
Total	26800	18900	5900	18500	6800	4800	1220	1240	45	50

* Micrograms per 100 sq. cm.
† G = green.
‡ C = chlorotic.

TABLE VIII

IRON, CALCIUM, POTASSIUM, MAGNESIUM, PHOSPHORUS, AND MANGANESE FRACTIONS* IN CHLOROTIC BARTLETT PEAR LEAVES AND LEAVES FROM THE SAME TREE BUT ON THE SIDE THAT HAD GREENED AFTER SOIL TREATMENT WITH IRON SULPHATE

FRACTION	Fe		Ca		K		Mg		P		Mn	
	G†	C‡	G	C	G	C	G	C	G	C	G	C
	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ
Water	4	4	1450	1240	8600	13600	1470	2270	130	380	22	10
"Ether"	8	2	0	0	900	1100	60	0	70	40	0	0
0.1 N HCl	17	3	9140	5040	3400	7600	1610	640	170	430	66	21
0.5 N HCl	25	6	6270	7370	1000	0	160	80	70	370	0	0
1.0 N HCl	17	2	4610	430	0	0	0	0	40	80	0	0
Insoluble	23	18	1450	0	700	0	60	0	380	580	0	0
Total	94	35	22920	14080	14600	22300	3360	2990	860	1880	88	31

* Micrograms per 100 sq. cm.
† G = green.
‡ C = chlorotic.

From the data in table VI it might be assumed that the 0.5 N acid fraction contained the iron associated with chlorophyll formation. The total iron in this fraction, however, was not directly proportional to the chlorophyll content.

The water-soluble fraction in both green and chlorotic leaves was found to be low in iron, as was found to be the case by electrodialysis (table I). About half the total iron in the leaf was in a form insoluble in the solvents used.

In table VII the results of other analyses are presented. There was much more potassium in all fractions of the chlorotic leaves than in corresponding fractions of the green leaves, with the exception of the 1 N acid fraction which did not yield any potassium even from chlorotic leaves. The chlorotic condition increased the water-soluble form of all other elements. The chlorotic leaves again show a somewhat lower total calcium content than do the

TABLE IX

IRON, CALCIUM, POTASSIUM, MAGNESIUM, PHOSPHORUS, AND MANGANESE FRACTIONS* IN PREVIOUSLY CHLOROTIC BARTLETT PEAR LEAVES THAT WERE GREENED BY THE TREATMENT OF LEAVES LOWER DOWN ON THE SAME BRANCH WITH IRON CITRATE

FRACTION	Fe	Ca	K	Mg	P	Mn
	γ	γ	γ	γ	γ	γ
Water	6	2000	17700	2900	250	18
"Ether"	2	0	0	100	50	0
0.1 N HCl	40	8900	1900	1100	170	32
0.5 N HCl	55	8200	0	0	70	0
1.0 N HCl	24	5900	0	0	30	0
Insoluble	71	800	0	0	560	0
Total	198	25800	19600	4100	1130	50

* Micrograms per 100 sq. cm.

green leaves. Total magnesium, likewise, is somewhat lower in chlorotic leaves, while phosphorus and manganese seem to be essentially the same in both green and chlorotic leaves. It is of interest to note that manganese seems to be present in forms which are either water soluble or easily dissociated. It must form compounds in the plant which are quite different from those of iron.

In order to determine whether the 0.5 N acid fraction had increased in iron when chlorotic leaves had been greened by a soil treatment with iron sulphate, green and chlorotic leaf samples were taken from a Bartlett pear tree in August. One side of the tree had been given a soil treatment by trenching in iron sulphate in May. Only the treated side of the tree had become green, and thus it was possible to obtain both green and chlorotic samples from the same tree. The results of the analyses of these samples are shown in table VIII. In the green leaves all fractions, except the water-soluble fraction, were higher in iron than in the chlorotic leaves. The total amounts of calcium, magnesium, manganese, and iron were higher in the green leaves, while the total amounts of potassium and phosphorus were

lower. Here again the calcium/potassium relation appeared to be associated with chlorosis.

Somewhat similar effects were obtained when chlorotic leaves were greened by dipping them in solutions of iron salts. The solutions were strong enough to burn the leaves that were dipped, but enough iron was absorbed and translocated to other leaves on the same branch to cause them to turn green. In table IX are presented the results obtained from the analyses of leaves that had become green after leaves lower down on the same branch had been dipped in iron citrate solution. The dipping was performed on May 23, 1941, and leaf samples were taken on August 23, 1941. These data may be compared with the August samples in the two previous tables (VII, VIII). The chlorophyll content of this sample was 2.20 mg. per 100 sq. cm. Here again the calcium and the iron content were markedly greater than in chlorotic leaves. The 0.5 N acid fraction was relatively high in iron. The total potassium content, however, was not markedly lower.

Leaf fractionations in true iron deficiency

It became of interest to determine whether these nutrient relations would be found in the case of true iron deficiency as well as in lime-induced chlorosis. Apple seedlings were grown for three months in the laboratory in water culture with and without added iron. Leaves on the plants growing in the solutions from which the iron was withheld soon turned quite yellow. These yellow leaves, and comparable leaves from green plants, were fractionated as in the previous tests. Table X shows the results of these analyses. Since the chlorotic leaves were only about one-half the size of the green leaves, the results were expressed on a per leaf basis as well as on an area basis. The relative distribution of the elements in the various fractions should be considered here rather than the absolute amounts in these fractions. The area basis should probably not be used because of the difference in leaf size. In all previous samples the normal and chlorotic leaves were of about the same size.

As seen in table X, the calcium/potassium relations found to prevail in lime-induced chlorosis do not seem to hold for true iron deficiency. The iron is low in all the acid soluble fractions in the chlorotic leaves, including the 0.5 N acid fraction. It is also interesting to note that the water soluble iron is very low in the chlorotic leaves. The chlorophyll content was 79 micrograms per leaf in the yellow leaves and 362 micrograms in the green leaves. The differences in chlorophyll content are much greater than any differences in iron content.

Leaf fractionations in genetically yellow Spiraea

The question then arose as to whether genetically variegated leaves had the same nutrient relations as leaves affected with lime-induced chlorosis. A comparison was made of fully green and completely yellow Spiraea sp. leaves from the same plant. The data are shown in table XI. In the case

TABLE X

IRON, CALCIUM, POTASSIUM, MAGNESIUM, PHOSPHORUS, AND MANGANESE FRACTIONS IN GREEN AND CHLOROTIC LEAVES OF APPLE SEEDLINGS GROWING IN WATER CULTURES WITH AND WITHOUT ADDED IRON

FRACTION	Fe		Ca		K		Mg		P		Mn	
	G*	C†	G	C	G	C	G	C	G	C	G	C
	MICROGRAMS PER 100 SQ. CM.											
Water	20	1	670	920	8320	8410	610	1310	260	550	20	38
"Ether"	11	32	20	0	140	880	130	0	40	80	0	0
0.1 N HCl	22	20	1580	2730	1330	440	410	340	10	60	25	28
0.5 N HCl	11	10	310	0	380	880	0	0	0	20	0	0
1.0 N HCl	9	11	60	0	0	660	0	0	10	0	0	0
Insoluble	12	49	50	0	0	0	0	0	130	240	0	0
Total	85	123	2690	3650	10170	11270	1150	1650	450	950	45	66
	MICROGRAMS PER LEAF											
Water	2.7	0.1	91	57	1137	518	84	81	36	34	2.8	2.4
"Ether"	1.6	2.0	2	0	19	54	18	0	5	5	0.0	0.0
0.1 N HCl	3.0	1.2	217	169	182	27	56	21	1	4	3.4	1.5
0.5 N HCl	1.3	0.6	42	0	52	54	0	0	0	1	0.0	0.0
1.0 N HCl	1.2	0.7	9	0	0	40	0	0	1	0	0.0	0.0
Insoluble	1.7	3.0	7	0	0	0	0	0	17	15	0.0	0.0
Total	11.5	7.6	368	226	1390	693	158	102	60	59	6.2	3.9

* G = green (iron added).
† C = chlorotic (iron not added).

TABLE XI

IRON, CALCIUM, POTASSIUM, MAGNESIUM, PHOSPHORUS, AND MANGANESE FRACTIONS* OF GREEN AND GENETICALLY YELLOW SPIRAEA LEAVES

FRACTION	Fe		Ca		K		Mg		P		Mn	
	G†	Y‡	G	Y	G	Y	G	Y	G	Y	G	Y
	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ
Water	11	21	370	100	7490	11550	103	551	737	939	3	10
"Ether"	20	18	0	0	0	0	78	0	94	44	0	0
0.1 N HCl	18	16	1290	560	0	1520	514	388	43	44	11	10
0.5 N HCl	14	14	0	40	0	0	103	0	35	21	0	0
1.0 N HCl	14	15	0	20	0	0	0	0	43	33	0	0
Insoluble	28	45	0	70	0	0	154	0	343	248	0	0
Total	105	129	1660	790	7490	13070	952	939	1295	1329	14	20

* Micrograms per 100 sq. cm.
† G = green.
‡ Y = genetically yellow.

of lime-induced chlorosis, leaves which appeared almost white were found to contain small amounts of chlorophyll. The yellow Spiraea leaves, however, were completely devoid of chlorophyll, a fact which indicated some basic upset in chlorophyll manufacture. The green leaves had 3.85 mg. of chlorophyll per 100 sq. cm. There was less calcium in the yellow leaves but more potassium. This condition is much like that of lime-induced chlorosis. The 0.5 N acid fraction, however, was not low in iron in the yellow leaves, indicating that some factor other than iron is probably responsible for the lack of chlorophyll formation in these leaves.

Discussion

In the work reported here, no consistent correlation was found between the total iron content of the leaves and lime-induced chlorosis. MILAD (**16**), ALLYN (**1**), WALLACE (**32**), and OSERKOWSKY (**21**) have all reported similar results. Furthermore, there seemed to be no correlation between lime-induced chlorosis and either the phosphate/iron balance or the manganese/iron balance. It would thus appear that lime-induced chlorosis is not associated with these other types of iron chlorosis. In some cases, a somewhat lower iron content may be a contributing factor in lime-induced chlorosis, but other factors must also be in play. The data presented here may be criticized in that samples were usually taken during June, July, and August instead of earlier in the season when chloroplasts are thought to be more active. However, under our conditions solutions of iron salts applied to chlorotic foliage as late as mid-July will show a greening effect on the leaves by late August, indicating that the chloroplasts are still able to synthesize chlorophyll during this period.

Iron undoubtedly forms many different compounds within the tissues of the plant. Long ago, MACALLUM (**13**) demonstrated that there existed firmly combined iron in plant tissue, particularly associated with nuclei. More recently, REED and DUFRENOY (**24**) demonstrated that in orange leaves iron was mainly associated with the plastids and nuclei. NOACK and LIEBICH (**18**) reported that 82 per cent. of the total iron in spinach leaves was associated with the chloroplasts. They separated this iron into various fractions, and concluded that five-sixths of the firmly bound iron was absorbed on phosphorus-containing proteins. THEORELL (**30**) recently summarized the known iron porphyrin enzymes and listed them as: (a), Warburg's respiratory ferment; (b), the cytochromes—a, b, and c; (c), the catalases; and (d), the peroxidases. SAYRE (**26**) studied the iron deposits in corn stems and concluded that they were complex hydrous iron oxides combined with proteins.

These several investigations, in addition to the fractionation results reported in this paper, suggest that in the iron nutrition of a plant there is a competition between the various compounds in the plant for the available iron. There is probably a dynamic equilibrium in leaf tissue between such forms of iron as iron ions, iron salts of organic acids, iron-phosphate com-

plexes, iron-hydroxide complexes, iron-silica complexes, lipoidal iron, hematin iron, iron-nucleoproteins, and possibly the iron-containing "chlorophyll enzyme." In addition, there probably is an equilibrium between the two oxidation states of iron. When the nutrition of the plant is upset in any way, there could easily be a shift in the equilibrium between the various iron compounds.

Even though there seems to be many iron compounds in the plant, there are, as yet, only two known functions of iron. One of these functions is as a constituent of many enzymes that take part in the respiratory system. The other rôle appears to be as a catalyst in chlorophyll formation. This iron-containing "chlorophyll enzyme" seems to be one of the least stable of the iron compounds, at least its deficiency is one of the most readily observed results of an upset iron metabolism. It is quite probable that some or all of the other iron compounds play certain specific rôles in plant nutrition, but their functions have not as yet been ascertained.

One difficulty in working with an element, such as iron, that is present in very low concentrations in plant material is that the methods of analysis may not be sensitive enough to detect certain very important compounds containing the element. It is quite conceivable that an enzyme may be present in low enough concentration in plant material to allow for less than 1 p.p.m. of iron, for 1 p.p.m. of iron is about equal to 1×10^{16} molecules per gram of leaf tissue.

When iron was withheld from apple seedlings in water culture, the water-soluble fraction from the leaves of these plants was low in iron, a fact which would seem to indicate that substances in this fraction are not able to compete with those of other fractions for available iron. The 0.5 N acid fraction is also low under conditions of true iron deficiency, and it is also consistently low in the case of lime-induced chlorosis. This suggests that 0.5 N HCl removes the iron from the enzyme that is responsible for chlorophyll formation. The iron content of this fraction is not directly proportional to the chlorophyll content of the leaf. This might be expected, however, because iron other than that of the chlorophyll enzyme could very well be present in this fraction. Furthermore, the rate of enzyme action is not necessarily directly proportional to the amount of enzyme present; even though this fraction contained only that iron present in the chlorophyll enzyme, it would not necessarily follow that the iron content would be directly proportional to the chlorophyll content.

A high potassium content was also correlated with lime-induced chlorosis. It would appear that the high potassium content of chlorotic leaves caused the iron to be displaced from the enzyme responsible for chlorophyll formation, resulting in the inactivation of this enzyme. However, further proof is needed for this hypothesis. In this regard, WALSH and CLARKE (**33**) have recently reported a chlorosis of tomato plants associated with a luxury consumption of potassium.

The magnesium content was found to be low in the "ether" fraction of

chlorotic leaves, but this was probably due to the fact that the chlorophyll content was low rather than to a derangement in magnesium metabolism.

The calcium/potassium relations of lime-induced chlorosis have been pointed out before by other workers, but no great significance has been attached to them. As long ago as 1886, CHURCH (**4**) reported that chlorotic oak leaves were high in potassium and low in calcium. COLIN and GRANDSIRE (**6**) have shown the same relation in chlorotic leaves of chestnuts and elms and WALLACE and MANN (**31**) for apple. In later work, WALLACE (**32**) has shown the same relations for lime-induced chlorosis of apple, pear, plum, and raspberry. GRANDSIRE (**7**) has also reported a high potassium and low calcium content of albino leaves. The fact that genetic albinism induces an upset in calcium/potassium relations does not necessarily prove that these relations are not connected with the cause of lime-induced chlorosis. The work reported here, and the work of OLSEN (**19**), indicates that chlorosis brought about by true iron deficiency or by an upset in phosphate/iron balance is not associated with an upset in calcium/potassium relations. Thus leaves may be low in chlorophyll and still not accumulate large amounts of potassium. Furthermore, MCGEORGE (**14**) has shown that plants growing in calcareous soil pick up very large amounts of potassium. Thus it is conceivable that lime-induced chlorosis may be brought about in part by a high potassium level of the leaves. In any nutritional explanation of this type of chlorosis, it will be necessary to take into account the fact that only parts of a tree may be chlorotic and that even adjacent leaves may vary markedly in their degree of chlorosis.

CHAPMAN (**3**) and HAAS (**8**) have shown that the maintenance of a high moisture level in calcareous soils tended to increase the incidence of chlorosis, while a low moisture level tended to reduce it. According to their explanation, an increased water supply increased the hydrolysis of calcium carbonate and resulted in an increase of hydroxyl ion concentration. However, PARSCHE (**22**) has shown that calcium chloride, as well as calcium carbonate, may induce iron chlorosis of lupine, even though the calcium chloride did not affect the pH of the soil appreciably. Thus it is possible that soil pH plays a rôle in lime-induced chlorosis only insofar as it affects the calcium/potassium relations. This is further indicated in the work of HOFFER (**9**) and others who have shown that corn plants deficient in potassium accumulate large amounts of iron.

Lime-induced chlorosis is a complex disorder and needs more investigation before much light can be thrown on its physiology. Further experiments are in progress and it is hoped they will help elucidate some of the problems discussed here.

Summary

1. Lime-induced chlorosis of plants is distinguished from other types of iron chlorosis such as true iron deficiency, upset manganese/iron balance, and upset phosphate/iron balance.

2. Leaves affected with lime-induced chlorosis are high in potassium but

somewhat low in calcium and magnesium. It is not definitely established whether these nutrient relations are associated with the cause or are a result of chlorosis.

3. Data are presented on the distribution of Ca, K, Mg, P, Fe, and Mn in various fractions of green and chlorotic pear and apple leaves and of green and variegated Spiraea leaves. The potassium was found to be mostly water-soluble. Magnesium and manganese were either water-soluble or readily dissociated by 0.1 N HCl (except for the small amount of magnesium present in the ether fraction as part of the chlorophyll molecule). Calcium, phosphorus, and iron, on the other hand, were distributed more generally in all of the fractions.

4. Over half the iron in the leaves examined was present in a form insoluble in 1 N HCl; presumably as iron-hematin, iron-nucleoprotein, or in other complex organic compounds.

5. Neither the total iron, insoluble iron, nor the iron soluble in water, ether, alcohol, 1 N acetic acid, 1 N ammonium hydroxide, 0.1 N HCl, or 1 N HCl was found to be correlated with lime-induced chlorosis. Likewise, electro-dialyzable iron could not be correlated with chlorosis.

6. The iron extracted by 0.5 N HCl was found to be low in chlorotic leaves. The 0.5 N HCl probably removed the iron from an enzyme that plays a rôle in chlorophyll formation.

7. Lime-induced chlorosis is probably brought about by a complex of causes whose interrelations are not yet fully established. The data suggest that a relatively high potassium level induces chlorosis by replacing the iron on the enzyme responsible for chlorophyll formation, thereby inactivating the enzyme.

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