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THE EFFECT OF DIFFERENT IRON AND MANGANESE NUTRIENT LEVELS ON THE CATALASE AND CYTOCHROME OXIDASE ACTIVITIES OF GREEN AND ALBINO SUNFLOWER LEAF TISSUES[1]

L. H. WEINSTEIN AND W. R. ROBBINS
DEPARTMENT OF PLANT PHYSIOLOGY, RUTGERS UNIVERSITY, NEW BRUNSWICK, NEW JERSEY

From a physiological standpoint, it is difficult to assess separately the role of iron and manganese in plant nutrition because these elements have been shown to be mutually antagonistic. Shive et al (16, 20, 21) and Twyman (25, 26) have reported that a high nutrient level of manganese has a depressing effect on the absorption of iron from nutrient solutions and upon the maintenance of a high level of water-soluble iron in plant tissues, inducing symptoms of iron deficiency chlorosis. High nutrient levels of iron were likewise shown to have a depressing effect on the absorption of manganese from nutrient solutions and upon the maintenance of a high level of water-soluble manganese in plant tissues, although to a much lesser degree than the effect of manganese on iron. Oulette (14) found that the severity of manganese toxicity symptoms in soybeans decreased as the nutrient level of iron was increased in the substrate. Other instances of manganese-induced iron deficiency have been reported by Sideris and Young (18), Sideris (17) and Hewitt (8, 9, 10, 11) for several plants.

Sideris and Young (18) and Twyman (26) have suggested that manganese-induced iron deficiency in plants is a result of substitution of manganese for two hydrogen atoms or for iron in porphyrin molecules. In vitro substitution of iron by manganese in purified horseradish peroxidase with resulting feeble peroxidatic activity has been reported by Gjessing and Sum-

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ner (7). Theorell (23) reported that such a substitution eliminated all peroxidatic activity.

It has been reported that activities of the enzymes catalase and peroxidase are related to the presence in plant tissues of photoreceptive pigments such as chlorophyll (6) and flavines (5). Catalase seems to be involved in photosynthesis (6) and peroxidase in the light-activated indoleacetic acid oxidase system in peas (5).

To study the effect of different levels of iron and manganese nutrition on the activity of iron-containing enzymes of chlorophyll-deficient and chlorophyll-bearing tissues, albino as well as green plants were employed in the present studies.

Materials and Methods

In order to continue the active growth of chlorophyll-deficient leaf tissue during the experimental period albino scions were grafted into the stems of plants having green leaves. Green and albino seedlings of Russian sunflower (*Helianthus annuus* L.) seeds [2] which originated as a result of mutations induced by ultrasonic vibrations (29, 30), were used in these experiments. Green seedlings used for stock plants to which green and albino scions were grafted were siblings from an earlier generation of the originally treated seeds. Seeds for the green stock plants were sown on September 30, 1952, and seeds for the albino seedlings were sown 20 days later in white quartz sand. The normal seasonal daily sunlight period was extended to 16 hours with incandescent light. A minimum intensity of about 6 fc was provided.

Soon after the albino and green seedlings from the second sowing emerged from the sand, they were cleft-grafted, one per plant, into the apical portions of the stems of the older green sunflower stock plants. Both albino and green seedlings were grafted into the green stock plants in order to provide albino and green tissues which were produced under as similar conditions of growth as possible. During the period from November 5th to 13th, 55 albino and 34 green seedlings were grafted. Twenty-seven albino and 24 green grafts united successfully.

The grafted plants were placed in culture solutions on December 12th. The culture vessels were one-gallon wide-mouth glass jars, each containing two albino-grafted plants and one green-grafted plant. Composition of the nutrient solution used is given as follows: Macronutrient salts—0.001 M KH_2PO_4, 0.0045 M $Ca(NO_3)_2 \cdot 4\,H_2O$, 0.002 M $MgSO_4 \cdot 7\,H_2O$, 0.0015 M K_2SO_4, and 0.0013 M HNO_3; micronutrient elements—0.10 ppm Zn, 0.10 ppm B, 0.01 ppm Cu, and 0.01 ppm Mo. During the eight-day period prior to the initiation of the different iron and manganese nutrient levels, the concentrations of iron and manganese supplied was 0.20 ppm and 0.05 ppm, respectively, in all cultures. When the different nutrient treatments were started, concentrations of 0.001 ppm, 0.25 ppm, and 10.0 ppm were chosen as the low, adequate, and high levels of each element, respectively. Plants grown with a level of 0.25 ppm of both elements will be referred to as control plants. These nutrient levels together with the nutrient ratios of iron to manganese are given in table I. All nutrient salts were of reagent grade. Stock solutions of macronutrient salts were purified by the alkaline adsorption method of Stout and Arnon (22), followed by further purification with dithizone and carbon tetrachloride (15). All nutrient solutions were prepared with deionized water which had a specific resistance immediately after deionization of from 11 to 15 megohms.

Cultures receiving a nutrient level of either low iron, high manganese, or an adequate level of both elements were run in triplicate, and those receiving a nutrient level of high iron or of low manganese were run in duplicate. The nutrient solutions were renewed twice weekly by complete replacement.

Albino and green leaf tissues for catalase and cytochrome oxidase activity determinations were removed from the grafted scions at desired times after distinct leaf symptoms associated with different nutrient levels of iron and manganese became apparent. Activity determinations were carried out over a period of about 30 days. A final harvest of grafted scions was made on February 3, 1953. Aliquots of leaf tissue were dried in a forced-draft oven at 70° C for the

[2] Seeds were provided through the courtesy of Dr. Raymond H. Wallace, Department of Botany, University of Connecticut, Storrs, Connecticut.

Table I

Iron and Manganese Contents of Leaf Tissues of Green-grafted Scions of Russian Sunflower Plants Grown under Different Iron and Manganese Nutrient Levels

Nutrient level		Fe/Mn nutrient ratio	Tissue content *					
Fe	Mn		Water-sol. Fe	Water-insol. Fe	Total Fe	Water-sol. Mn	Water-insol. Mn	Total Mn
ppm	*ppm*		*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*
0.001	0.25	0.004	9.0	67.5	76.5	55.5	95.5	151.0
0.25	0.25	1.0	56.5	113.0	169.5	51.0	111.5	162.5
10.0	0.25	40.0	63.3	127.7	191.0	40.5	117.5	158.0
0.25	0.001	250.0	49.0	99.2	148.2	6.0	60.0	66.0
0.25	10.0	0.025	12.5	83.0	95.5	372.0	387.0	759.0

* Expressed on dry wt basis.

determination of total and percentage dry weights. Small aliquots of leaf tissue were freeze-dried for protein nitrogen determinations, and other aliquots were frozen for determination of water-soluble, water-insoluble, and total iron and total manganese contents.

Soluble iron and soluble manganese contents of frozen tissue samples were determined on the basis of sap expressed in an aluminum cylinder and piston under a pressure of 2000 lbs/in^2 applied by means of a Carver press (20). Digestion of samples and subsequent color development in the determination of iron and manganese were carried out according to the method of Toth et al (24).

Protein nitrogen was determined by extracting small samples of freeze-dried leaf tissues for 16 hours with a refluxing water-alcohol mixture (27). Extracted tissue was then wet-digested and the total insoluble nitrogen content was determined by the semi-micro Kjeldahl procedure.

Tissue samples were prepared for enzyme activity determinations by grinding one gram of young leaf blade tissue in a cold mortar and pestle with a small quantity of cold deionized water. Maceration was completed in a cold Ten Brock glass homogenizer. For cytochrome oxidase, the homogenate was then diluted 1 : 25 with ice-cold deionized water, and for catalase 1 : 100 with ice-cold 0.05 M phosphate buffer, pH 7.0. Just prior to use of the enzyme preparation for catalase activity measurements, an aliquot of the homogenate was diluted to an appropriate volume with the cold phosphate buffer.

Catalase activity was determined manometrically in a Warburg apparatus at 25° C in triplicate by a method similar to that described by Sizer (19). Each reaction vessel contained 2.0 ml of 0.10 M hydrogen peroxide made up in 0.05 M phosphate buffer, pH 7.0. The sidearm contained 1.0 ml of enzyme preparation. In each series of determinations, one reaction vessel received 1.0 ml of the phosphate buffer in the sidearm instead of enzyme; this reaction vessel served to measure the amount of spontaneous decomposition of hydrogen peroxide in the absence of catalase. Oxygen evolution was followed over a period of 5 minutes.

Cytochrome oxidase activity was determined spectrophotometrically by measuring the rate of oxidation of ferrocytochrome c at a wavelength of 550 mμ in a Beckman Model DU spectrophotometer (3, 28, 32). The reaction mixture was composed of 2.0 ml of 4.0×10^{-5} M ferrocytochrome c – 0.15 M phosphate buffer at pH 6.0, 0.9 ml of deionized water, and 0.10 ml of enzyme preparation, making a total volume of 3.0 ml. Each activity determination was carried out in triplicate.

Results

Growth Responses of Plants: Within 12 days after initiation of the different nutrient treatments, severe symptoms of iron-deficiency chlorosis were apparent on young green leaves of plants supplied with a low nutrient level of iron or with a high nutrient level of manganese. The striking similarity in leaf symptoms induced by these different treatments can be seen in figure 1. The only visual means of distinguishing between the symptoms of low iron and of high manganese on the plants as a whole was a brown color of the trichomes along the stems, petioles, and lower surfaces of older leaves of plants subjected to a high nutrient level of manganese. This symptom was not present on plants receiving a low nutrient level of iron or in any other nutrient treatment. Plants subjected to a high level of iron were somewhat stunted in growth as compared with the control plants. Leaves were small and dark green in color, and stems were spindling. Symptoms of manganese deficiency were evident on leaves of green plants grown with a low nutrient level of manganese as a yellowing of the youngest leaves and some interveinal yellow mottling of the older leaves. Control plants were large and well developed. There were no visual differences in the albino scions in the different nutrient treatments with the exception of the browning of

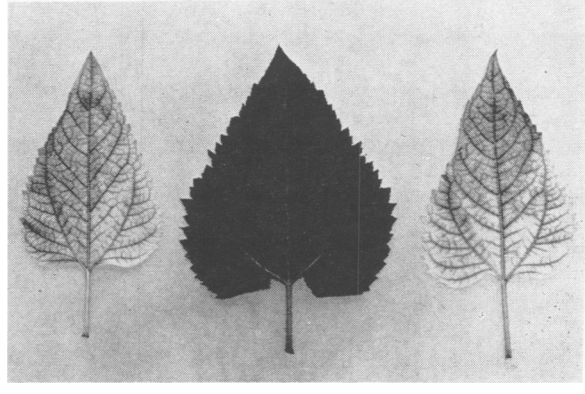

Fig. 1. Leaves of sunflower plants grown in culture solutions supplied with different iron and manganese nutrient levels. *Left.* Iron deficiency induced by a low nutrient level of iron. *Center.* Control. *Right.* Iron deficiency induced by a high nutrient level of manganese.

trichomes under conditions of high manganese already noted. Green scions attained heights of from 54 to 105 cm before harvesting, the average height being about 90 cm. Albino scions attained heights ranging from 9 to 50 cm with the majority averaging about 34 cm. Figure 2 shows albino grafts on green stock plants.

Iron and Manganese Contents of Plants: Results of chemical determinations of water-soluble, water-insoluble, and total iron and manganese contents of leaf tissues of green-grafted scions are shown in table II. No analyses were made for these elements in albino leaves because of the limited amount of tissue that was available.

As may be observed in table I, a low nutrient level of iron and a high nutrient level of manganese each resulted in very low water-soluble, and relatively low water-insoluble and total iron contents of the tissues as compared with the corresponding tissue fractions of iron with other nutrient treatments. With a low nutrient level of manganese, the water-

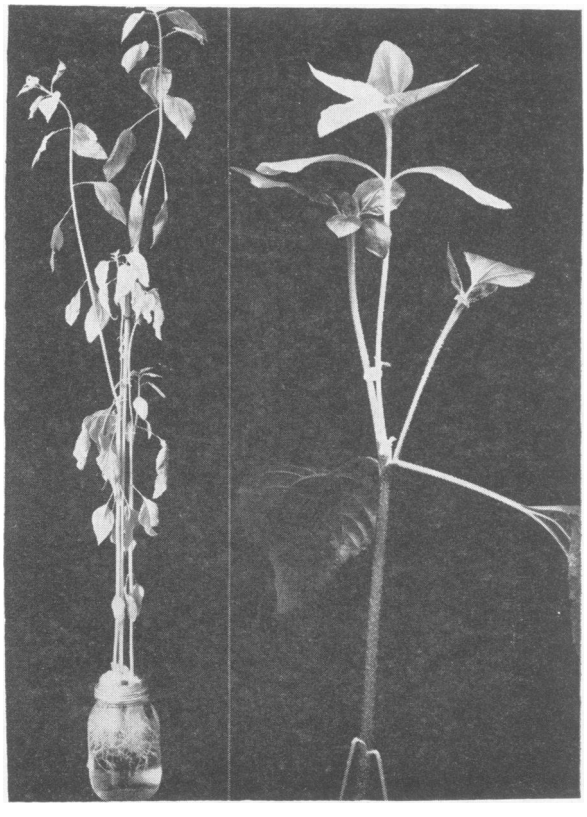

FIG. 2. Albino sunflower scions grafted to green sunflower plants. *Left.* Grown with a low nutrient level of iron. *Right.* Grown with control nutrient treatment.

soluble iron content (49.0 ppm) was much higher than with a high nutrient level of manganese (12.5 ppm). There were small, if any, differences between the water-soluble manganese contents of high iron, control, and low iron tissues, but with a high nutrient level of manganese water-soluble manganese was nearly 7 times higher (372.0 ppm) than in any other nutrient treatment. These results agree, in general, with those reported by Somers and Shive (20) for soybeans.

PROTEIN NITROGEN: The protein nitrogen content of albino and green leaf tissues grown with different levels of iron and manganese nutrition are shown in table II. In both green and albino tissues, the low nutrient level of iron resulted in relatively low protein nitrogen content. In green tissues, the high manganese treatment also resulted in a low protein nitrogen content but this was not the case in the albino tissues.

ENZYME ACTIVITY: Catalase and cytochrome oxidase activty data are shown in table II. Comparative determinations for nutrient treatments were carried out on the same day, but a series of replicate determinations for each treatment, run in triplicate, was carried out on different days over the 30-day harvest period. This was done to compare the relative activities under different light and other environmental conditions. Work to be reported in another paper has shown that catalase activity values of tissues grown with the same nutrient treatment varied considerably with differences in light intensity.

The data disclose that low levels of catalase and of cytochrome oxidase were found in both green and albino leaf tissues of plants grown with substrate levels of low iron as well as of high manganese. The highest activities of catalase were associated with substrate levels of both high iron and of low manganese. Enzyme activity of control plant tissues usually were intermediate in value. Catalase and cytochrome oxidase activities of albino tissues were usually less than those found for green tissues grown with the same nutrient treatments. In the case of low iron treatment there was no difference between the catalase activities of green and albino tissues.

TABLE II

PROTEIN NITROGEN CONTENT AND CATALASE AND CYTOCHROME OXIDASE ACTIVITIES OF LEAF TISSUES OF ALBINO- AND GREEN-GRAFTED SCIONS OF RUSSIAN SUNFLOWER PLANTS GROWN UNDER DIFFERENT IRON AND MANGANESE NUTRIENT LEVELS

NUTRIENT LEVEL		PROTEIN NITROGEN *		CYTOCHROME OXIDASE **		CATALASE ***	
FE	MN	ALBINO	GREEN	ALBINO †	GREEN ††	ALBINO ‡	GREEN ‡‡
ppm	*ppm*						
0.001	0.25	3.78	3.18	0.350	0.371	4.18	4.14
0.25	0.25	4.67	4.39	0.436	0.683	12.31	17.81
10.0	0.25	4.61	4.88	....	....	15.87	20.69
0.25	0.001	4.35	4.81	....	....	13.25	20.59
0.25	10.0	4.43	3.59	0.337	0.412	5.48	7.85

* Values expressed as % dry wt.
** Values expressed as K sec^{-1}; mg protein N in 3 ml.
*** Values expressed as ml O_2 evolved/min × mg protein N. Average of 5 (albino) and 4 (green) replicate determinations.
† L.S.D. (least significant difference) at 0.05 = 0.043 (differences not significant at 0.01).
†† L.S.D. at 0.05 = 0.153 (difference not significant at 0.01).
‡ L.S.D. at 0.05 = 6.93; at 0.01 = 9.50.
‡‡ L.S.D. at 0.05 = 3.03; at 0.01 = 4.25.

In both green and albino plants, average catalase activity of control leaf tissue was several times as great as those of low iron and of high manganese leaf tissues. Catalase activity of high iron leaf tissue was not significantly different from those of low manganese or of control tissues.

Cytochrome oxidase activity of albino leaf tissue of control plants was significantly higher than the activities of tissues grown with either low iron or high manganese. In the case of green tissues, cytochrome oxidase activity of controls was also significantly higher than that found for low iron or for high manganese.

Discussion

The results of this experiment substantiate the findings of Brown and Hendricks (2) that the nutrient deficiency of an element will be evident in a changed activity of enzymes requiring this element for function. This has also been shown by Nason et al (13). In accordance with this concept, low iron in the nutrient substrate resulted in a low activity of the iron-containing enzymes catalase and cytochrome oxidase in green-grafted and albino-grafted scions. Of perhaps more significance is the strikingly low activity of these enzymes in the presence of a high tissue content of manganese. The similarity in response of each enzyme to both low nutrient levels of iron and to high nutrient levels of manganese suggests that both treatments induce similar alterations in the metabolic pattern of these plants. This suggestion is substantiated by the similarity in the characteristic visual leaf symptoms, water-soluble iron, and in protein nitrogen contents of the leaf tissues. Furthermore, an earlier experiment with Havana Seed tobacco plants (31) demonstrated that the relative distribution of citric, malic, oxalic, and total organic acids of the leaf tissues was similar in low iron and in high manganese plants, and in high iron and in low manganese plants. The exact mechanism associated with these responses is not known. In the case of iron-containing enzymes, it is entirely plausible that in high manganese tissues there is direct competition between manganese and iron for a position in the heme nucleus of the enzyme as suggested by other workers (18, 26).

The inhibition of chlorophyll production in albino sunflower leaf tissues is not paralleled by the lack of production of the porphyrin nucleus for iron enzymes, since these enzymes are present in significant quantities. However, the relatively low catalase activity of albino tissues confirms the results published by Eyster (4) and Appleman (1) who found catalase activity of albino tissues to be less than that of green tissues. The presence of cytochrome oxidase in albino leaf tissues suggests that this enzyme mediates at least a portion of terminal respiration in these tissues. Definite proof of this, however, awaits a study of other terminal oxidases in these tissues.

From the information gathered in this and in previous experiments in this laboratory, it is concluded that on the basis of biochemical data presented and in the species of plants used, a high nutrient level of manganese in the presence of low iron induces a true iron deficiency. Thus, for each set of cultural conditions and for each nutrient level of iron, there is a nutrient level of manganese which will induce iron deficiency symptoms on many species of plants. If this level is surpassed, symptoms of manganese toxicity in addition to those of iron deficiency may become apparent. The browning of trichomes on sunflower plants observed in these experiments is believed to have resulted from a nutrient level of manganese which was higher than that required to induce only iron deficiency symptoms. It is recognized, however, that the general pattern of response to high manganese is not the same on all species of plants (8, 9, 10, 11, 12).

Summary

1. A solution culture experiment was carried out in order to study the effects of different iron and manganese nutrient levels on the water-soluble, water-insoluble, and total tissue content of both iron and of manganese, on protein nitrogen content, and on catalase and cytochrome oxidase activities of leaf tissues. Green and albino sunflower scions grafted to green stock sunflower plants were used.

2. Symptoms of iron deficiency chlorosis were evident on green portions of plants supplied with a high nutrient level of manganese, as well as with a low nutrient level of iron.

3. Symptoms of manganese deficiency chlorosis appeared on green plants supplied with a low nutrient level of manganese, but not on plants supplied with a high nutrient level of iron.

4. A low nutrient level of iron and a high nutrient level of manganese each resulted in a very low water-soluble iron content of green tissues as compared with controls.

5. In both green and albino tissues, the low nutrient level of iron resulted in a relatively low protein nitrogen content. In green tissues, the high manganese treatment also resulted in a low protein nitrogen content but this was not the case in the albino tissues.

6. Low levels of catalase and cytochrome oxidase activities were found in both green and in albino leaf tissues of plants grown with low nutrient levels of iron or of high nutrient levels of manganese.

7. Catalase and cytochrome oxidase activities of albino tissues were usually less than those found for corresponding green tissues grown with the same nutrient treatments.

8. Results support an hypothesis by other workers that induction of iron deficiency by high manganese may be due to a direct competition between manganese and iron for a position in the heme nucleus of the iron-containing enzymes.

9. It is concluded, on the basis of the biochemical data presented, that in the species of plant used, a high nutrient level of manganese in the presence of low iron induces a true iron deficiency.

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