Effect of Iron Supply on Growth, Chlorophyll, Tissue Iron and Activity of Certain Enzymes in Maize and Radish [1, 2]

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Introduction

Iron is a constituent of the heme of catalase, cytochrome oxidase and peroxidase and iron deficiency is known to depress iron porphyrin enzymes (1, 3, 9, 14, 22, 27, 35). There is hardly any information on the effect of iron supply on enzymes for which iron is not a cofactor. The work presented here was primarily carried out to investigate the effect of graded levels of iron supply, ranging from acute deficiency to excess, on nonheme enzymes, starch phosphorylase, fructose-1,6-diP aldolase, acid phosphatase, transaminase and ribonuclease, in addition to that on the hematin enzymes catalase and peroxidase. Investigation was also carried out to find out whether additional iron supply would restore chlorophyll and enzyme concentration in iron deficient plants to the levels found in plants receiving normal iron supply.

Materials and Methods

Maize (*Zea mays* L. var. Deccan, hybrid) and radish (*Raphanus sativus* L.) plants were raised at 6 graded levels of iron supply, 0.004, 0.02, 0.1, 0.2, 0.5, and 1 mM for maize; 0.005, 0.01, 0.02, 0.1, 0.2, and 0.5 mM for radish. Iron was supplied as ferric ethylenediamine tetraacetic acid (Fe-EDTA) prepared from equimolar solutions of ferrous sulphate and disodium salt of ethylenediamine tetraacetic acid. The other macro and micronutrient elements were supplied as: 4 mM $Ca(NO_3)_2$; 4 mM KNO_3; 2 mM $MgSO_4$; 1.33 mM NaH_2PO_4; 10 μM $MnSO_4 \cdot 4H_2O$; 1 μM $CuSO_4$; 1 μM $ZnSO_4$; 33 μM H_3BO_3; 0.2 μM Na_2MoO_4; 0.1 μM $CoSO_4$ and 0.1 μM $NiSO_4$.

Plants were raised in acid washed silica sand contained in bitumen painted clay flower pots. Sand was purified according to the procedure worked out by Agarwala and Sharma (2). Deionised water containing 0.1 μM iron was used for culture. Macronutrient stock solutions were depleted of iron by phosphate adsorption technique described by Hewitt (20) and micronutrient solutions were prepared from recrystallised analytical reagent grade salts. The diluted nutrient solution was supplied daily except on weekends, when pots were flushed with deionized water. The pH of the nutrient solution which drained out of the culture vessels ranged between 6.7 to 7. For each treatment there were 30 to 50 pots randomized in 2 blocks.

Maize and radish plants were sampled at 20 and 23 days growth respectively and estimations were made for yield, chlorophyll, tissue iron, protein nitrogen and for enzymes; aldolase, acid phosphatase, ribonuclease, catalase, peroxidase, starch phosphorylase and alanine-glutamate transaminase. The last 2 enzymes were estimated in radish only. The enzyme assay was carried out in the extracts prepared from the top parts, the youngest expanded leaf and the one next to it, of both the plant species and in roots from maize.

At 25 days in case of maize and 23 days in case of radish, 1 mM Fe-EDTA was supplied to some of the pots in which plants showed effects of iron deficiency; 0.02 and 0.1 mM iron in case of maize and 0.005 and 0.01 mM iron in case of radish. After the commencement of additional iron supply to the deficient plants suitable samples for chlorophyll estimations and the assay of enzymes, found to be affected by iron supply, were drawn at 24 and 72 hours in case of maize and 48 and 96 hours in case of radish to find out to what extent iron deficiency had been reversed.

Enzymes were estimated on crude tissue extracts. For acid phosphatase, ribonuclease, transaminase, starch phosphorylase and aldolase assay, finely chopped leaf lamina or root material was chilled and ground with acid washed silica sand in a chilled pestle and mortar in glass distilled water in the proportion of 1 g fresh material to 10 ml of glass distilled water. Temperature during the grinding procedure was maintained below 5°. The crude extract was strained through 2-fold muslin. Extract for catalase and peroxidase was prepared as for the other enzymes except that the fresh chilled tissue was ground in 0.005 M potassium phosphate buffer pH 7 instead of glass distilled water.

Ribonuclease was determined by the method of McDonald (24). pH Optimum for ribonuclease in maize and radish was found to be 6. Ribonuclease activity was measured in citrate buffer pH 6.

Aldolase was estimated by an adaption of the method described by Stumpf (33). 0.2 ml of suitably diluted enzyme preparation was added to 0.2 ml of 0.2 M potassium cyanide acidified to pH 9 and

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0.2 ml of 0.1 M veronal buffer pH 8.5 in a centrifuge tube maintained at 30° for 5 minutes. 0.2 ml of 0.05 M fructose-1, 6-diP was then added. The reaction was allowed to continue for 20 minutes. The reaction was stopped by adding 1 ml of chilled 10 % trichloroacetic acid. Blanks with trichloroacetic acid added before adding fructose-1, 6-diP were run simultaneously. The reaction mixture was centrifuged for 10 minutes at 400 × g at 4° in a refrigerated centrifuge. To 1 ml of the supernatant solution was added 1 ml of 2 % NaOH. After standing for 20 minutes at room temperature the alkaline mixture was neutralized with 0.1 N H_2SO_4 and Pi determined in a suitable aliquot by the method of Fiske and Subbarow (16).

Starch phosphorylase in radish was estimated by the method of Green and Stumpf (19) as modified by Srivastava and Krishnan (32).

Acid phophatase was estimated by a modification of the method of Schmidt (31). The reaction mixture contained 0.5 ml of 0.1 M acetate buffer pH 6, 0.2 ml of 0.1 M sodium β-glycerophosphate, 0.2 ml of glass distilled water and 0.2 ml of suitably diluted enzyme preparation. The reaction was carried out at 30° in a water bath for 20 minutes when the reaction was stopped by adding 1 ml of chilled 10 % trichloroacetic acid and the mixture centrifuged at 400 × g. The supernatant fraction was appropriately diluted and Pi determined in a suitable aliquot by the method described by Fiske and Subbarow (16).

Alanine-glutamate transaminase activity in radish was assayed by an adaptation of the method of Aspen and Meister (5). The standard test assay consisted 0.1 ml of 0.1 M α-ketoglutaric acid; 0.2 ml of 0.1 M alanine; 0.2 ml of 0.1 M potassium phophate buffer pH 8; and 0.1 ml of 0.01 % pyridoxyl phosphate in a centrifuge tube. The reaction was started by adding 0.4 ml of suitably diluted enzyme preparation and the incubation was carried out at 37° for 1 hour. The reaction was stopped by adding 2 ml of hot ethanol and the contents were centrifuged at 400 × g. Glutamic acid formed was estimated in a convenient volume of the supernatant after separation by paper chromatography by the method of Giri et al. (18).

Catalase was estimated by the method of Euler and Josephson (14).

Peroxidase was determined by the method of Willstätter and Stoll, as modified by Sumner and Gjessing (34). The reaction was carried out in a water bath at 15°. The purpurogallin formed was extracted in ether and measured colorimetrically.

The activity of the different enzymes was estimated in the range in which the activity was found to be proportional to the enzyme concentration in the crude extract.

Dry matter yield was determined by drying the plants in a forced draft oven at 70° for 24 hours. Since the dry matter was required for the estimation of tissue iron, fresh matter kept for drying was thoroughly cleaned against surface contamination by first washing in running deionized water for a short duration and then rinsing with glass distilled water.

Chlorophyll was estimated colorimetrically in acetone extracts by the method of Petering et al. (29). The calibration curve for chlorophyll was prepared by the method of Comar and Zscheile (12).

Total iron was determined colorimetrically as ferrous-orthophenanthroline complex by the method described by Humphries (19) after wet digestion of the oven-dried samples with nitric-perchloric acids carried out according to Piper (30).

For expressing the enzymes on the protein nitrogen basis estimation was made of the protein nitrogen by the micro-Kjeldahl method in the crude enzyme preparations made for enzyme assay after precipitation with 20 % trichloroacetic acid and centrifugation at 710 × g. The residue was treated in cold for 2 hours with 2 % salicylic acid in sulphuric acid. The salicylic acid was removed by heating with powdered sodium thiosulphate. The sample was then digested by the method of Chibnall et al. (11). The digest was made to volume. The ammonia produced was distilled by steam distillation in a Markham apparatus into a boric acid buffer in an ammonia-free atmosphere and estimated by titration with 0.007 N H_2SO_4 containing Conway and O'Malley indicator (13).

Results

At low levels of iron supply (0.004–0.1 mM in maize and 0.005–0.02 mM in radish) the growth of plants, both tops and roots, was significantly restricted (fig 1). The optimum yield of maize was obtained at 0.5 mM and that of radish at 0.1 mM iron supply. Plants raised at low levels of iron supply developed chlorosis of the younger growths. In maize, chlorosis appeared in 4-day-old seedlings raised at 0.004 to 0.2 mM iron supply. While at 0.004 and 0.02 mM iron supply the entire second and the subsequently emerged leaves were severely chlorotic or bleached, at 0.1 and 0.2 mM iron supply chlorosis was restricted to intervenal areas of the younger growths. At later stages of growth the severity of chlorosis at 0.2 mM iron supply decreased. In radish, at 0.005 to 0.02 mM iron supply, right from the emergence seedlings had large and fleshy pale cotyledons and the leaf lamina was markedly restricted and severely chlorotic. Later the entire lamina became almost bleached and intervenal areas became necrotic.

Within the range 0.004 to 0.5 mM in maize and 0.005 to 0.2 mM in radish increase in iron supply brought about an increase in protein nitrogen. Marked and significant depression in protein nitrogen was found at an iron supply of 0.004 and 0.02 mM in maize and 0.005 and 0.01 mM in radish (fig 2).

At the suboptimal levels of iron supply, < 0.5 mM in maize and < 0.1 mM in radish, chlorophyll content was markedly and significantly depressed (fig 3).

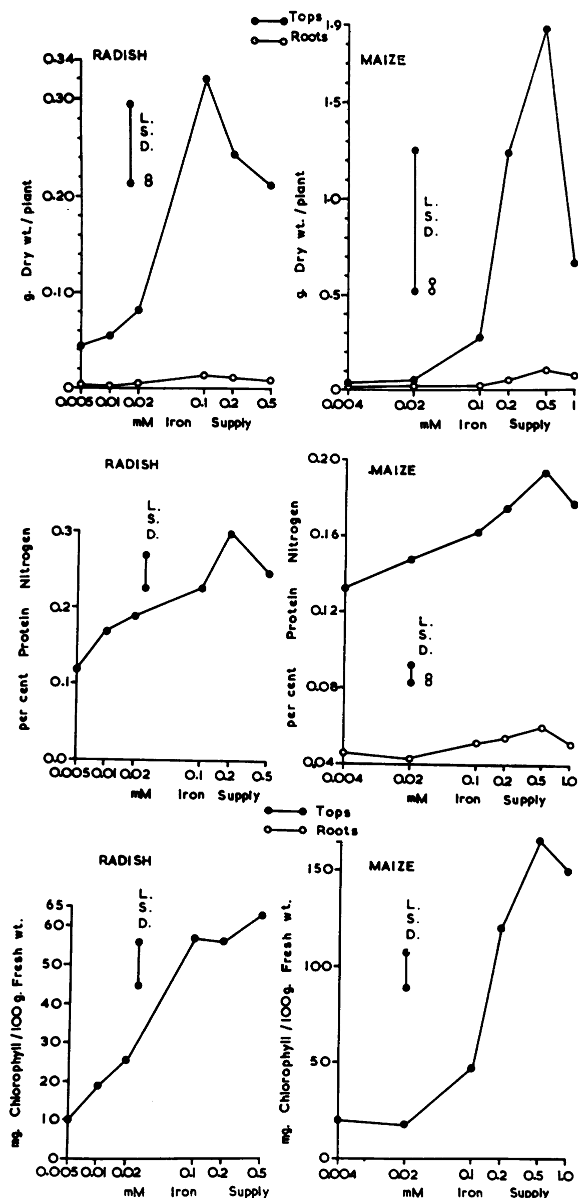


FIG. 1 (*top*). Effect of iron supply on the yield of radish and maize plants.

FIG. 2. (*middle*). Effect of iron supply on protein nitrogen content of radish and maize plants.

FIG. 3. (*bottom*). Effect of iron supply on the chlorophyll content of radish and maize plants.

The iron content of maize leaves did not show any definite trend in relation to iron supply. In radish leaves iron content increased with an increase in iron supply but even in this plant species the increase was large enough to be significant only at the highest iron supply. In both radish and maize iron content of roots increased with an increase in the iron supply; but the increase reached the level of significance only at 0.2 and 0.5 mM iron supply in radish and at 1 mM iron supply in maize.

In the extracts of leaves of plants raised at low levels of iron supply (0.004–0.1 mM in maize and 0.005–0.01 mM in radish) the activity of catalase was significantly reduced (fig 4, 5). The activity of catalase in the extracts of maize roots did not show any definite trend in relation to iron supply.

In the leaves of radish plants raised at 0.005 mM iron supply peroxidase activity was almost equal to that at 0.1 mM iron supply but at 0.01 and 0.02 mM iron supply peroxidase activity was significantly depressed (fig 5). Significant depression in peroxidase activity was also found in the crude extracts of leaves of maize plants raised at 0.004 to 0.1 mM iron supply; but in maize roots peroxidase was stimulated by iron deficiency, the stimulation at 0.004 and 0.02 mM iron supply being marked and significant (fig 4).

In the extracts of leaves of maize plants raised at 0.004 to 0.02 mM iron supply and radish plants raised at less than 0.1 mM iron supply the activity of aldolase was significantly lower than at higher levels of iron supply. In the latter, at lower levels of iron supply (0.005–0.02 mM) the activity of phosphorylase was also significantly depressed (fig 5).

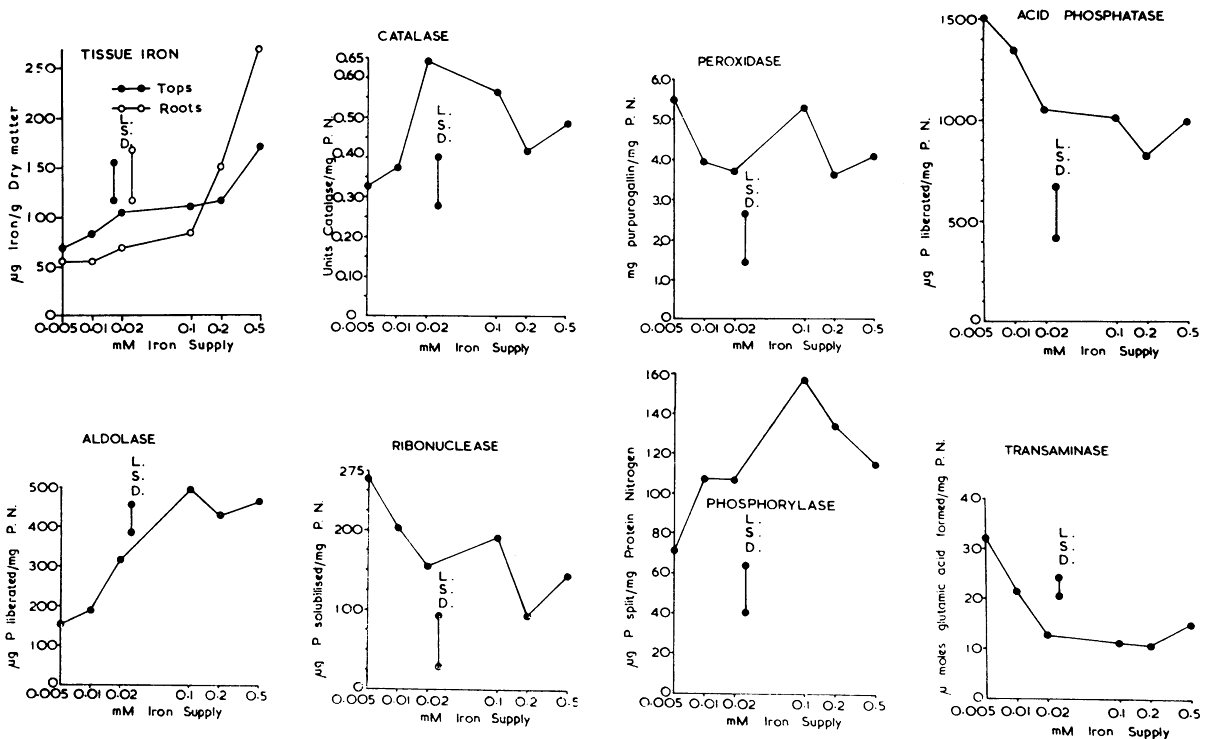
In the leaves of both maize and radish iron deficiency caused a significant increase in the activity of acid phosphatase. Significant stimulation was also found in ribonuclease activity of maize plants grown in the range of iron supply 0.004 to 0.01 mM and of radish plants in the range of iron supply 0.005 to 0.02 mM (fig 4, 5). In radish alanine-glutamate transaminase also showed marked and significant stimulation at low (0.005–0.01 mM) iron supply (fig 5).

Neither the depression in aldolase in maize and radish nor that of phosphorylase in radish was mitigated by the addition of Fe^{++} or Fe^{+++} iron to the crude extracts of plants grown at low levels of iron supply. The Fe^{++} complexing agents, *a, á*-dipyridyl and *o*-phenanthroline did not inhibit the activity of aldolase and phosphorylase in radish and maize plants.

Supply of additional iron (1 mM) to plants raised at deficient iron levels (maize at 0.02 and 0.1 mM, and radish at 0.005 and 0.01 mM iron) brought about a marked and significant increase in chlorophyll and with 1 exception increase in protein nitrogen (fig 6). The enzymes, catalase, peroxidase and aldolase (fig 8, 9) in both the plant species investigated and phosphorylase in radish raised at low levels of iron supply also showed a marked and significant increase on application of additional iron (fig 7, 8). The increase was appreciable even after 24 hours of the recovery treatment in maize and 48 hours of the recovery treatment in radish. In the former, 72 hours after the commencement of the recovery treatment to plants raised at 0.02 mM iron supply, chlorophyll, catalase, peroxidase and aldolase became as much or even more than in plants raised throughout at 0.1 mM iron supply; and in plants raised at 0.1 mM iron supply, chlorophyll, peroxidase and aldolase became almost as much as and catalase even more than that in the leaves of plants raised at normal (0.5 mM)

FIG. 4. Effect of iron supply on the tissue concentration of iron and on the specific activity of enzymes in the extracts of maize plants.

FIG. 5. Effect of iron supply on the tissue concentration of iron and on the specific activity of enzymes in the extracts of radish plants.



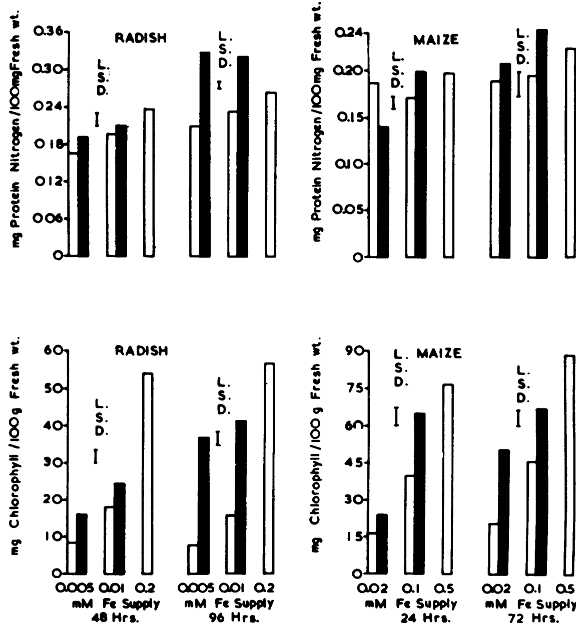
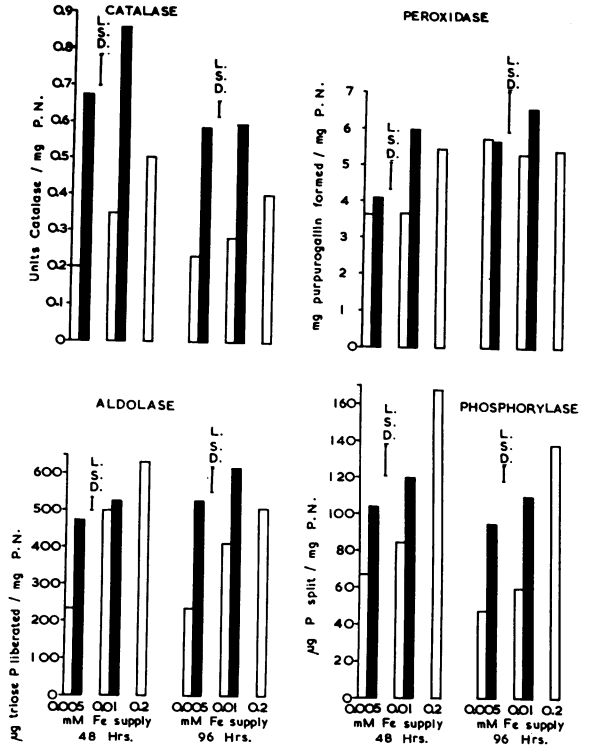


FIG. 6. Effect of additional (1 mM) iron supply on the protein nitrogen and chlorophyll content of maize and radish plants raised at low levels of iron.



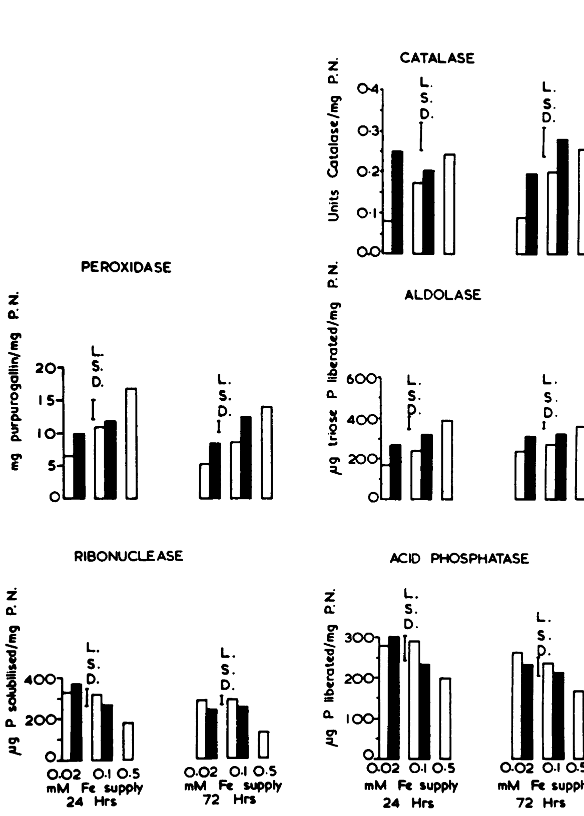


FIG. 7. Effect of additional (1 mM) iron supply on the specific activity of enzymes in the extracts of maize plants raised at low levels of iron.

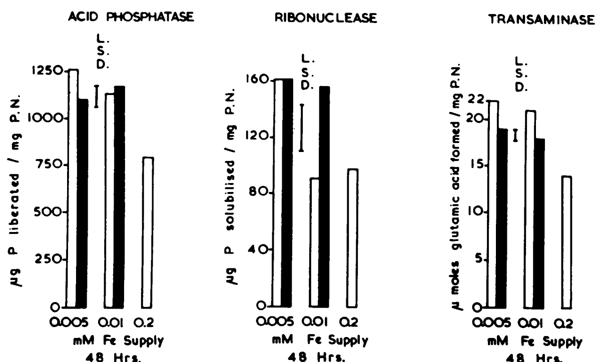


FIG. 8. Effect of additional (1 mM) iron supply on the activity of enzymes in the extracts of radish plants raised at low levels of iron. In figures 6, 7 and 8 the shaded bars represent samples drawn from plants supplied additional (1 mM) iron.

iron supply (fig 6, 7). The effect of additional iron supply was often even more marked in radish than in maize. In radish 48 hours after the supply of additional iron to plants raised at 0.005 mM iron, the activity of catalase in these plants exceeded that in plants raised at normal (0.2 mM) iron supply (fig 8).

Acid phosphatase and ribonuclease which in the extracts of maize and radish leaves showed a stimulation at low levels of iron supply were not brought back to normal by the additional iron supply (fig 7, 8). The stimulation in transaminase in radish became less marked 48 hours after the supply of additional iron to iron deficient plants (fig 8).

Discussion

A poor correlation in the iron content and iron supply was found in top parts of maize plants as has also been recently reported for some other plants (1, 10, 22).

In respect to the depression in both catalase and peroxidase at the deficient levels of iron supply the results obtained here are in accord with DeKock et al. (14) and Nicholas and Goodman (27), and in respect to catalase only, with those of Weinstein and Robbins (35), Banerjee (9), Agarwala and Sharma (1), Marsh et al. (22), and Agarwala et al. (3). The depression in catalase and peroxidase along with that of chlorophyll could be, as has been pointed out by several workers, due to depression in the synthesis of protoporphyrin (1, 6, 23).

The results indicate that synthesis of aldolase and phosphorylase, for which iron has not been shown to be a cofactor in higher plants, is affected by iron deficiency. Recovery experiments on radish carried out by supplying 1 mM additional iron to plants showing characteristic iron deficiency effects, show that the optimum iron requirement for the synthesis of aldolase and starch phosphorylase is even higher than for the iron porphyrin enzymes, catalase and peroxidase.

The possibility that in the plant species investigated ferrous iron may be a cofactor of aldolase, as has been shown for bacteria (6) and very recently for blue green algae by Gibbs et al. (17), is partly discounted by the fact that neither the aldolase activity in the extracts from iron deficient plants increased on addition of Fe^{++} iron nor iron complexing agents e. g., *a, á*-dipyridyl or *o*-phenanthroline inhibited the activity of aldolase in plant extracts. It is possible that iron or some compound, the synthesis or activity of which is dependent on iron supply is necessary for the formation of the specific proteins, aldolase and phosphorylase in radish and aldolase in maize. The depression in protein nitrogen was not large enough to account for the depression found in the various enzymes, catalase, peroxidase and aldolase in both maize and radish and phosphorylase in radish. Besides, acid phosphatase and ribonuclease in both maize and radish and transaminase in radish were found to be stimulated by iron deficiency. It, therefore, appears that iron deficiency upsets the normal balance in the formation of different specific proteins, the enzymes, some of which are stimulated and others depressed. This would also account for the results reported by several workers (8, 25, 26, 27, 28).

Summary

A study was made of the effect of graded levels of iron supply ranging from acute deficiency to excess, on growth, tissue concentration of iron, chlorophyll and the specific activity of enzymes, catalase, peroxidase, fructose-1, 6-diphosphate aldolase, acid phosphatase, ribonuclease, phosphorylase, transaminase, in maize (*Zea mays* L.) and radish (*Raphanus sativus* L.) plants grown in sand culture.

The optimal yield of maize and radish was obtained at 0.5 and 0.1 mM iron supply, respectively. In both plant species characteristic iron deficiency effects were produced at lower levels of iron supply. The chlorophyll content of the 2 plant species was related to the level of iron supply. In radish tissue iron increased with an increase in iron supply but it was not always so in the top parts of maize plants.

In both plant species, catalase, peroxidase and aldolase were depressed by iron deficiency. Phosphorylase in radish was also depressed by iron deficiency. All the 4 enzymes which were depressed due to iron deficiency were to a large extent restored to the normal level within 72 to 96 hours of the supply of additional iron to the deficient plants. Acid phosphatase and ribonuclease in both maize and radish and transaminase in radish were stimulated by iron deficiency. This would show that iron is involved not only in the synthesis of enzymes of which it is a cofactor as heme, but also in enzymes of which it is not a cofactor. The synthesis of the different specific proteins appears to be affected by iron supply.

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