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Reduction of Iron by Leaf Extracts and Its Significance for the Assay of Fe(II) Iron in Plants[1]

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ABSTRACT

"Active Fe" in plants has generally been interpreted to be ferrous iron. This study evaluated some of the commonly followed active Fe determination procedures based on Fe(II) measurements. In each of 12 species examined, leaf extracts exhibited strong reducing activity and quickly reduced any added Fe(III) with or without light. The reducing activity was attributed to ascorbic acid and phenols in the plant extracts. The reliability of the Fe(II) iron determination procedures in plants and the interpretation that active Fe is Fe(II) are questionable.

Total Fe of plants is generally not taken as a reliable indicator of the Fe status of plants (13). Oserkowsky (17) first showed that the 1 N HCl-soluble fraction of plant Fe is involved in Chl biosynthesis and provides a reliable indication of plant Fe status. He termed this fraction "active Fe." This fraction has been suggested to comprise Fe in the ferrous form (24). A number of methods using different extractants, chiefly weak acids or chelating agents, are now available for the determination of different fractions of Fe in plants (1, 3, 9, 11, 12, 14, 18, 19, 24).

Several investigators have suggested that the metabolically active form of Fe is Fe(II) (4–7, 9, 16, 21, 22). In dicots and non-graminaceous monocots, it appears that Fe is absorbed primarily as the ferrous ion (5). Katyal and Sharma (9) used *o*-phe[2] as a chelating agent to describe a procedure for the assay of Fe(II) in plants. These authors interpreted active Fe to be Fe(II) and they did not take into account the specificity of *o*-phe for Fe(II) (18) nor the error that could be caused by photoreduction of Fe(III) in their assays (2, 4, 10). The current investigation presents evidence for the presence of reducing substances in leaf extracts, used to assay active Fe, based on Fe(II) methods, that could cause the reduction of Fe(III) to Fe(II) during the extraction process.

MATERIALS AND METHODS

Plant Material

Fresh leaves of barley (*Hordeum vulgare* L.), bonavist bean (*Dolichos lablab* L.), eggplant (*Solanum melongena* L.), cabbage (*Brassica oleracea* L. var Capitata), chickpea (*Cicer arietinum* L.), citrus (*Citrus aurantium* L.), grape (*Vitis vinifera* L.), maize (*Zea mays* L.), mulberry (*Morus alba* L.), radish (*Raphanus sativus* L.), rice (*Oryza sativa* L.), and ridge gourd (*Luffa acutangula* (L.) Roxb.) from 10 to 12 randomly selected field-grown plants were combined and cleaned against surface contamination by successive washings with 0.1 N HCl, tap water, deionized water, and finally glass distilled water. After blotting, the leaf samples were chopped finely (1–2 mm) using stainless steel scissors, and suitable quantities were drawn. The weighed samples were stored at 4°C and utilized for extraction within 1 h.

Assay of Fe(II) and Reduction of Added Fe(III)

For each of the 12 species, 1 N HCl extracts were prepared by homogenizing the leaf material in the extractant in the proportion of 1 g tissue per 10 mL 1 N HCl, following the Mehrotra *et al.* (14) procedure. Iron (II) concentration in these extracts were quantified by the addition of *o*-phe, a ferrous chelating agent that forms a stable reddish orange complex (ferroin) in association with Fe(II), and the concentration of this complex, with and without the addition of the reducing agent OHam, was determined spectrophotometrically at an absorbance of 510 nm (8). To the HCl extracts of each species containing *o*-phe without the reducing agent (OHam), a standard solution of Fe(III) (2 μg mL^{-1} as $FeCl_3$) was added and the increase in A at 510 nm was recorded at different time intervals under normal laboratory light conditions. The difference in A before and after the addition of Fe(III), in reference to a standard calibration curve, provided an estimate of the amount of Fe(III) reduced by the extract. Under similar assay conditions, the rate of photoreduction was also determined in an aliquot of the standard solution of Fe [100 μg Fe(III), o-phe, pH 4.8].

The 1 N HCl extracts in rice were also prepared by the Takkar and Kaur (24) procedure where the leaf material, instead of being homogenized, was immersed in the extractant (1 g tissue, 10 mL 1 N HCl, 24 h incubation), shaken occasionally, and filtered. Iron(II) concentration in a 3-mL aliquot of the extract, buffered at pH 3.0, was determined spectrophotometrically using *o*-phe as the chelating agent and measuring A at 510 nm. Reduction of 40 μg Fe(III), added to a 3-mL aliquot of the extract containing *o*-phe, was determined under normal light conditions as described earlier. Rice and mulberry leaf extracts were also prepared in a solution of 1.5% *o*-phe following the procedure of Katyal and Sharma (9) [1 g tissue, 10 mL 1.5% *o*-phe (pH 3.0), 16 h incubation] and the Fe(II) iron was quantified by directly measuring the

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[2] Abbreviations: *o*-phe, *o*-phenanthroline; OHam, hydroxylamine.

Table I. *Concentrations of Fe, Ascorbic Acid, Total Phenols, and Reducing Sugars in 1 N HCl Extracts of Leaf Samples from 12 Plant Species*

Plant Species	Tissue Fe		Ascorbic Acid	Total Phenols	Reducing Sugars
	− OHam	+ OHam			
	μg/g fresh wt		*mg/g fresh wt*		
Barley	14.8	15.0	0.34	2.50	0.13
Bonavist bean	12.3	12.3	0.42	4.50	0.30
Eggplant	15.0	15.5	0.59	4.32	0.50
Cabbage	21.0	21.5	0.55	3.92	0.10
Chickpea	34.5	34.0	0.29	18.74	0.30
Citrus	33.0	32.0	0.17	7.50	0.40
Grape	24.0	23.0	0.42	11.04	1.02
Maize	11.0	13.0	0.34	4.60	0.55
Mulberry	18.5	19.0	0.29	10.46	0.28
Radish	10.5	11.0	0.71	4.06	0.30
Rice	24.0	24.0	0.55	3.96	0.28
Ridge gourd	20.0	20.0	0.29	5.52	0.75
LSD (P = 0.05)	2.2		0.12	1.40	0.12

A of the filtered extracts at 510 nm. To the leaf extract of mulberry thus prepared 100 μg Fe(III) using a standard Fe(III) solution was added at the onset of the incubation period. The increase in *A* of the extracts at 510 nm, measured at different time intervals, provided an estimate of the rate of Fe(III) reduction. The reduction rates were determined under both light and dark conditions. The effect of adding Fe(III) after the completion of the incubation period was studied in rice, using similar quantities of the extract and standard Fe solution.

For chickpea, 2,2′-bipyridyl extracts were prepared by the Abadia *et al.* (1) procedure (1 g tissue, 10 mL 1.5% 2,2′-bipyridyl [pH 3.0], 24 h incubation in the dark). Direct measurement of the *A* of the filtered extracts at 522 nm provided an estimate of the Fe(II) iron. A sample of 100 μg Fe(III) using a standard Fe(III) solution was added to the 1 g leaf extract after the completion of the incubation period, and the increase in *A* with time provided an estimate of the reduction rate.

In all the assay procedures due corrections were made for the respective tissue and reagent blanks. Data are based on two to three replications drawn from the composite leaf samples and the coefficients of variation ranged from 2 to 10% in different experiments.

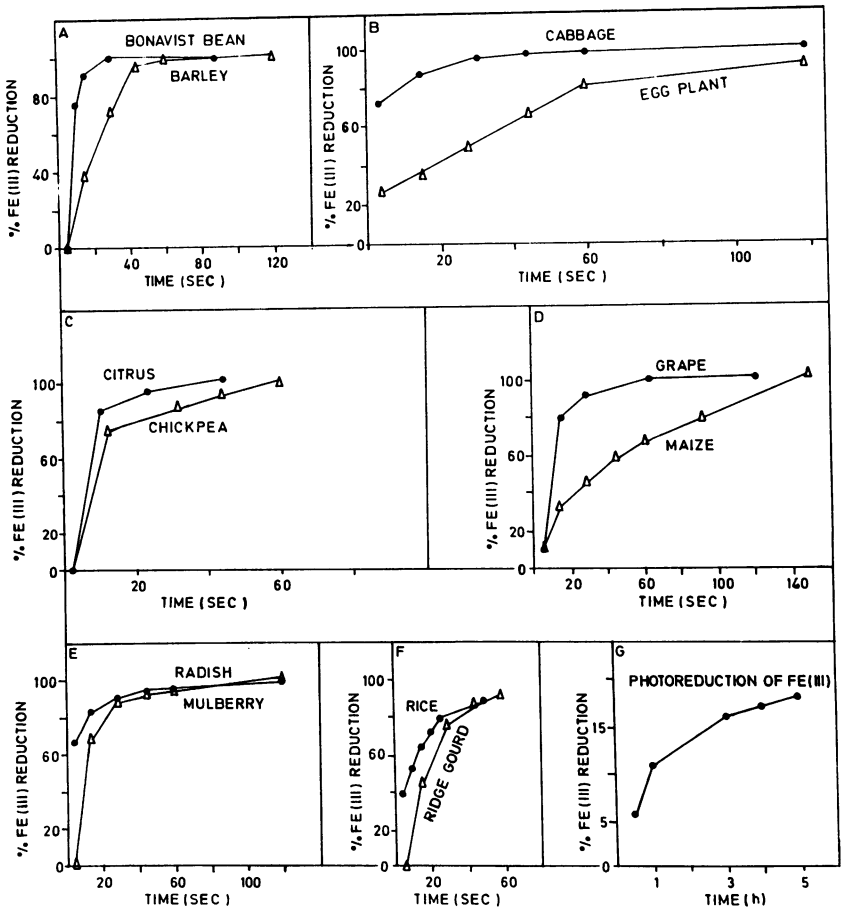

Figure 1. Reduction of added Fe(III) by 1 N HCl-leaf extracts of 12 plant species. The extracts were prepared by the Mehrotra *et al.* (14) procedure and the reduction of Fe(III) added (2 μg mL^{-1} as $FeCl_3$) was measured in the different plant species by the *o*-phe method (−OHam) (8), under normal laboratory light conditions (A–F). Under similar assay conditions, the rate of photoreduction was also determined (G).

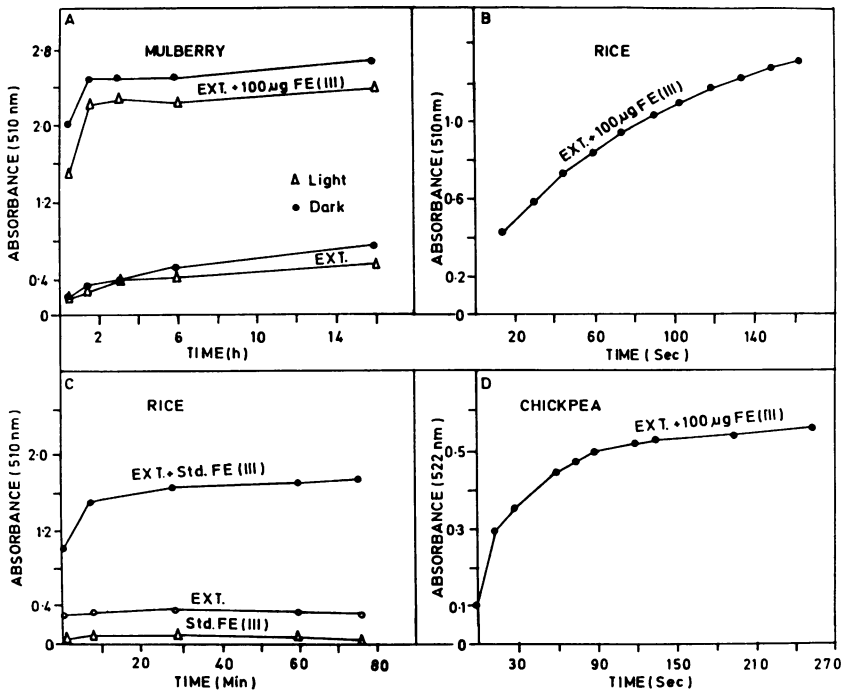

Figure 2. Reduction of added Fe(III) by leaf extracts, prepared for Fe(II) assay by different methods. (A) Fresh mulberry leaf extracts were prepared in *o*-phe by the Katyal and Sharma (9) procedure and reduction of 100 µg of Fe(III), added at the onset of the incubation period, was measured under both normal light and dark conditions. (B) Rice leaf extracts were prepared as in (A) but 100 µg of Fe(III) was added to the extracts after the incubation period, and the rate of reduction was measured in normal light. (C) Rice leaf extracts were prepared in 1 N HCl by the Takkar and Kaur (24) procedure and reduction of 40 µg Fe(III), added to a 3-mL aliquot of the extract (1 g tissue/10 mL 1 N HCl) containing *o*-phe, was measured in normal light. Also shown are the rates of Fe(III) reduction for the plant extract and the standard Fe solution under similar assay conditions. (D) Chickpea leaf extracts were prepared in 2,2′-bipyridyl by the Abadia *et al.* (1) procedure and reduction of 100 µg of Fe(III) added to 1 g leaf extracts after the incubation period was measured under normal light conditions.

Assay of Ascorbic Acid, Total Phenols, and Reducing Sugars

The concentration of ascorbic acid in 1 N HCl extracts was determined titrimetrically (20), and reducing sugars (15) and total phenols (23) were determined colorimetrically. All chemicals used were analytical grade.

RESULTS AND DISCUSSION

Concentrations of Fe in 1 N HCl extracts of the 12 species examined are shown in Table I. Because the Fe concentration did not change significantly after addition of the reducing agent OHam, it would suggest that all the Fe in the 1 N HCl-extracts was in the reduced [Fe(II)] state which support the observations made earlier (24). However, when Fe(III) was added to HCl-extracts which contained *o*-phe (without OHam), it was completely reduced within 60 to 120 s in each different species (Fig. 1, A to F). These rates of reduction were not due to photoreduction of Fe(III), based on the results presented in Figure 1G and from earlier studies (2, 10). These results indicate the presence of strong reducing substances in the leaf extracts.

We also measured the reduction of added Fe(III) using other methods for Fe(II) determination (1, 9, 24). Iron (100 µg) added as Fe(III) at the onset of the incubation period was completely reduced in 1.5 h by 1 g leaf extracts in *o*-phe under both light and dark conditions (Fig. 2A), using the Katyal and Sharma (9) procedure. The slight increase in *A* beyond 1.5 to 2 h shows that some Fe was being continuously extracted from the tissue, in support of observations made earlier (9, 24). It appeared that within 1.5 h sufficient reductants had been released into the extract to reduce all Fe(III) in the extract. When the same quantity of Fe(III) was added to 1 g leaf extracts after the initial incubation period, almost a complete reduction was evident within 3 min (Fig. 2B). A similar reduction of added Fe(III) was observed in the leaf extracts prepared by incubating the material in 1 N HCl according to Takkar and Kaur (24) (Fig. 2C), or by incubating the material in 2,2′-bipyridyl, following the Abadia *et al.* (1) procedure (Fig. 2D). Sufficient reductants appeared to be present in the extracts to reduce Fe(III) within 30 min.

The results showed that the plant extracts had considerable reducing properties, irrespective of the extractant used for the assay of Fe(II) iron. The reduction of Fe(III) occurred even in the dark. In an earlier study with peach [*Prunus persica* (L.) Batsch], Abadia *et al.* (1) observed that leaf extracts could reduce Fe(III) to Fe(II) in the presence of Fe(II) chelating agent (2,2′-bipyridyl) without light. They suggested that due to the stability of the amine-Fe(II) complexes a displacement of the equilibrium between Fe(II) and Fe(III) may occur.

Analysis of 1 N HCl-extracts from each species used showed the presence of such reducing substances as ascorbic acid,

Table II. *Reduction of 100 µg of Fe(III) by Ascorbic Acid (Asc.A) and Gallic Acid (Gal.A)*

Treatment	Reduction with Time (s)		
	15	30	60
	% *Fe(III) reduction*		
0.125 mg Asc.A	95	100	100
0.250 mg Asc.A	100	100	100
0.125 mg Gal.A	55	73	92
0.250 mg Gal.A	61	82	100
0.125 mg Asc.A + 0.125 mg Gal.A	100	100	100
0.250 mg Asc.A + 0.250 mg Gal.A	100	100	100

phenols and reducing sugars (Table I). After testing the effects of ascorbic acid and gallic acid (taken as a representative of phenols) on Fe(III) reduction by the *o*-phe method, omitting OHam (8) (Table II), it was concluded that the amounts of these substances present in 1 g leaf extracts were more than sufficient to reduce 100 μg of Fe(III) almost instantaneously.

Because of reducing substances in leaf extracts, it appeared that Fe(III) would be measured as Fe(II) in extractions. Therefore, we question the reliability of the Fe(II) determination procedures in plants (1, 9, 24), and the interpretation that Fe(II) is the active Fe.

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