



Ferric chelate reduction by sunflower (*Helianthus annuus* L.) leaves: influence of light, oxygen, iron-deficiency and leaf age

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Abstract

The presence of ferric chelate reducing activity in sunflower (*Helianthus annuus* L.) leaves has been studied by submerging leaf discs in a solution with Fe(III)-ethylenediaminetetra-acetate (FeEDTA), bathophenanthroline disulphonate (BPDS) and vacuum infiltration. The effect of different factors on the Fe(III) reduction rate was studied. Ferric reduction rate was about 10-fold higher in the light than in darkness. The light effect was greatly inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a photosystem II inhibitor. Several inhibitors of redox systems [*cis*-platinum (II) diamine dichloride (*cis*-platin), *p*-nitrophenylacetate (*p*-NPA) and *p*-hydroxymercuribenzoic acid (*p*HMB)] decreased the FeEDTA reduction rate. The greatest inhibition was produced by the –SH group reagent *p*HMB (17% of control, in light). The FeEDTA reduction rate was much higher in the absence of O₂ than with air or 100% O₂. Superoxide dismutase (SOD) decreased FeEDTA reduction with air in the light. Young leaves reduced Fe(III)-chelate at a higher rate than did older leaves. In iron-deficient plants, leaves did not exhibit enhanced ferric chelate-reducing activity as was observed in roots. It is suggested that at least two different redox systems or two states of the same redox system work in the light and in darkness.

Key words: Iron, leaves, plasma membrane-redox, light, oxygen level.

Introduction

At least seven oxidoreductase enzyme systems associated with plant plasma membrane (PM) have been proposed (Crane *et al.*, 1991). In the root-cell PM, Bienfait (1988) suggested the existence of two different oxidoreductases capable of transferring electrons from the cytosol to several external electron acceptors (e.g. ferricyanide or ferric chelates). One is the 'standard' oxidoreductase, which can reduce Fe(III) in ferricyanide but not in the synthetic ferric chelates, and the other is a 'turbo' oxidoreductase, capable of reducing both ferric chelates and ferricyanide. This latter reductase is induced or stimulated by iron-deficiency stress (Bienfait, 1988; Bienfait and Lüttge, 1988) and is responsible for generating Fe²⁺ prior to uptake by dicotyledonous and non-graminaceous monocotyledonous plants. For a recent review see Moog and Brüggemann (1994). A 3-fold increase in *in vivo* root Fe(III)-chelate reductase activity in tomato (Brüggemann *et al.*, 1990) and an almost 10-fold increase in cucumber (Alcántara *et al.*, 1991) and Fe-efficient sunflower plants (Alcántara and de la Guardia, 1987) was observed in plants grown under iron-deficiency stress.

The reduction rate of ferric chelates is normally lower than that of ferricyanide. Ferric chelate and ferricyanide reductase activities have been separated by isoelectric focusing in PM from Fe-stressed tomato roots (Holden *et al.*, 1991), and Serrano *et al.* (1994) have obtained two distinct NAD(P)H dehydrogenases from onion root PM and both reduced ferricyanide and ferric chelate although at different rates.

Inside the root cells, Fe(II) is oxidized to Fe(III) and

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Abbreviations: BPDS, bathophenanthrolinedisulphonate; *cis*-platin, *cis*-platinum (II) diamine dichloride; e⁻, electron; EDHHA, ethylenediamine-di(*o*-hydroxyphenylacetate); EDTA, ethylenediaminetetraacetic acid; FW, fresh weight; PCMB, *p*-chloromercuribenzoic acid; PCMPS, *p*-chloromercuriphenylsulphonate; *p*HMB, *p*-hydroxymercuribenzoic acid; PM, plasma membrane; *p*-NPA, *p*-nitrophenylacetate.

transported as Fe(III)-citrate, via xylem, to the leaves (Tiffin, 1970). It has been assumed that a second Fe(III) reduction step is needed for the uptake of apoplastic Fe²⁺ by leaf mesophyll cells (Böttger *et al.*, 1991).

Plasma membrane-bound redox systems are ubiquitous to all kind of plant tissues. In photosynthetic cells, and using ferricyanide as electron acceptor, PM redox systems have been found in leaf segments of oat (Dharmawardhane *et al.*, 1987), in free cells of *Asparagus* (Neufeld and Bown, 1987) and in PM vesicles from spinach and sugar beet leaves (Askerlund *et al.*, 1991). However a PM-bound Fe(III)-chelate reductase in leaf cells has only been studied by Brüggemann *et al.* (1993) in *Vigna unguiculata* which reduces Fe(III)-citrate and Fe(III)EDTA before its uptake as Fe²⁺.

The present work was initiated to study ferric chelate (FeEDTA) reduction by sunflower leaves (the 'turbo' oxidoreductase), to determine whether Fe-deficiency stress enhances Fe(III) reduction in leaves, as it does in roots. However, since other factors (level of light and oxygen, leaf age, inhibitors) affected leaf Fe(III)-chelate reducing activity to different degrees, the effects of such factors were studied further. Preliminary results of this research were presented elsewhere (de la Guardia and Alcántara, 1993).

Materials and methods

Plant material

Seeds of sunflower (*Helianthus annuus* L. cv. Sungrow 380) were germinated in darkness in perlite moistened with nutrient solution in a chamber at 27 °C. After 4 or 5 d seedlings were transferred individually to glass vessels containing 700 ml of a continuously aerated nutrient solution with the following composition (mM): 2 Ca(NO₃)₂, 0.75 K₂SO₄, 0.65 MgSO₄, 0.5 KH₂PO₄; (μM): 50 KCl, 10 H₃BO₃, 1 MnSO₄, 0.5 CuSO₄, 0.5 ZnSO₄, 0.05 (NH₄)₆Mo₇O₂₄, 10 FeEDDHA. The pH was adjusted to 6.0 with 0.1 N KOH. The solution was renewed every 5 d and 1 d before an assay was carried out. Plants were grown for 3 or more weeks in a growth chamber at 22 °C day/18 °C night with a 14 h photoperiod and PPF of 350 μmol m⁻² s⁻¹ provided by fluorescent lights (Sylvania Cool White VHO).

Fe(III) reduction by leaves and roots

The Fe(III) reduction assay method was based on an *in vivo* nitrate reductase assay (Mauriño *et al.*, 1986). Most of the work was done with the second pair of true leaves at the end of the expanding period. Leaf discs (5 mm diameter) of areas without major veins were obtained with a cork borer from leaves of identical positions in the plant and from several plants of the same age. Discs were pooled. Samples containing 0.1 g fresh weight (20 to 22 discs per sample and 4 samples per treatment) were placed in 10 ml glass test tubes containing 5 ml of 0.5 mM CaSO₄ solution, washed for 30 min, then the solution was replaced by 5 ml of assay medium. The standard assay medium consisted of 0.5 mM CaSO₄, 1 mM KCl, 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM FeEDTA, and 0.3 mM BPDS. The tubes containing the medium and leaf discs

were sealed with rubber stoppers (Arthur H. Thomas Co., USA) and were evacuated (2.6 kPa pressure) for solution infiltration during 10 min (two periods of 5 min with vacuum released between them). The evacuation was performed either in the light (Day-light Standard, 200 μmol m⁻² s⁻¹ or Cool White VHO, 350 μmol m⁻² s⁻¹) or in darkness, with the test tubes wrapped in aluminium foil. In the assays in darkness (Tables 1, 2, 3; Figs 5, 6), test tubes were incubated after infiltration in a water bath (incubation phase) in darkness at 25 °C for 1 or 2 h, and Fe(III) reduction was then determined. Reduction rates were determined as Fe(II)BPDS by measuring the absorbance at 535 nm, after subtraction of the appropriate blanks (without leaf tissue), using an absorption coefficient of 22.14 mM⁻¹ cm⁻¹. The vacuum method was necessary as without evacuation no Fe(III) reduction was detectable.

In some experiments the release of reducing compounds (leakage) from leaf tissues was determined. After incubation in a medium without Fe and BPDS, the solution was filtered to remove leaf discs and small debris; FeEDTA and BPDS were then added, and the solution was incubated for an additional 30 min in darkness.

To study the time-course of Fe(III) reduction, higher volumes of assay medium (75 ml) and higher amounts of leaf discs (0.75 g) were used in a 250 ml Erlenmeyer filtering flask connected to a vacuum pump. This system allowed periodic sampling for absorbance determination. Samples were returned to the flask thereafter.

Fe(III) reduction by roots of intact plants was determined as previously described (Alcántara *et al.*, 1991). In this case the volume of the solution for preincubation and reduction was 500 ml.

Inhibitors and enzymes

The effects of the following chemicals were studied: DCMU (10 μM) dissolved in methanol:water (80:20, v/v), *p*-hydroxymercuribenzoic acid (*p*HMB) (0.5 mM) dissolved in 0.1 M KOH, *p*-nitrophenylacetate (*p*-NPA) (0.1 mM) dissolved in methanol, *cis*-platinum (II) diamine dichloride (*cis*-platin) (0.2 mM) dissolved in 0.1 M KOH, catalase (120 IU ml⁻¹), SOD (60 IU ml⁻¹). All chemicals were from Sigma Chemical Co.

Proper controls were performed with methanol alone. In experiments with chemicals a 5 min pretreatment with the chemical was given with the vacuum procedure. In assays with oxygen (O₂), air or nitrogen (N₂), the gases were bubbled in each test-tube by a fine glass tube connected to a small pump (for air) or to an O₂ or N₂ cylinder.

The chlorophyll content in leaves was determined after 8 h extraction with methanol. All treatments had four replications, and each experiment was repeated at least twice.

Results

Fe(III) reduction in different media

Experiments were conducted following the method used for *in vivo* nitrate reductase assay (Mauriño *et al.*, 1986). In the first assays only one absorbance determination was recorded after the two phases of infiltration and incubation.

As shown in Table 1, sunflower leaf discs reduced externally applied FeEDTA. Higher Fe(III) reduction rates were obtained in the media with 50 mM potassium

Table 1 Fe(III)-EDTA reduction by sunflower leaves and by their leakages in different incubation media

Leaf discs were submerged in glass tubes containing different media which were vacuum evacuated for 10 min in natural laboratory light ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$), and were then incubated 2 h at 25°C in darkness. Fe(II)-BPDS complex was determined by measuring the absorbance at 535 nm. Parallel assays were performed with leaf discs in the same medium without FeEDTA and BPDS, which were added to the medium after incubation and filtration. Data are means \pm SE, $n=4$.

Incubation medium ^a	Fe(III) reduced by		Final pH
	Leaves ($\text{nmol g}^{-1} \text{ fresh wt min}^{-1}$)	Medium ^b ($\text{nmol g}^{-1} \text{ fresh wt min}^{-1}$)	
CaSO ₄ , KCl, pH 5.5	4.4 \pm 0.2	0.3 \pm 0.1 (7)	6.48 \pm 0.02 6.42 \pm 0.04
CaSO ₄ , KCl, phosphate buffer pH 5.5	5.3 \pm 0.1	1.4 \pm 0.2 (26)	5.50 \pm 0.01 5.50 \pm 0.02
CaSO ₄ , KCl, phosphate buffer pH 7.0 (standard)	5.3 \pm 0.3	0.5 \pm 0.1 (9)	7.00 \pm 0.01 7.00 \pm 0.01

^a Concentrations: 0.5 mM CaSO₄; 1 mM KCl; 50 mM K₂HPO₄/KH₂PO₄. Moreover, all media had 0.1 mM FeEDTA and 0.3 mM BPDS

^b In parentheses; the Fe(III) reduced by the medium (leakage) as a percentage of total Fe(III) reduced by leaves.

phosphate buffer (pH 5.5 or 7.0) than in the medium without phosphate, however, higher amounts of reducing agents (or leakages) appeared in the incubation medium buffered at pH 5.5 (26%) than at pH 7.0 (9%).

If the medium was unbuffered, the pH of the medium was increased from 5.50 to 6.48 or 6.42 (Table 1) by the leaf discs whether FeEDTA was present or not.

In experiments with Fe(III)-citrate instead of FeEDTA (data not shown), Fe(III) reduction was more variable, especially in light. Higher Fe(III) photoreduction was observed in the controls without leaves and less Fe(III) was reduced by leaves with citrate than with EDTA.

From the above results the medium with 0.5 mM CaSO₄, 1 mM KCl and 50.0 mM potassium phosphate buffer pH 7.0 plus FeEDTA and BPDS was chosen as the standard assay medium for further experiments.

Light, dark and DCMU

Since light during the vacuum phase influences Fe(III) reduction, experiments were performed to determine Fe(III) reduction either in the light or in darkness.

Results in Fig. 1 show that the reduction rate was about 10-fold higher in the light than in darkness. At higher irradiance, a higher Fe(III) reduction rate was observed. The addition of 10 μM DCMU (a PSII inhibitor) to the assay medium decreased the Fe(III) reduction in the light to 6.5 $\text{nmol g}^{-1} \text{ min}^{-1}$, which was almost the level of reduction in darkness (4.2 $\text{nmol g}^{-1} \text{ min}^{-1}$).

For the time-course of Fe(III) reduction, a different working system was designed with the leaf discs submerged in a larger volume of medium to allow periodical sampling. Results in Fig. 2 show that Fe(III) reduction in the light was nearly linear up to 30 min. In darkness, the Fe(III) reduction rate was so low that the period was extended for 120 min to increase the accuracy of the measurements. Leakage of Fe(III) reducing substances was similar in the light and in darkness, with most of them produced in the first 10 min and a small increase

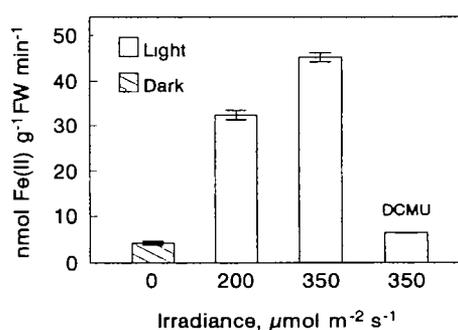


Fig. 1. Effect of irradiance level and DCMU treatments on Fe(III)-EDTA reduction by sunflower leaves during the 10 min of vacuum phase. Leaf discs were submerged in the standard medium: 0.5 mM CaSO₄, 1 mM KCl and 50 mM K₂HPO₄/KH₂PO₄, pH 7.0, 0.1 mM FeEDTA and 0.3 mM BPDS. DCMU concentration was 10 μM . Shown are means \pm SE ($n=4$).

was detected thereafter. In the light, after 30 min, leakage was responsible for less than 10% of the total Fe(III) reduced by the leaves, while in darkness, the leakage at 120 min, represented about 25%. Photoreduction of FeEDTA was observed in the medium without leaves (blank) at a steady rate that represented about 20%.

Effect of inhibitors and enzymes

Several substances with a possible effect on redox systems were used in two types of assay: (i) in the light during 10 min in vacuum, (ii) in darkness during 10 min vacuum plus 2 h incubation.

The results (Table 2) showed that all these substances inhibited Fe(III) reduction in both conditions, light and darkness. The addition of *p*HMB, which reacts with -SH groups of proteins, showed the highest degree of inhibition, the redox activity being 17% of control in the light and 42% in darkness.

The absolute value of Fe(III) reduced by the leakage of reducing agents was low and not very different to control value for all inhibitors.

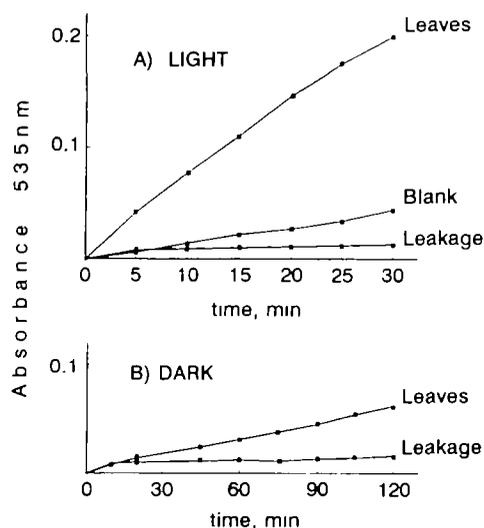


Fig. 2. Time-course of Fe(III)-EDTA reduction by leaves and their leakages in: (A) light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$) and (B) dark, and photoreduction of the incubation medium (Blank). Leaf discs (0.75 g) were submerged in 75 ml of standard medium in an Erlenmeyer filtering flask connected to a vacuum pump and 3 ml samples were taken periodically for absorbance determination. Reduction was followed for 30 min in light and 120 min in dark.

As Fe(III) reduction may be mediated by superoxide or peroxide radicals, superoxide dismutase (SOD) and catalase enzymes were added to the test tubes containing the samples and bubbled with air. The redox activity was inhibited by SOD in the light, to 61% of the control (Table 3), but increased in darkness. Catalase, in contrast, increased Fe(III) reduction, especially in the light.

Level of oxygen

The level of oxygen in the medium showed a large effect on the rate of Fe(III) reduction. The higher the O_2 concentration present, the less Fe(III) was reduced (Fig. 3). The net reduction rate was much higher with oxygen deprivation than with air or 100% O_2 . The effect was similar in the light and in darkness, but with lower rates of Fe(III) reduction in darkness.

Table 2. Effect of several inhibitors on Fe(III)-EDTA reduction by sunflower leaves and by their incubation medium

Light ($350 \mu\text{mol m}^{-2} \text{s}^{-1}$) and dark conditions correspond to independent assays. See other details in Materials and methods. Data are means \pm SE, $n=4$.

Treatment	Light, 10 min in vacuum				Dark, 10 min in vacuum + 2 h incubation			
	Leaves (nmol g^{-1} fr. wt min^{-1})	Medium (nmol g^{-1} fr. wt min^{-1})	Leaves only (nmol g^{-1} fr. wt min^{-1})	(%)	Leaves (nmol g^{-1} fr. wt min^{-1})	Medium (nmol g^{-1} fr. wt min^{-1})	Leaves only (nmol g^{-1} fr. wt min^{-1})	(%)
Control	44.7 ± 1.1	1.3 ± 0.1 (3) ^a	43.4	100	4.4 ± 0.2	0.9 ± 0.2 (20) ^a	3.5	100
<i>cis</i> -platin	37.2 ± 1.8	2.2 ± 0.3 (6)	35.0	81	3.8 ± 0.6	0.6 ± 0.2 (16)	3.2	91
<i>p</i> -nitrophenyl-acetate	30.3 ± 1.8	1.5 ± 0.1 (5)	28.8	66	3.1 ± 0.6 (39)	1.2 ± 0.4	1.9	54
<i>p</i> HMB	8.6 ± 0.3	1.3 ± 0.3 (15)	7.3	17	2.6 ± 0.8 (42)	1.1 ± 0.7	1.5	42

^aIn parentheses, Fe(III) reduced by the incubation medium as a percentage of total Fe(III) reduced by leaves.

Table 3. Effect of catalase and superoxide dismutase (SOD) on Fe(III)-EDTA reduction in the light and darkness

After 10 min of vacuum in dim light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) samples were incubated by bubbling air in each tube either in the light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$), for 20 min, or in darkness for 1 h. Data are means \pm SE, $n=4$.

Treatment	Light (nmol g^{-1} fr. wt min^{-1})	Darkness (nmol g^{-1} fr. wt min^{-1})
Control	29.0 ± 0.7 (100) ^a	1.3 ± 0.1 (100) ^a
Catalase (120 IU ml^{-1})	35.2 ± 1.7 (121)	1.5 ± 0.1 (113)
SOD (60 IU ml^{-1})	17.8 ± 1.0 (61)	1.9 ± 0.1 (144)

^aPercentage of control. Values are given for the period of incubation (20 min or 1 h), without leakage discount.

Although the Fe(III) reduction rates by the media are not presented in Fig. 3, in the light they were below 10% for all O_2 levels, and in darkness they were higher with N_2 (25%) than with the other two O_2 levels (less than 20%).

Iron deficiency

When plants were deprived of iron ($-$ Fe plants), the new leaves became chlorotic and young lateral roots showed the characteristic Fe-deficiency stress-response mechanisms: enhanced Fe(III) reducing capacity, subapical swelling and acidification of the medium. The Fe(III) reducing capacity was determined in roots and leaves of the same plants (Fig. 4). While roots increased Fe(III) reduction 4-fold upon iron deficiency, leaf discs did not increase activity either in darkness or in light. Moreover, in the light $-$ Fe leaves reduced at a lower rate than $+$ Fe leaves, although it must be considered that the mean chlorophyll content was 1.83 mg g^{-1} FW for $+$ Fe leaves and 0.95 mg g^{-1} FW for $-$ Fe leaves.

Leakage rates (not shown in Fig. 4) were similar to previous assays, without significant differences between $-$ Fe and $+$ Fe leaves.

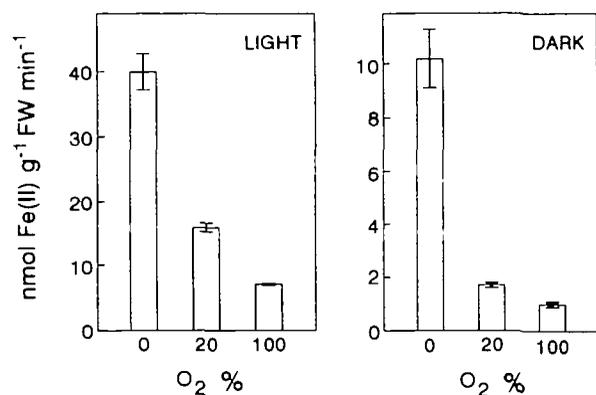


Fig. 3. Effect of the level of oxygen on Fe(III)-EDTA reduction by sunflower leaves in light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$) and dark. The three O₂ levels were obtained by bubbling N₂, air or O₂ in the tubes containing the samples, after the vacuum phase. Values are the net rates of Fe(III) reduced by leaves after discounting Fe(III) reduced by leakage. Bubbling duration was 30 min for light and 1 h for dark. Shown are means \pm SE ($n=4$).

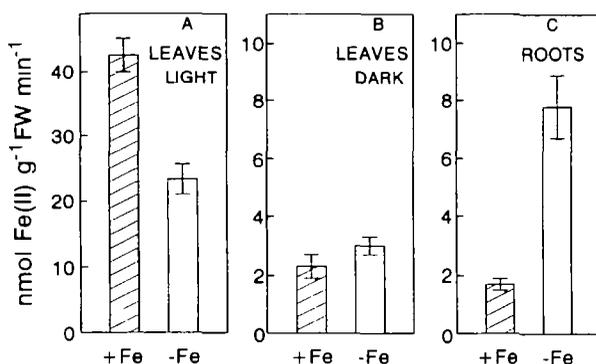


Fig. 4. Effect of iron deficiency on Fe(III)-EDTA reduction by sunflower leaves and roots. Plants were grown in nutrient solution with $20 \mu\text{M}$ FeEDHHA for 21 d. Then solutions were changed, half of the plants to the same solution (+Fe), and half of them to the same solution without iron (-Fe). After 5 d of growth in these solutions, FeEDTA reduction was determined in the following conditions: (A) 10 min of vacuum in the light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$). (B) 2 h of dark incubation after 10 min vacuum in darkness. (C) 4 h of dark incubation for roots, holding the shoot in light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$). Given values are without leakage rates subtracted. Shown are means \pm SE ($n=4$).

Leaf age

In order to investigate the influence of leaf age or leaf position on FeEDTA reduction, seeds were germinated every 10 d to get plants of different ages at the same time.

As Fig. 5 shows, the activity declined with age, especially in the light.

Discussion

The results of the present paper show that sunflower leaves can reduce external Fe(III) chelates, both in the light and in darkness, the Fe(III) reduction rate being 10 times higher in the light than in darkness (Fig. 1).

Iron-deficiency did not enhance Fe(III) reducing capa-

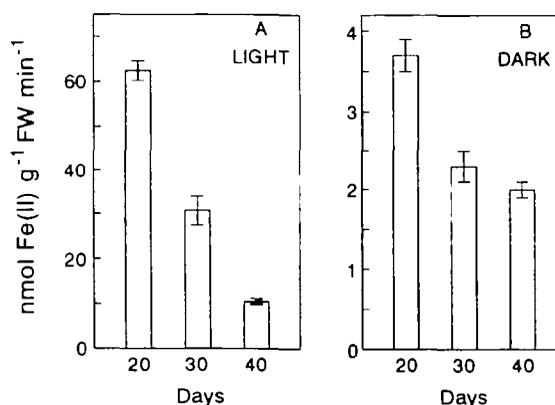


Fig. 5. Effect of sunflower leaf age on Fe(III)-EDTA reduction. Leaf number 3 (counted from cotyledons) from 20, 30 and 40-d-old plants was used. (A) During 10 min of vacuum in the light ($350 \mu\text{mol m}^{-2} \text{s}^{-1}$). (B) During 2 h of dark incubation after the vacuum. Given values are without leakage rates subtracted. Shown are means \pm SE ($n=4$).

city in leaves as it did in roots (Fig. 4). Furthermore, and in contrast to roots, the Fe(III) reduction rate in the light was lower in -Fe leaves than in +Fe leaves.

One question to be considered is the existence of substances capable of reducing ferric chelates inside leaf cells. Mehrotra and Gupta (1990) showed, by homogenizing leaf material from 12 species, that the extracts exhibited strong Fe(III) reducing activity, which was attributed to ascorbic acid and phenols. In these assays no extractions were done, but rather pieces of intact tissue incubated in a medium were used. During long incubation or with some chemicals, reducing agents may leak from the cells. For this reason, in most of the assays the reduction capacity of the medium was determined. Generally, the Fe(III) reduction by leakage was below 10% of the total Fe(III) reduced in short-term experiments in the light, but in certain conditions, especially in darkness, the percentage was higher (Tables 1, 2; Fig. 2).

Other workers that studied ferricyanide reduction by leaf cells related e⁻ transport in PM redox systems to H⁺ extrusion (Böttger and Lüthen, 1986). In these studies a redox-related pH decrease was not observed during ferric chelate reduction. By contrast, the pH of the external medium increased from 5.50 to 6.42 or 6.48 (Table 1), with or without Fe(III) reducing activity.

The stimulation of ferricyanide reduction by light in leaves or mesophyll cells has been shown by others (Dharmawardhane *et al.*, 1987; Neufeld and Bown, 1987), and a strong inhibition of the stimulating effect of light was produced by DCMU (Dharmawardhane *et al.*, 1987). Brüggemann *et al.* (1993), using FeEDTA as electron acceptor, found a 3-fold increase in Fe(III) reduction rate in red light with respect to darkness. In the present work a stimulating effect of light and inhibition by DCMU on Fe(III) reduction was also observed (Fig. 1).

The reason for the light-dark difference in Fe(III)

reduction could be similar to the one given for nitrate reduction in leaves. In the light, chloroplasts produce and export to the cytosol triose phosphates, which generate NAD(P)H, the e^- donor for NO_3^- reduction by nitrate reductase (Huppe and Turpin, 1994) or for redox systems in PM to reduce external e^- acceptors, as FeEDTA (Bienfait, 1988). In darkness, the e^- for reduction can be provided by several sources: glycolysis, pentose phosphate pathway, ascorbate or glutathione (Böttger *et al.*, 1991; Huppe and Turpin, 1994).

The strong inhibition of Fe(III) reduction produced by the sulphhydryl-group reagent *p*HMB (Table 2) suggests that this PM redox system has functional SH-groups. Luster and Buckhout (1989) showed a strong inhibition of ferricyanide reducing activity by PCMPS (a sulphhydryl-group inhibitor), in an electron transport protein isolated from maize root PM, and Serrano *et al.* (1994) also found inhibition of two dehydrogenases from onion root PM by *N*-ethylmaleimide or by *p*HMB.

Peroxidases can catalyse the reduction of Fe(III) on the surface of plant cells, and catalase suppress peroxidase activity due to its effect on H_2O_2 (Penel and Castillo, 1991). These results show that catalase did not inhibit Fe(III) reduction (Table 3), which suggests no participation of peroxidase in Fe(III) reduction by sunflower leaves. However, the inhibition produced by SOD in air and light (Table 3) suggests that superoxide radical formation may contribute at least partially to Fe(III) reduction activity in the light.

The different reduction rates in the light and darkness (Fig. 1) and the different effect of inhibitors and catalase and SOD enzymes (Tables 2, 3) on both conditions, suggests that different redox systems are operating in the light and in darkness, without discarding a possible change of state in a unique redox system, produced by light conditions. Darmawardhane *et al.* (1987) proposed two classes of redox activities for ferricyanide reduction at the surface of oat mesophyll cells, and only one of these redox systems was stimulated by the light.

The strong inhibition of Fe(III) reduction by O_2 (Fig. 3) could be explained either by competition for NAD(P)H between mitochondria (in the presence of O_2) and the PM redox systems or by competition for electrons in the reduction process between Fe(III) and O_2 . Both possibilities could occur in darkness. Inhibition of NO_3^- reduction by O_2 , in darkness, has been observed and explained as competition between NO_3^- reduction and dark respiration for cytoplasmic-reducing equivalents (Mauriño *et al.*, 1986). Oxygen has been proposed as one electron acceptor competing with ferricyanide in PM redox systems of maize roots (Böttger and Lüthen, 1986; Döring *et al.*, 1990). Fe(III) ion and O_2 reduction have been found at the surface of protoplasts and cells of *Acer pseudoplatanus* (Macri *et al.*, 1992). The results in the light, when respiratory activity is partially inhibited

(Kröner, 1995), give support to the idea of competition between Fe(III) and O_2 in the reduction process. However, another possibilities must be considered: (i) the redox mechanisms depend on functional $-\text{SH}$ groups that can be oxidized at low rates by molecular oxygen (Bienfait and Lüttge, 1988), (ii) re-oxidation of Fe(II) by high O_2 level, before being trapped by BPDS.

In Fe-deficient plants ($-\text{Fe}$ plants, Fig. 4A, B), the low level of iron in the leaves decreased the chlorophyll content (from 1.83 to 0.95 mg g^{-1} FW) and, consequently, photosynthesis rate. Terry (1980) found that the maximum rate of photosynthesis per unit leaf area was linearly related to chlorophyll content in sugar beet leaves. So, in chlorotic leaves in the light, presumably fewer compounds capable of generating NAD(P)H would be exported from the chloroplast to the cytosol. In this condition, reduction of external Fe(III)-chelate (expressed on a leaf fresh weight basis), which depends on cytosolic NAD(P)H (Bienfait, 1988), would also decrease (Fig. 4A). Brüggemann *et al.* (1993) did not obtain differences in ferric chelate reduction between $-\text{Fe}$ and $+\text{Fe}$ leaves of *Vigna unguiculata*, but they expressed the results on a chlorophyll content basis. In darkness, where Fe(III) reduction occurred at a lower rate, no significant difference was found between $-\text{Fe}$ and $+\text{Fe}$ leaves (Fig. 4B). These results suggest that Fe(III)-chelate PM redox systems are in the same amount in both $-\text{Fe}$ and $+\text{Fe}$ leaves, the Fe(III) reduction rate depending on the level of cytosolic NAD(P)H.

The change in FeEDTA reduction activity with leaf age (Fig. 5) could be related to the change in photosynthesis rate, to ontogenic transition of leaf from sink to source or to stage of growth.

In conclusion, the results confirm the existence of Fe(III)-chelate reductase activity in intact sunflower leaf mesophyll, as demonstrated by Brüggemann *et al.* (1993) in the leaf mesophyll of *Vigna unguiculata*, as a prerequisite for Fe(III) reduction before iron uptake by leaf cells.

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