

## Effect of iron deficiency induced changes on photosynthetic pigments, ribulose-1,5-bisphosphate carboxylase, and photosystem activities in field grown grapevine (*Vitis vinifera* L. cv. Pinot noir) leaves

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### Abstract

The effect of iron deficiency on photosynthetic pigments, ribulose-1,5-bisphosphate carboxylase (RuBPC), and photosystem activities were investigated in field grown grapevine (*Vitis vinifera* L. cv. Pinot noir) leaves. The contents of chlorophyll (Chl) (*a+b*) and carotenoids per unit fresh mass showed a progressive decrease upon increase in iron deficiency. Similar results were also observed in content of total soluble proteins and RuBPC activity. The marked loss of large (55 kDa) and small (15 kDa) subunits of RuBPC was also observed in severely chlorotic leaves. However, when various photosynthetic electron transport activities were analysed in isolated thylakoids, a major decrease in the rate of whole chain ( $H_2O \rightarrow$  methyl viologen) electron transport was observed in iron deficient leaves. Such reduction was mainly due to the loss of photosystem 2 (PS2) activity. The same results were obtained when  $F_v/F_m$  was evaluated by Chl fluorescence measurements in leaves. Smaller inhibition of photosystem 1 (PS1) activity was also observed in both mild and severely chlorotic leaves. The artificial electron donors, diphenyl carbazide and  $NH_2OH$ , markedly restored the loss of PS2 activity in severely chlorotic leaves. The marked loss of PS2 activity was evidently due to the loss of 33, 23, 28-25, and 17 kDa polypeptides in iron deficient leaves.

*Additional key words:* carotenoids; chlorophyll; fluorescence; electron transport; photosystem 1 and 2; proteins; ribulose-1,5-bisphosphate carboxylase.

### Introduction

Iron deficiency limiting crop productivity is a major problem in high-value fruit tree crops in the Mediterranean region and in other semi-arid environments (Sanz *et al.* 1992). Iron deficient plants are characterised by the development of a pronounced interveinal chlorosis similar to that caused by magnesium deficiency but occurring first on the youngest leaves. Interveinal chlorosis is sometimes followed by chlorosis of the veins, so the whole leaf then becomes yellow. In severe cases, the leaves become white with necrotic lesions (Abadía 1992). The reason why iron deficiency results in a rapid inhibition of Chl formation is not fully understood, even though this problem has been studied for many years.

Iron deficiency lowers the amount of photosynthates formed (Srivastava *et al.* 1998). The inhibition of Chl formation under iron deficiency is, at least in part, the result of an impaired protein synthesis. The requirement of protein synthesis is reflected in the leaves by a drastic decline in the number of ribosomes, the sites of protein synthesis (Lin and Stocking 1978). A peculiarity of iron deficiency is a greater decline in protein synthesis in the chloroplasts of leaf cells than in cytoplasm (Shetty and Miller 1966). As the severity of iron deficiency increases the protein content per leaf area, the leaf cell volume and the number of chloroplasts remain unaffected, whereas the chloroplast volume and the amount of protein per

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*Abbreviations:* Chl - chlorophyll; DCBQ - 2,6-dichloro-*p*-benzoquinone; DCPIP - 2,6-dichlorophenol indophenol; DPC - diphenyl carbazide; DTT - dithiothreitol;  $F_0$  - minimal fluorescence;  $F_m$  - maximum fluorescence; kDa - kilodalton; LHCP - light-harvesting chlorophyll protein; LSU - large subunit; MV - methyl viologen; PPFD - photosynthetic photon flux density; PS - photosystem; RuBPC - ribulose-1,5-bisphosphate carboxylase; SDS-PAGE - sodium dodecylsulphate polyacrylamide gel electrophoresis; SiMo - silicomolybdate; SSU - small subunit.

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chloroplast decline (Terry 1983).

The most obvious characteristic of the leaves from iron deficient plants is chlorosis, due to low concentrations per area of Chls and carotenoids (Abadía and Abadía 1993, Morales *et al.* 1994). However, not all photosynthetic pigments are decreased to the same extent by iron deficiency, xanthophylls being less affected than Chls and  $\beta$ -carotene (Morales *et al.* 1990, 1994). One of the distinctive characteristics of iron deficiency in field crops is the lack of correlation between leaf iron content and chlorosis (Morales *et al.* 1998). This has been termed the 'chlorosis paradox' (Romheld 1999). Therefore, leaf Chl contents are generally used to monitor iron chlorosis.

Changes in the structure and composition of photosynthetic membranes caused by iron deficiency were investigated in different plants (Terry and Abadía 1986). At the structural level, a drastic reduction in thylakoid membranes containing few grana stacks was reported in maize (Stocking 1975), sugar beet (Platt-Aloia *et al.* 1983), and bean (Pushnik and Miller 1982). Lower efficiency of PS2 photochemistry ( $F_v/F_m$ ) was found in plants affected by iron deficiency. The ratio of variable to maximum fluorescence decreased in iron deficient cyanobacteria (Riethman and Sherman 1988) and sugar beet grown in controlled environments (Morales *et al.* 1990, 2000). Leaves of iron deficient higher plants have a reduced number of grana and stroma lamellae per chloro-

plast (Spiller and Terry 1980). This is accompanied by a decrease in all membrane components, including electron carriers in the photosynthetic electron transport chain (Spiller and Terry 1980, Terry 1983) and the light-harvesting Chls and carotenoids (Morales *et al.* 1990, 1994, Abadía and Abadía 1993, Abadía *et al.* 2000).

Iron chlorosis in sugar beet also decreases the RuBPC capacity, through diminished ribulose-1,5-bisphosphate carboxylase/oxygenase activity (Taylor and Terry 1986, Winder and Nishio 1995) and down-regulation of gene expression (Winder and Nishio 1995). Winder and Nishio (1995) proposed that changes in light harvesting, electron transport, and carbon fixation caused by iron deficiency are well co-ordinated.

Most of the knowledge about iron deficient plants has been obtained with annual plants (bean, sugar beet, barley, and sunflower) grown in hydroponics. These plants are usually grown in greenhouses or cultivation chambers under controlled irradiance, photoperiod, temperature, and humidity. Only few studies have been focused on the consequences of iron deficiency on the photosynthetic performance and structural consequences of plants developed in natural environments. In the present work we determined the contents of photosynthetic pigments and RuBPC, and photosynthetic electron transport activities in iron deficient grapevine plants grown in the field.

## Materials and methods

**Plants:** Leaves were harvested on grapevine (*Vitis vinifera* L. cv. Pinot noir) plants grown under field conditions in San Michele all' Adige, Italy. We classified leaf samples into three groups according to their Chl content per leaf unit area as severely chlorotic [less than 100  $\mu\text{mol}(\text{Chl}) \text{ m}^{-2}$ ] and mild chlorotic [average content of ca. 200  $\mu\text{mol}(\text{Chl}) \text{ m}^{-2}$ ]; leaves above 300  $\mu\text{mol}(\text{Chl}) \text{ m}^{-2}$  were classified as control.

**Pigment analysis:** Chl content was estimated using the SPAD-502, Minolta system which was calibrated against total Chl measured by extraction. Chl was extracted with 100 % acetone from liquid  $\text{N}_2$  frozen leaf discs and stored at  $-20^\circ\text{C}$ . Chl and carotenoids were analysed spectrophotometrically according to Lichtenthaler (1987).

**Modulated Chl fluorescence in leaves** was measured on leaf discs using a PAM-2000 fluorometer (H. Walz, Effeltrich, FRG).  $F_0$  was measured by switching on the modulated radiation 0.6 kHz; PPFD was less than 0.1  $\mu\text{mol} \text{ m}^{-2} \text{ s}^{-1}$  at the leaf surface.  $F_m$  was measured at 20 kHz with a 1-s pulse of 6000  $\mu\text{mol} \text{ m}^{-2} \text{ s}^{-1}$  of 'white light'.

**Activities of electron transport:** Thylakoid membranes were isolated from the leaves as described by Berthold *et al.* (1981). Whole chain electron transport ( $\text{H}_2\text{O} \rightarrow \text{MV}$ ) was measured according to Armond *et al.* (1978) and partial reactions of photosynthetic electron transport mediated by PS2 ( $\text{H}_2\text{O} \rightarrow \text{DCBQ}$ ;  $\text{H}_2\text{O} \rightarrow \text{SiMo}$ ) and PS1 ( $\text{DCPIP} \rightarrow \text{MV}$ ) were measured as described by Noorudeen and Kulandaivelu (1982). Thylakoids were suspended at 10  $\text{g}(\text{Chl}) \text{ m}^{-3}$  in the assay medium containing 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM  $\text{MgCl}_2$ , 5 mM  $\text{NH}_4\text{Cl}$ , and 100 mM sucrose supplemented with 500  $\mu\text{M}$  DCBQ and 200  $\mu\text{M}$  SiMo.

**The rate of DCPIP photoreduction** was determined as decrease in absorbance at 590 nm using a Hitachi spectrophotometer. The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 5 mM  $\text{MgCl}_2$ , 10 mM NaCl, 100 mM sucrose, 100  $\mu\text{M}$  DCPIP, and thylakoid membranes equivalent to 20  $\mu\text{g}$  of Chl. Where mentioned, the concentrations of  $\text{MnCl}_2$ , DPC, and  $\text{NH}_2\text{OH}$  were 5, 0.5, and 5 mM, respectively.

**Total soluble proteins** were extracted by grinding two leaves (0.3-0.5 g fresh mass) in a mortar with 6 cm<sup>3</sup> of 100 mM Tris-HCl, pH 7.8 containing 15 mM MgCl<sub>2</sub>, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 10 mM PMSF in the presence of liquid nitrogen. Homogenates were filtered through nylon cloth. After centrifugation at 11 000×g for 10 min, the content of soluble proteins was determined in the supernatant according to Lowry *et al.* (1951).

**Extracts and assay of RuBPC activity:** Fully expanded leaves were cut into small pieces and homogenised in a grinding medium of 50 mM Tris-HCl, pH 7.8, 10 mM MgCl<sub>2</sub>, 5 mM DTT, and 0.25 mM EDTA. The extract was clarified by centrifugation at 10 000×g for 10 min.

## Results

**Changes in contents of Chl and carotenoids:** When determined per unit fresh mass, the Chl (*a+b*) content was drastically reduced in severely chlorotic leaves (Table 1).

The clear supernatant was decanted slowly and used as the RuBPC. The RuBPC activity was measured as described by Nedunchezian and Kulandaivelu (1991).

**SDS-PAGE:** Thylakoids and crude leaf extracts were separated using the polyacrylamide gel system of Laemmli (1970), with following modifications. Gels consisted of 12 % linear polyacrylamide containing 4 M urea. Samples were solubilised at 20 °C for 5 min in 2 % (m/v) SDS, 60 mM DTT, and 8 % sucrose using SDS-Chl ratio of 20:1. Electrophoresis was performed at 20 °C with constant current of 5 mA. Gels were stained in methanol/acetic acid/water (4 : 1 : 5, v/v/v) containing 0.1 % (m/v) Coomassie brilliant blue R and de-stained in methanol/acetic acid/water (4 : 1 : 5, v/v/v).

As much as 42 and 84 % reduction was observed in mild and severely chlorotic leaves, respectively. Similar trend was noticed for carotenoids (Table 1).

Table 1. Differences in contents of chlorophyll (Chl) and carotenoids, fluorescence induction characteristics  $F_0$ ,  $F_v$ , and  $F_v/F_m$ , photosystem and ribulose biphosphate carboxylase (RuBPC) activities, and protein contents in control and different stages of iron deficient plants. Values represent averages of 5 experiments. Means and  $\pm$  standard errors (SE).

		Control	Mild chlorotic	Severely chlorotic
Chl ( <i>a+b</i> )		2.25 $\pm$ 0.19	1.30 $\pm$ 0.11	0.36 $\pm$ 0.08
Carotenoids		0.85 $\pm$ 0.08	0.56 $\pm$ 0.06	0.20 $\pm$ 0.04
$F_0$		98 $\pm$ 2.40	153 $\pm$ 5.10	139 $\pm$ 2.80
$F_v$		528 $\pm$ 9.20	418 $\pm$ 6.80	298 $\pm$ 4.60
$F_v/F_m$		0.81 $\pm$ 0.06	0.63 $\pm$ 0.04	0.50 $\pm$ 0.02
Whole electron transport chain	H <sub>2</sub> O $\rightarrow$ MV	148.6 $\pm$ 5.0	96.6 $\pm$ 2.0	50.5 $\pm$ 2.0
Photosystem 1	DCPIP <sub>H</sub> $\rightarrow$ MV	368.5 $\pm$ 8.0	351.8 $\pm$ 6.4	324.7 $\pm$ 6.0
Photosystem 2	H <sub>2</sub> O $\rightarrow$ DCBQ	240.2 $\pm$ 5.1	165.7 $\pm$ 2.9	129.8 $\pm$ 3.0
	H <sub>2</sub> O $\rightarrow$ SiMo	124.3 $\pm$ 3.0	119.3 $\pm$ 2.0	48.4 $\pm$ 1.5
	H <sub>2</sub> O $\rightarrow$ DCPIP	192.2 $\pm$ 6.0	149.9 $\pm$ 6.2	73.0 $\pm$ 4.1
	DPC $\rightarrow$ DCPIP	205.8 $\pm$ 7.5	164.6 $\pm$ 4.6	174.9 $\pm$ 5.6
	MnCl <sub>2</sub> $\rightarrow$ DCPIP	194.9 $\pm$ 6.2	153.9 $\pm$ 5.4	77.9 $\pm$ 3.8
	NH <sub>2</sub> OH $\rightarrow$ DCPIP	209.6 $\pm$ 8.1	169.9 $\pm$ 4.9	167.6 $\pm$ 5.1
RuBPC		56.4 $\pm$ 3.2	32.7 $\pm$ 2.8	14.6 $\pm$ 1.2
Proteins		48.6 $\pm$ 3.4	36.9 $\pm$ 2.6	23.3 $\pm$ 2.2

**Changes in photosynthetic activities:** To obtain information on PS2 activity,  $F_v/F_m$ , that reflects the quantum yield of PS2 photochemistry (Krause and Weis 1991), was determined *in vivo* using leaf discs which had been dark-adapted for 30 min. At the beginning of the experiment,  $F_v/F_m$  in control leaves was 0.810. As shown in Table 1, the  $F_v/F_m$  ratio decreased to around 0.630 in mild chlorotic and to 0.500 in severely chlorotic leaves. In mild chlorotic leaves an increase of  $F_0$  was observed. Similar  $F_0$  was observed in leaves in anaerobic condition or in leaves from plants grown in deficiency of Mg and S. In both cases, the increase of  $F_0$  was ascribed to the

presence of reduced  $Q_A^-$  (Šetlik *et al.* 1990, Godde and Dannehl 1994). However, when photosynthetic electron transport was studied using isolated thylakoids from control and iron deficient leaves, photosynthetic electron transport from DCPIP<sub>H</sub>  $\rightarrow$  MV (PS1) was reduced by about 9 and 16 % in mild and severely chlorotic leaves, respectively. PS1 activity is thus less sensitive to iron deficiency than PS2 (Misra and Srivastava 1994, Nedunchezian *et al.* 1997).

The PS2-mediated electron transport was measured in thylakoids isolated from the iron deficient leaves. Photosynthetic electron transport from H<sub>2</sub>O  $\rightarrow$  DCBQ

and  $\text{H}_2\text{O} \rightarrow \text{SiMo}$  was reduced by about 31 and 4 % in mild chlorotic leaves and by 46 and 61 % in severely chlorotic leaves, respectively (Table 1). A similar trend was also detected in the activity of whole chain electrontransport ( $\text{H}_2\text{O} \rightarrow \text{MV}$ ) in both mild and severely chlorotic leaves (Table 1).

To locate the possible site of inhibition in the PS2 reaction, we followed the DCPIP reduction supported by various exogenous electron donors in thylakoids of control and iron deficient leaves. Wydrzynski and Govindjee (1975) show that  $\text{MnCl}_2$ , DPC,  $\text{NH}_2\text{OH}$ , and HQ may donate the electrons to the intermediates  $\text{Z}_1$  and

$\text{Z}_2$  of the PS2 reaction. Table 1 shows the electron transport activity of PS2 in the presence and absence of three of the above compounds. In severely chlorotic leaves, the PS2 activity was reduced to about 63 % when water served as electron donor. A similar trend was also found when using  $\text{MnCl}_2$  as donor. In thylakoids from severely chlorotic leaves a significant restoration of PS2-mediated DCPIP reduction was observed when  $\text{NH}_2\text{OH}$  and DPC were used as electron donor. In contrast, in mild chlorotic leaves PS2 activity was not restored, neither using DPC nor  $\text{NH}_2\text{OH}$  (Table 1).

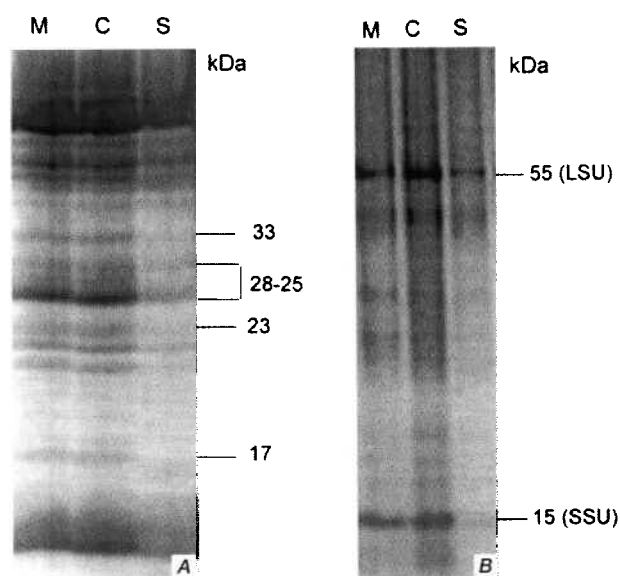


Fig. 1. Coomassie blue stained polypeptide profiles of thylakoid membranes (A) and SDS-PAGE of polypeptides in the crude leaf extracts (B) isolated from control and different stages of iron deficient leaves. Gel lanes were loaded with equal amount of proteins (100  $\mu\text{g}$ ). C, control; M, mild chlorotic; S, severely chlorotic.

**Changes in thylakoid membrane proteins:** Since the changes in photosynthetic electron transport activities could be caused primarily by the changes or reorganisation of thylakoid components, the polypeptide profiles of control and iron deficient thylakoids were analysed by SDS-PAGE. A comparison of iron deficient thylakoid polypeptides with those of the respective control indicated a specific loss in the contents of 33, 28-25, 23, and 17 kDa polypeptides (Fig. 1A). The loss of these polypeptides was more pronounced in severely chlorotic leaves.

**Changes in RuBPC activity and total soluble proteins:** A significant reduction (42 and 74 % in mild and severely chlorotic leaves) of RuBPC activity on protein basis was observed in severely chlorotic leaves (Table 1). Similar situation was also found for content of total soluble proteins (Table 1).

Iron deficiency markedly affected the level of both LSU (55 kDa) and SSU (15 kDa) of RuBPC present in crude leaf extracts (Fig. 1B). The contents of RuBP LSU and SSU polypeptides decreased with decreasing Chl content when measured per leaf area basis (Fig. 1B).

## Discussion

Iron deficiency is a common abiotic stress for many photosynthetic organisms on earth (Terry and Abadia 1986, Straus 1994). Iron deficiency markedly affects photosynthesis (Misra and Srivastava 1994, Nedunchezian *et al.* 1997, Morales *et al.* 2000). Species affected

range from high value crops in arid and semi arid environments (Mortvedt 1991) to sea phytoplankton (Behrenfeld *et al.* 1996). In our experiments, the contents of total Chl and carotenoid were progressively decreased upon increase in chlorotic conditions. Similar reduction

in total Chl and carotenoid contents was reported in various iron deficient plants (Pushnik and Miller 1982, Terry 1983, Terry and Abadía 1986, Morales *et al.* 1990, Misra and Srivastava 1994, Nedunchezian *et al.* 1997). Perez *et al.* (1995) reported that iron deficient field-grown peach leaves had reduced amounts of photosynthetic pigments per unit of leaf area; the increase of the carotenoid/Chl ratio lead to characteristic greenish-yellow colour.

Thylakoids isolated from grapevine chlorotic leaves showed a decrease in whole chain photosynthetic electron transport, mostly due to impairment of PS2, PS1 usually remaining unaffected. The PS2 activity of iron deficient grapevine leaves depended on the degree of chlorosis. Grapevine control leaves showed good PS2 activity, measured as the  $F_v/F_m$  ratio. Decrease in Chl content leads to decrease in  $F_v/F_m$  ratio; leaves with Chl content as low as  $40 \mu\text{mol(Chl)} \text{ m}^{-2}$  showed the lowest  $F_v/F_m$  ratio. Similar results were found in iron deficient pear and sugar beet leaves (Morales *et al.* 1994, 1998).

Analysis of electron transport in thylakoids isolated from mild chlorotic leaves showed that  $\text{O}_2$  evolution was inhibited when the used electron acceptor was DCBQ but not inhibited when the electron acceptor was SiMo. This indicates that thylakoids isolated from mild chlorotic leaves are affected at the reducing side of PS2. In contrast to this, in thylakoids isolated from severely chlorotic leaves, the rate of PS2 activity observed with SiMo was lower than that observed with DCBQ. Hence the donor side is more impaired than the acceptor side of PS2. Similar changes were observed in iron-deficient and copper-induced PS2 inactivation in peach and sugar beet thylakoids (Yruela *et al.* 1991, Nedunchezian *et al.* 1997).

Measurement of PS2-mediated DCPIP reduction in the presence of various artificial exogenous electron donors acting at the oxidising side of PS2 was made to locate the possible site of iron deficiency induced inhibition. DPC and  $\text{NH}_2\text{OH}$  were effective in restoring PS2 activity in severely chlorotic leaves. This indicates that severely chlorotic leaves induced changes on the oxidising side of PS2, prior to the  $\text{NH}_2\text{OH}$  donation side and perhaps close to or after the DPC donation side. This is supported by our earlier reports in sugar beet and peach (Nedunchezian *et al.* 1995, 1997).

The most likely explanation for the inactivation of electron transport activity is that the related protein(s) is (are) exposed at the thylakoid surface (Seidler 1994). A comparison of iron deficient thylakoids with those of the control showed specific loss of 33, 28-25, 23, and 17 kDa polypeptides. The loss was more pronounced in severely chlorotic thylakoids. The extrinsic proteins of 33, 23, and 17 kDa associated with the lumen surface of the thylakoid membranes are required for optimal functioning of the

oxygen evolving machinery. The three proteins are present in equimolar amounts (Murata *et al.* 1984, Enami *et al.* 1994), but it is still disputed whether one copy or two copies of each of the proteins are associated with the PS2 unit (Murata *et al.* 1984, Millner *et al.* 1987). Solubilisation of the proteins is associated with partial or total inactivation of  $\text{O}_2$  evolution. In particular, removal of the 33 kDa protein from PS2 membrane preparations by treatments with  $\text{CaCl}_2$ , NaCl (Enami *et al.* 1994) results in strong inhibition of  $\text{O}_2$  evolution and the loss is subsequently restored by reconstitution of the protein depleted membranes (Kuwabara *et al.* 1985). Marked reduction in the contents of 33, 23, and 17 kDa polypeptides showed the observed loss of PS2 activity in severe chlorotic leaves. The mild chlorotic leaves showed only marginal reduction in these polypeptides; this is one of the reasons for less inhibition of PS2 activity in these leaves. Our results indicate that the significant loss of 33, 23, and 17 kDa polypeptides could be the major reason for significant loss of  $\text{O}_2$  evolution induced by iron deficiency. Thus we confirmed that iron deficiency induced changes on the donor side and acceptor side of PS2 depend on chlorotic condition.

Light-harvesting complexes play important role in radiation absorption, thylakoid stacking, and energy distribution. Any damage to these complexes has multiple effects on the photosynthetic system. In our experiment, a significant loss of LHCP2 (28-25 kDa) polypeptides was observed in severely chlorotic leaves. This could induce the observed marked loss of PS2 activity and yellowish leaves in iron deficient leaves.

Total soluble protein contents were reduced markedly in severely chlorotic leaves. The relatively low contents of soluble proteins may have been due to decrease in the synthesis of RuBPC, the major soluble protein of leaf. A loss of leaf proteins in grapevine would partially account for damaged chloroplasts or be the result of inhibition of protein synthesis (Winder and Nishio 1995).

The reduction in overall photosynthetic rates correlated well with the decrease of RuBPC activity in iron deficient grapevine leaves. Our results indicate a gradual reduction of RuBPC enzyme activity with increasing chlorosis. Such reduction in the RuBPC was due to inhibition of protein synthesis under iron deficiency. This is supported by SDS-PAGE analysis of crude leaf extracts of RuBP proteins that show a significant loss of both LSU and SSU polypeptides in severely chlorotic leaves.

Our experiments show that iron deficiency induced chlorosis is brought about by a complicated interaction of damage to and degradation of the photosynthetic apparatus. Iron deficiency induced a fast degradation of LHCP2, which became visible as chlorosis in grapevine leaves.

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