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Signs of oxidative stress in the chlorotic leaves of iron starved plants

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Abstract

Studies were made to relate signs of oxidative stress in chlorotic and non-chlorotic leaves of plants receiving deficient supply, either nil (chlorotic leaves) or 10 μM (non-chlorotic leaves) of Fe, with thiobarbituric acid reactive substance (TBARS) and Fe status of leaf tissues. Total Fe concentration in the young leaves of mulberry, maize and cauliflower plants supplied deficient Fe did not show any significant change. Deficient supply of Fe caused decreases in the activities of catalase, peroxidase and ascorbate peroxidase and increases in the activity and number of isoforms of superoxide dismutase (SOD), accumulation of superoxide anion radical ($O_2^{\bullet -}$) and concentration of H_2O_2 in the young leaves of each mulberry, maize or cauliflower plants. While the chlorotic leaves of Fe-starved mulberry, maize and cauliflower plants had significantly lower TBARS compared to green leaves of the controls or non-chlorotic leaves of cauliflower plants supplied low Fe (10 μM). While TBARS concentration in the young leaves of these plants was well correlated with the concentrations of chloroplastic pigments, and activities of catalase and ascorbate peroxidase, its relationship with leaf tissue Fe or H_2O_2 concentration was rather poor. Progressive increase in the activity and induction of new isoforms of SOD and higher accumulation H_2O_2 and $O_2^{\bullet -}$ may be considered as signs of oxidative stress, whereas, increased ascorbate content along with lower mole fraction of ascorbic acid/total ascorbate are indicative of disturbed cellular redox environment in Fe-starved plants. It is concluded that chlorotic leaves of Fe-starved plants though have elevated titre of reactive oxygen species they are less susceptible to oxidative damage probably due to lower chloroplastic pigments/or low functional Fe in the tissue.

Keywords: *Morus alba*; *Zea mays*; *Brassica oleracea* var. *botrytis*; Functional Fe; ROS; TBARS; Fe-starvation; Fe-deficiency

1. Introduction

Iron is a constituent of several components of cellular electron transport system (ETS) [1]. Deficiency of iron is expected to cause hindrance in electron transport process due to decreases in the Fe-dependent components of ETS resulting in saturation of ETS intermediates. Under such reduced condition, high potential electrons could pass-on to O_2 and generate superoxide radical ($O_2^{\bullet -}$) and other reactive oxygen species (ROS) [2]. Though plants are well equipped with antioxidant defence systems comprising of antioxidant enzymes: superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione peroxidase (GPX) and enzymes of ascorbate-glutathione pathway and molecular antioxidants: ascorbate, α-tocopherol, carotenoids, flavonoids and glutathione [3–5]. Nevertheless, oxidative damage results when rate of ROS generation far exceeds to capacity of antioxidants to detoxify them efficiently.

Iron starved plants may be expected to be more prone to oxidative damage since Fe is a constituent of some of key antioxidant enzymes associated with detoxification of H_2O_2 (CAT, POD and APX) and with dismutation of $O_2^{\bullet -}$ (Fe-SOD) [1]. Deficiency of Fe has been reported by several workers to decrease the activities of H_2O_2 decomposing enzymes—CAT [6], POD [6], and APX [6–8]. Ranieri et al. [9] concluded, on the basis of increased accumulation of

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; EDTA, ethylenediamine tetraacetic acid; ETS, electron transport system; GPX, glutathione peroxidase; MDA, malondialdehyde; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance; TCA, trichloroacetic acid

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H_2O_2, that Fe deficiency induced secondary oxidative stress in sunflower plants showing decreased activities of POD and APX. On the other hand, lack of catalytic Fe, instrumental in generation of $OH^{\bullet}$ radicals, has been suggested to protect Fe-deficient plants against oxidative damages [6,9–10]. Moreover, Deák et al. [11] observed protection from oxidative damage in the transgenic tobacco plants showing ectopic expression of ferritin, the Fe-binding protein associated with sequestering intracellular Fe. Low tissue Fe concentration is suggested to account for the decreased Fe-SOD activity in Fe-deficient citrus [12], pea [6] and tobacco [13]. While Kurepa et al. [13] did not observe any significant effect of Fe deficiency on Cu/Zn-SOD, increased Cu/Zn-SOD activity in Fe-deficient pea observed by Iturbe-Ormaetxe et al. [6] supports a compensatory increase in the expression of another SOD form when the expression of one SOD form is decreased [14], and is suggestive of increased generation of $O_2^{\bullet -}$. However, high GSH/GSSG ratio, unaltered status of glutathione, α-tocopherol, malondialdehyde and carbonyl contents as observed by Iturbe-Ormaexte et al. [6] do not suggest induction of oxidative stress in Fe-deficient plants. Estevez et al. [15] though did not observe any change in lipid radicals, recorded decreases in the contents of α-tocopherol and ascorbyl radical and ascorbyl/ascorbate ratio in Fe-deficient *Chlorella vulgeris*.

Even though a large body of evidence in the literature suggests that Fe-deficient plants are better protected against oxidative stress, controversy persists regarding oxidative status of Fe-deficient plants. In the present study we have attempted to assess the oxidative status of three different plant species subjected to Fe-deficiency on the basis of accumulation of $O_2^{\bullet -}$, H_2O_2 and TBARS, and activities of SOD and H_2O_2 decomposing enzymes.

2. Materials and methods

2.1. Plant material

Four separate experiments including a time course experiment were conducted with mulberry *(Morus alba* L. cv. Kanva-2), maize (*Zea mays* L. cv. GSF-2), and cauliflower (*Brassica oleracea* var. *botrytis* L. cv. Snowball-16) grown in solution culture in glasshouse. Initially plants were grown in complete nutrient solution [16]: 2.0 mM KNO_3, 2.0 mM $Ca(NO_3)_2$, 1.0 mM $MgSO_4$, 0.67 mM NaH_2PO_4, 0.05 mM NaCl, 0.05 mM Fe–K_2EDTA, 5.0 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 16.5 μM H_3BO_3, 0.1 μM Na_2MoO_4, 0.05 μM $CoSO_4$ and 0.05 μM $NiSO_4$. The pH of the nutrient solution was maintained at 6.7 ± 0.2. After a week of transplantation, pots of maize and mulberry plants were grouped into two and those of cauliflower plants into three lots having four pots each. Whereas plants in lot 1 continued to receive complete nutrient solution (control), those in lot 2 of all the three plant species were supplied nutrient solution with nil Fe (−Fe). To the cauliflower plants in lot 3 supply of Fe was mildly deficient (10 μM; Fe-D), adjusted so it would not produce chlorotic leaves. The volume of nutrient solution was made-up daily by deionised water. The nutrient solution was refreshed every alternate day. Studies were made in the fully expanded young leaves of plants after a week, except for those otherwise stated, of initiating differential Fe supply.

For time course study, similar to other three experiments, maize plants were initially cultivated for a week in a complete nutrient solution and after a week, plants were grouped into two lots, lot 1 continued to receive complete nutrient solution, and plants in lot 2 received nutrient solution lacking in Fe–EDTA as described earlier. Leaf samples were drawn at different time interval (as given in the figures) for various analysis.

2.2. Iron

Iron (Fe) was estimated in HNO_3:$HClO_4$ (10:1, v/v) digest of young leaves atomic absorption spectrophotometrically.

2.3. Hydrogen peroxide and lipid peroxidation

H_2O_2 concentration was determined as H_2O_2–titanium complex formed by reaction of tissue–H_2O_2 with titanium tetrachloride by the method of Brennan and Frenkel [17]. Lipid peroxidation was determined by method of Heath and Packer [18] in terms of malondialdehyde (MDA) content by thiobarbituric acid (TBA) reaction. The amount of TBA reactive substance (TBARS) was calculated from the difference in absorbance at 532 and 600 nm using extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.4. In situ staining of superoxide radicals and hydrogen peroxide

Histochemical staining of tissues for $O_2^{\bullet -}$ and H_2O_2 was performed as described by Hernández et al. [19] with minor modification. Detection of $O_2^{\bullet -}$ was made by vacuum infiltrating leaf discs (8 mm diameter) with 0.1 mg mL^{-1} solution of *p*-nitroblue tetrazolium (NBT) in 0.2 M sodium phosphate buffer (pH 7.6) for 15 min and then incubating at 25 °C in the dark for 2 h.

For in situ detection of H_2O_2 an endogenous peroxidase dependent histochemical staining was used. The leaf discs (8 mm diameter) were vacuum infiltrated with 0.1 mg mL^{-1} 3,3′-diaminobenzidine in 50 mM Tris–acetate buffer (pH 5.0) for 15 min and then incubated at 25 °C in dark for 24 h on an orbital shaker (200 rpm). Pigments present in the leaf discs were cleared in 80% (v/v) ethanol for 20 min at 70 °C and then mounted in lactic acid, phenol and water mixture (1:1:1, v/v). The mounted leaf discs were photographed using Nikon advanced research microscope (Model E-400) with photographic system (H-III).