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Effect of Fe Deficiency on Antioxidant System in Leaves of Three Flax Cultivars

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Abstract

The potential role of antioxidant enzymes as well as antioxidant compounds in protecting plant from the deleterious effect of iron deficiency was examined in different flax *Linum usitassimum* L. cultivars. Three flax cultivars (Sakha 1, Sakha 2 and Giza 8) were grown in water culture with (Fe-sufficient plants, +Fe) or without (Fe-deficient plants,-Fe) iron supply for 40 days. The obtained results showed that, iron deficiency severely decreased dry weight and concentration of iron in all cultivars leaf tissue. Besides this, to verify whether iron deficiency could induce alteration in reactive oxygen species, high concentration of lipid peroxidation (TBARS) and H_2O_2 content in leaves of flax cultivars were detected under (-Fe) compared to (+Fe) treatments. Iron deficiency can also modulate the content of glutathione (GSH) level, which were significantly increased in Fe deficient treatment compared to Fe-sufficient treatment among cultivars. Activity of superoxide dismutase (SOD) was increased under deficient treatment. In contrast, significant differences were observed between cultivars in the activity of Fe containing enzymes such as ascorbate peroxidase (APX), peroxidase (POD) and catalase (CAT) that was greater under Fe sufficient treatment, suggesting higher amounts of physiological iron in leaf tissue of all cultivars. In addition, some changes in POD isoenzyme profile was detected under iron stress.These results suggested that, these antioxidant compounds are the key compounds to protect cell from oxidative injury.

Keywords: Antioxidant enzymes, Flax, Fe-deficiency, lipid peroxidation, peroxidase isoenzyme.

Introduction

Iron is considered as an essential nutrient for plant growth and plays a central role on overall physiology of the plants. Iron deficiency is one of the major abiotic stresses affecting crops growing in calcareous soils in the Mediterranean area. The most obvious effect of Fe-deficiency is the yellowing of the young leaves, and Fe deficiency is usually called 'Fe chlorosis. Iron chlorosis is one of the limiting factors for fruit crop production, since growers not using Fe treatments face major yield and quality losses and marked reductions in orchard longevity (Sanz *et al*., 1992). When plants subjected to iron deficiency stress, a range of deleterious effects including inhibition of photosynthesis processes, pigment synthesis and other metabolic disturbances such as change in the levels of several metaloenzymes activity were established (Manthey and Crowely, 1997).

The relationship between decrease iron availability in the nutrient media and the possible onset of oxidative stress is becoming more evident, because of the dual role played by iron in cell metabolism as either an antioxidant or a pro-oxidant factor. In fact, iron is a constituent or a cofactor of many antioxidant enzymes but, on the other hand, it can act as pro-oxidant because it catalysis free radical generation through the Fenton's reaction (Minotti and Aust, 1987). However, it has been shown that many enzymes require iron in order to function correctly in particular iron is present in the active sites of catalase and superoxide dismutase involved in the scavenging of reactive oxygen species (ROS) as described previously by Elstner and Osswald, (1994). The generation of (ROS) such as superoxide anion radical ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxide radical ($^{\cdot}OH$) can damage many cellular components including protein, membrane lipids and nucleic acids, so plant cells respond to the formation of ROS by increasing the production of metaloenzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), peroxidase (POD; EC 1.11.1.7) and especially (APX, EC 1.11.1.6), and non-enzymatic antioxidant substances, such as glutathione (GSH), α-tocopherol, carotenoids and flavonoids that protecting cells against oxidative injury caused by many biotic and abiotic stresses (Halliwell and Gutteridge, 1987). SOD is a major scavenger of $O_2^{\cdot-}$ and its enzymatic action results in the formation of H_2O_2 and O_2. POD decomposes H_2O_2, by oxidation of co-substrates, such as phenolic compounds and/or antioxidants, whereas CAT breaks down H_2O_2 into water and molecular oxygen (Mittler, 2002). As an abiotic stress for plants, iron deficiency was shown to affect the expression and the activity of certain peroxidase isoenzymes and induces secondary oxidative stress in dicotyledonous species (Ranieri *et al*., 2001). Recently, Zaharieva *et al*. (2004)

found in sugar beet roots that iron deficiency resulted in the decreased activity of APX and increased content of GSH. On the other hand, excessive iron is also harmful to plant. The free ionic form, due to its catalytic action in one electron redox reactions, can catalyze the formation of ROS through the metal-dependent Haber-Weiss and Fenton reactions (Asada and Takahashi, 1987). Thus, plants must tightly control the iron concentration and iron homeostasis is essential for plants.

The objective of this work is to investigate the effect of iron deficiency stress on leaves of three Flax cultivars by using antioxidant component (GSH) content, antioxidative enzyme activities and peroxidase isozyme profile as laboratory biochemical techniques.

Materials and methods

Plant growth condition and Fe treatments

Three cultivars of flax Linum usitatissiimum L. namely (Sakha 1, Sakha 2 and Giza 8) were used in this study. These cultivars were kindly obtained from Field Crops Research Department in National Research Center, Egypt. Seeds were germinated in sand in dark at 18°C. After 4-5 days, seedlings were transferred into 1 liter plastic vessels containing an aerated nutrient solution. Seedlings were grown under controlled conditions (14/10h light / dark period at 18/20oC, relative humidity 65-70%, and light intensity of 300 µ Mol m-2s-1). The composition of nutrient solution and other growth conditions were described according to Hoagland and Arnon (1950). Seedlings were applied with two levels of Fe as Na-Fe-EDTA at a concentration of 40 µ mol and Zero Fe. The plants were grown in nutrient solution for 40 days.

Plant analysis

Leaves of each treatment were gathered after 40 days then mixed thoroughly and were taken for the following analysis:

1- Fe-content

Leaves were washed and drying at 70o C then tissues were ground to a fine powder and were digested by wet digestion according to Chapman and Pratt (1978). Then Fe concentration was determined by atomic absorption spectroscopy, Perken Elmer model.

2- Preparation of enzyme extracts

Samples of 1 g were homogenized in 3 ml of 50 mM phosphate buffer pH 7.0, 1% PVP (Sigma), 1 mM ascorbate (Sigma) at 4°C. After centrifugation at 15,000×g for 15 min the supernatant was collected.

3- Assay of protein content

Protein was determined according to the method of Bradford (Bradford, 1976) with standard curves prepared using bovine serum albumin

4- Lipid peroxidation (TBARS contents)

Lipid peroxidation was determined as described by Heath and Packer (1968). Fresh leaves (200 mg) were homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 12,000×g for 20 min. The supernatant (1 ml) obtained was mixed with an equal volume of TCA (10%) containing 0.5% (w/v) TBA or no TBA as the blank, and heated at 95°C for 30 min and then cooled in ice. The reaction product was centrifuged at 12,000×g for 15 min and the supernatant absorbance was measured at 532 nm. The TBARS equivalent was derived from the absorbance according to Hodges, *et al.*, (1999).The level of lipid peroxidation was expressed as nmol MDA/mg protein/hr.

5- Assay of hydrogen peroxide concentration

Hydrogen peroxide was measured by the method described by (Capaldi, and Taylor, 1983) with a slight modification. The ground leaves in 5% TCA with 50 mg active charcoal at 0°C, and centrifuged for 10 min at 15,000 × g. Supernatant was collected, neutralized with 4 N KOH to pH 3.6 and used for H2O2 assay. The reaction mixture contained 200 µl of leaf extract, 100 µl of 3.4mM 3-methylbenzothiazoline hydrazone (MBTH). The reaction was initiated by adding 500 µl of horseradish peroxidase solution (90 U per 100 ml) in 0.2M sodium acetate (pH 3.6). Two minutes later 1400 µl of 1 N HCl was added. Absorbance was read at 630 nm after 15 min.

6 - Determination of total glutathione

The level of total acid-soluble SH compound (glutathione GSH) was determined with Ellman's reagent according to (De Vos, *et al.*, 1992). The buffer was mixed with 630µl of 0.5 M K2HPO4 and 25 µl of mM 5, 5'–dithiobis (2-nitrobenzoic acid) (final pH 7). The absorbance at 412 nm was read after 2 min. GSH was used as a standard.

6- Determination of antioxidant enzymes activity

6.1- Assay of SOD activity

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of (Beauchamp, and Fridovich, 1971). The 3 ml reaction mixture contained 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 1.0 mM EDTA and 20 µl enzyme extract. Riboflavin was added last and the reaction was initiated by placing the tubes 30 cm below 15 W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 10 min. Switching off the light stopped the reaction and the tubes were covered with black cloth. Non-

illuminated tubes served as control. The absorbance at 560 nm was read. One unit of SOD is the amount of extracts that gives 50% inhibition the rate of NBT reduction.

6.2- Assay of ascorbate peroxidase (APX) activity

Ascorbate peroxidase activity was determined spectrophotometrically by a decrease in the absorbance at 265 nm (Σ = 13.7mM^{-1} cm^{-1}) using the method of (Nakano, *et al*., 1981). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 5 mM ascorbate, 0.5 mM H_2O_2 and enzyme extract. Addition of started the reaction. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm [coefficient 2.8 (mM^{-1} cm^{-1})].

6.3- Assay of peroxidase (POD) activity

Peroxidase activity was determined spectrophotometrically by increasing in the absorbance at 430 nm (Σ = 2.47mM^{-1} cm^{-1}) using the method of (Amako *et al*., 1994). The reaction mixture contained 100 mM potassium phosphate buffer pH 6.8, 60 mM pyrogallol, 60 mM H_2O_2 and enzyme extract. One unit is defined as the amount of enzyme forming 1 mg of purpurogallin from pyrogallol in 20 s and cause 0.01 absorbance increases per min to H_2O_2.

6.4- Assay of catalase (CAT) activity

Catalase activity was determined by consumption of H_2O_2 using the method of (Dhindsa, *et al*., 1981). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 15 mM H_2O_2 and enzyme extract. The consumption of H_2O_2 was monitored spectrophotometrically at 240 nm (e = 45.2m M^{-1} cm^{-1}). Enzyme activity was expressed in μM H_2O_2 min^{-1}.

7- POD isoenzyme detection

Native PAGE was performed for isoenzyme in vertical polyacrylamide gels with a discontinuous buffer system. Peroxidase (POD) was stained according to Stegmann *et al*. (1987).

Statistical analyses

All determinations done in triplicate. Statistical analysis were done using SPSS (version 10) program. Mean and standard error were descriptive measures of quantitative data using the analysis of variance test (ANOVA) for independent samples. P-values <0.05 were considered significant.

Results and Discussions

Leaf dry weight and Fe contents

Forty days after Fe-deficiency was imposed, all cultivars developed chlorosis with the duration of the deficiency stress. The leaf of Giza 8 and Sakha 2 showed symptoms of mild chlorosis, which became severe after. In contrast, Sakha 1 showed only minor symptoms of Fe-deficiency (-Fe). On the other hand, the three cultivars which were grown on the (+Fe) treatment showed no symptoms of chlorosis through out the experimental period. Plants growth, estimated from leave dry weight during 40 days, in either sufficient or deficient levels of Fe, was used as a preliminary evaluation for the response to Fe-deficiency (Fig. 1). The results indicated that all flax cultivars showed less value for dry weight with Fe-deficient treatment. Slight decrease in leaf dry weight of Sakha 1 cultivar under Fe starvation conditions while the respective values for Sakha 2 and Giza 8 It was found that under (-Fe) treatment. Fe content in leaves varied among the three cultivars (Fig. 2). The total iron content was dramatically reduced under Fe-starvation in the leaf of flax cultivars Sakha 1, Sakha 2 and Giza 8 respectively as compared with sufficient Fe treatments. From obtained results showed that Sakha 1 cultivar was less affected compared with Sakha 2 and Giza 8 cultivars under Fe-stress. Fe deficiency involved in the biosynthesis pathway of chloroplast (Marschner, 1995) owing to the fact that most of leaf iron is localized in chloroplast, mainly in complexes involved in the photosynthetic electron transport chain, which contain about 60% of total leaf iron content (Terry and Abadia, 1986). Fe deficiency primary affects structure and function of the chloroplasts thus, under Fe deficiency the reduction of leaf Fe content accompanied by a marked reduction of chlorophyll level (Gogor Cena *et al*., 2004).Such a large decrease in chlorophyll content would be attributed to the role of Fe for the formation of aminolevulinc acid and photochlorophylid the precursors of Chlorophyll biosynthesis (Marschner, 1995). It can be concluded that Fe-deficiency caused reduction in dry matter accumulation and iron content, which may be due to the decrease of chlorophyll content and photosynthesis rate (Mengel, 1995; Raniari *et al*., 2001). In addition, the decrease of dry weight may be attributed to peroxide radicals, which enhanced and caused photoxidation damage in leaves, leading to lower leave growth under Fe-deficiency as stated by (Von wiren *et al*., 1994). The reduction in the dry weight may be also due to the alteration in the activity of carboxylating enzymes such as PEPcarboxylase, RuBPC and carbonic anhydrase. 1985). This preliminary evaluation procedure had been used previously to reliably assort plant cultivars according to their efficiency to tolerate Fe-deficiency (Von Wiren *et al*., 1994; El-Bendary *et al*., 1999).

Lipid peroxidation and H_2O_2 content:

Chlorosis that became visible at leaf after extended exposure to iron deficiency pointed to the possible involvement of oxidative stress in iron deficiency. Lipid peroxidation is one of the first consequences of oxidative damage,

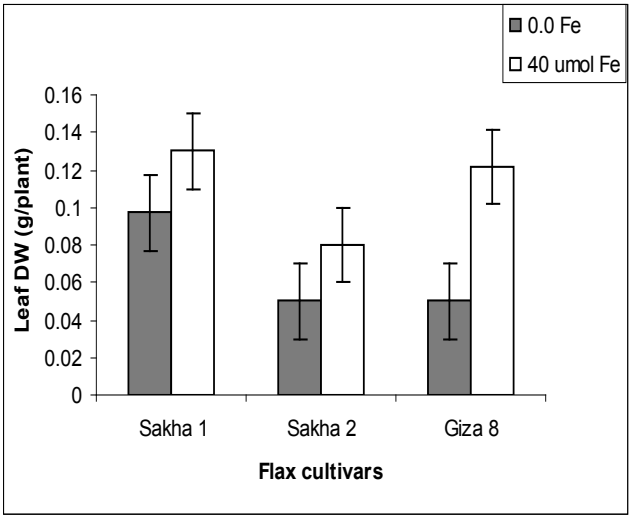

Fig. 1. Changes in leaf dry weight (g / plant) of flax cultivars (Sakha 1, Sakha 2 and Giza 8) grown in the presence of Fe and absence Fe of which on for 40 days. Values with different letters in the same experiment and same column are significantly different (P ≤ 0.05).

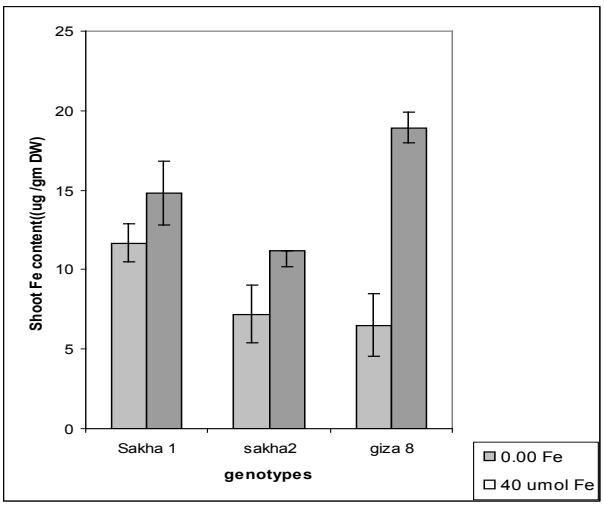

Fig. 2. Changes in leaf Fe content (µg / g DW) of flax cultivars (Sakha 1, Sakha 2 and Giza 8) grown in the presence of Fe and absence Fe of which on for 40 days. Values with different letters in the same experiment and same column are significantly different (P ≤ 0.05).

and production of thiobarbituric acid-reacting substances (TBARS) can be used as an indicator of oxidative stress. In iron deficient-flax cultivar (Sakha 1, Sakha 2 and Giza 8) leaves, TBARS showed the higher values than that in +Fe leaves (Tab. 1). The increases in TBARS under iron deficiency indicate that iron deficiency induced oxidative damage on lipids and proteins (Sun *et al*., 2007). The contents of H_2O_2 were higher in iron-deficient leaves than in iron-sufficient of all cultivars (Tab. 1). Because iron is an important component of the electron transport chains in chloroplasts and mitochondria, iron deficiency impairs the electron transport and probably leads to the production of ROS (Graziano and Lamattina, 2005). Ranieri *et al.,* (2001) have reported that iron deficiency enhanced H_2O_2 accumulation in sunflower leaves. Our results also demonstrated that the contents of H_2O_2 increased in iron-deficient flax leaves.

Glutathione content

Fe-deficient leaves showed higher GSH content in flax cultivars (Sakha 1, Sakha 2 and Giza 8) against Fe-sufficient treatments. In addition, ascorbate leaf content showed marked increase at the same previous flax cultivars under Fe starvation treatments. Our results are agreement with (Zaharieva, *et al*., 1999; Zaharieva, Abadia 2003) who found marked increases in GSH content in cucumber and sugar beet under Fe-deficiency. Moreover, the fact that GSH concentrations are also rapidly affected by Fe deficiency suggests that this compound may have a role in the nutrient stress response of the plant. The GSH cycle is a key component in detoxifying ROS in plants, and NADPH-dependent GSSG reductase (GR) is the key rate-limiting enzyme in this cycle (Noctor and Foyer, 1998). Accumulation H_2O_2 appear to activate antioxidant machinery including increases of GSH levels in Fe-starvation plants Zaharieva *et al.* (2004). Plant responses to Fe deficiency stress may include a shift in the redox balance inside the root cell towards more reduced state, along with the increased capacity of the leaves and roots to reduce extracellular Fe. It has been proposed that a redox-based sensory system may activate mechanisms to organize plant responses to stress (Foyer *et al*., 2001). The ascorbate–glutathione cycle plays a central role in the maintenance of the cellular homeostasis and includes three independent redox couples: ASC/DHASC, GSH/GSSG, and NADPH/NADP. Recent experimental evidence has indicated that the levels of GSH could be used to sense the environmental changes and trigger an up-regulation of mechanisms involved in co-regulation of GSH and NADPH pools during environmental stress (Maggio, *et al*., 2002; May *et al*., 1998).

Activities of antioxidant enzymes

Since iron deficiency caused oxidative stress in flax leaves, the behavior of the antioxidant enzymes were examined. SOD activity showed remarkable increase respectively at flax cultivars (Sakha 1, Sakha 2 and Giza 8) (Tab. 2). SOD, an important enzyme involved in antioxidation processes and present in different organelles in plants, catalyzes the superoxide radical conversion to H_2O_2. The increment of SOD activity may account for the increased accumulation of superoxide radicals ($O_2^{\cdot-}$) in iron-deficient leaves (Sun *et al*., 2007). H_2O_2 accumulation (Tab. 1), resulting from SOD mediated dismutation $O_2^{\cdot-}$ in Fe-starved plants having feeble H_2O_2-scavenging machinery. The data in Tab. 2 showed that the activities of APX, POD and CAT decreased significantly under iron deficiency in all cultivars while the activities of SOD increased. The activity of APX and POD in leaves of flax cultivars (Sakh 1,

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Tab. 1. Effects of Fe treatments on the contents of lipid peroxidation (MDA), H2O2, GSH in leaves of flax cultivars

Flax cultivars & Fe treatments	MDA (n molMDA/ mgprotein/hr)	%	H_2O_2 (μ mol/g FW)	%	GSH (μ mol/g FW)	%
Sakha 1 (-Fe)	18.7 [a]	286.8	200.73 [a]	165.8	170.33 [a]	180.2
Sakha 1 (+Fe)	6.52 [b]	100	121.07 [b]	100	94.50 [b]	100
Sakha 2 (-Fe)	19.8 [a]	342.5	243.00 [a]	179.3	161.6 [a]	174.9
Sakha 2 (+Fe)	5.78 [b]	100	135.53 [b]	100	92.40 [b]	100
Giza 8 (-Fe)	21.60 [a]	337.5	250.03 [a]	179.1	150.7 [a]	166.5
Giza 8 (+Fe)	6.40 [b]	100	139.60 [b]	100	90.50 [b]	100

Sakha 2 and Giza 8) were reduced. In addition Fe starvation caused highly significant decrease at CAT activity at previous flax cultivars. Overproduction of ROS can lead to oxidative injury such as membrane lipidperoxidation, protein oxidation, enzyme inhibition and DNA and RNA damage (Mittler, 2002). CAT, POD and APX are important H_2O_2 detoxifying enzymes in plants. Because they are all heme-containing enzymes, their activities are likely to be affected by iron deficiency (Ranieri *et al*., 2001; Shigeoka *et al*., 2002). Our results show that the activities of APX, POD and CAT were reduced under iron deficiency (Tab. 2). The decreased activities of these three enzymes indicate that they may not play essential roles in detoxifying ROS under iron deficiency (Sun *et al*., 2007). Our results in consonance with several earlier reports (Sun *et al*., 2007; Tewari *et al*., 2005) and are suggestive of decreased functional Fe in the leaf of maize, mulberry and cauliflower, and decrease the activities of iron containing antioxidative enzymes APX, POD and CAT, and increased the SOD activity.

POD isoenzyme

Peroxidase isoforms, which were detected in the cytosolic extract of leaves in flax cultivars grown under iron stress condition, were photographed and presented in (Fig. 3). The separation of POD isoforms has confirmed the data obtained through spectrometric analysis showing a general reduction in POD activity in the flax cultivars. Reduction in the staining intensity of POD isoform bands (A, B, C and D) was noticed in iron-deprived samples in comparison with iron-sufficient ones. It is also interesting to note that under Fe-deficiency the electrophoretic POD isoenzyme bands showed differences in its mobility, compared to Fe-sufficient treatments in the three cultivars. Leidi and Gomez (1989), Ranieri *et al*. (2001) reported that POD isoenzyme patterns of soybean and sunflower leaves grown in nutrient solution are affected by iron-starvation and showed preferential reduction in their activity in the detoxification process.

Conclusion

The present data revealed that Sakha1 was the most efficient cultivars compared with Sakha2 and Giza8 cultivars under Fe deficiency treatments. In addition this study suggested that quantification of antioxidant enzymes activity, antioxidants metabolites, the isoenzyme polymorphism and EF band intensity can be used as reliable biochemical and molecular biomarkers for assessing the Fe efficiency in flax cultivars.

Tab. 2. Effect of Fe treatments on the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD) and catalase (CAT) in leaves of flax cultivars

Flax cultivars & Fe treatments	SOD activity (Unit/mg protein/min)	%	APX activity (Unit/mg protein/min)	%	POD activity (Unit/mg protein/min)	%	CAT activity (n mol/mg protein/min)	%
Sakha 1 (-Fe)	185.3 [a]	-56	8.63 [b]	62	406.50 [b]	78	139.20 [b]	40
Sakha 1 (+Fe)	118.6 [b]	100	13.87 [a]	100	518.86 [a]	100	345.47 [a]	100
Sakha 2 (-Fe)	132.6 [a]	-46	4.83 [b]	48	376.90 [b]	75	112.43 [b]	35
Sakha 2 (+Fe)	91.13 [b]	100	10.13 [a]	100	500.40 [a]	100	324.71 [a]	100
Giza 8 (-Fe)	176.87	-35	6.30 [b]	52	269.24 [b]	51	130.87 [b]	33
Giza 8 (+Fe)	131.03 [b]	100	12.10 [a]	100	527.46 [a]	100	398.27 [a]	100

SOD:One unit of SOD is the amount of extracts that gives 50% inhibition the rate of NBT reduction.

APX: A decrease in the absorbance at 290 nm [coefficient 2.8 (mM−1 cm−1)].

Cat: One unit is expressed as the amount of enzyme forming H2O2 min−1. POD: One unit is defined as the amount of enzyme forming 1 mg of purpurogallin from pyrogallol in 20 s and cause 0.01 absorbance increases per min to H2O2. Data represent the mean ± S.E. of three experimental replicates.Values with different letters in the same experiment and same column are significantly different ($P \leq 0.05$).

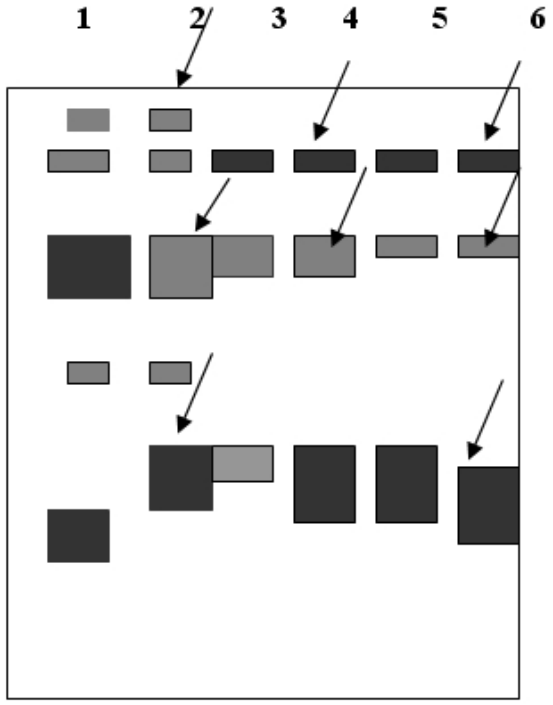

Fig. 3. Peroxidase (POD) isoenzyme profile of leaves in three flax cultivars as affected by Fe levels in grown medium. Effect of Fe Deficiency on antioxidant system

Lane 1. (+ Fe) in sakha 1. Lane 2. (-Fe) in sakha 1.

Lane 3. (+ Fe) in sakha 2. Lane 4. (-Fe) in sakha 2.

Lane 5. (+ Fe) in giza 8. Lane 6. (-Fe) in giza 8.

Arrows indicate isoforms which are most affected by iron deficiency.

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