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Genotypic Differences of Two Wheat Cultivars for the Enzymes Activity, Amino Acids and Protein Profile under Fe Deficiency

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Abstract: A pot experiment was carried out at the National Research Centre, Dokki, Cairo-Egypt using water culture technique. The possibility of using some biochemical and molecular markers as a tool for the diagnosis of iron deficiency was studied in two bread wheat cultivars (***Triticum aestivum*** L.) cv, Sids I and Giza 164 which were different in their response to iron deficiency. When iron was supplied at two levels (0 and 80 μM Fe-EDTA) the results indicated that, at 15 days old, the concentration of active iron (ferrous form) in leaves were closely correlated with visual iron deficiency symptoms of both cultivars. Generally chlorophylls concentration in leaves of both cultivars were decreased in deficient plant compared to sufficient one. On the other hand a distinct differences in the amount and types of amino acids was found in both cultivars whereas the total amount of amino acids of chlorotic leaves were higher than non chlorotic one in Giza 164 cultivar. Activities of peroxidase and catalase enzymes in leaves were also determined. Fe deficiency treatment resulted in decrease enzyme activities. Furthermore the profile of isoperoxidase and proteins were modified in leaves and roots of both cultivars during stress condition as evident from the electrogram.

Key words: Wheat, Fe-deficiency, amino acids, enzymes activity, isoperoxidase and protein patterns

Introduction

Iron deficiency is a major mineral disorder due to low Fe-solubility rate and low iron concentration in the soil solution, that decrease Fe- mobility which required for normal plant growth. In many situations, agricultural extension workers would like to have rapid and simple assessment for diagnosis of micronutrient deficient plant. Iron is a catalitical microelement for the life of plants, entering in the composition of various enzymes, regulating many biochemical processes like chlorophyll synthesis, photosynthesis and respiration. Therefore several biochemical and molecular markers could be tested for evaluating iron deficiency in wheat plant depend on the function role of iron in plant metabolism.

Several investigators found that it is often difficult to identify micronutrient deficiency on the basis of total element concentrations in the leaves tissue (Valenzuela and Romero, 1988 and El-Baz ***et al.***, 1998). The determination of active fraction of Fe has been suggested to be useful because the total iron concentration does not reveal nutrient deficiency (Pierson and Clark, 1984).

In addition, iron stressed plants showed visible deficiency symptoms in the youngest leaves, which become yellow and chlorotic due to a decline of the chlorophyll content. However, ferrous form was more important for the synthesis of chlorophyll and consequently occurrence of chlorosis (Monge ***et al.***, 1993).

Protein synthesis is impaired under Fe-deficiency, whereas (Veliksar ***et al.***, 1997) studied the content of free amino acids in relation to iron stress in grape leaves.

The possibility of using metalloenzymes such as peroxidase and catalase as sensitive biological parameters for diagnosis of iron imbalances in young maize plants had been previously reported (Nenova and Stoyanov, 1995).

Other biochemical markers like isozymes polymorphism could be used. Peroxidase, catalase, its isoforms expression and the change of protein profile patterns were used as a markers to evaluate iron deficiency in bean plants (El-Baz and Mohamed, 2002).

In addition (Guikema and Sherman, 1984) studied the profile patterns of membrane proteins isolated from iron deficient and sufficient cells of cyanobacterium, they found that iron deficiency caused major differences in the protein profile. The main aim of this work was to explore the possibility of using active iron content, chlorophylls concentration, amino acids content, enzymes activity of peroxidase, catalase, isoperoxidase and protein profile as biochemical markers for the diagnosis of iron deficiency in two wheat cultivars.

Materials and Methods

Plant material and growth conditions

Two cultivars of bread wheat (***Triticum aestivum*** L.cvs, Sids 1 and Giza, 164) were grown as previously described by (Erenoglu ***et al.***, 1996). After 4 days in the dark at 22°C, seedlings were transferred into full strength aerated nutrient solution in plastic vessels of 2L volume of the following composition: (mM) 0.7 K_2SO_4 ; 0.1 KCl ; 2.0 $Ca(NO_3)_2$; 2.0 $MgSO_4$; 0.2 KH_2PO_4; (μM) 1.0 H_3BO_3; 0.5 $MnSO_4$; 1.0 $ZnSO_4$; 0.2 $CuSO_4$; 0.01 $(NH_4)_6$ Mo_7O_{24}, with (+Fe) or without (-Fe) the addition of 80 μM of Fe-EDTA.

The pH of the nutrient solution was adjusted to 6.2, the nutrient solution were completely renewed every 2-3 days. Plants were grown at control condition with the following environmental regime, 16/8 hr day/night, 420 μmol $m^{-2}s^{-1}$ light intensity at 25/23°C and 65% relative humidity.

Plant analysis

Plants were harvested after 15 days of the growth in nutrient solution, and plants were separated into roots and shoots.

Determination of active Fe

The method of (Takkar and Kaur, 1984) was used. Two grams of fresh leaves tissue were washed and cut into pieces of 1mm. Fe^{2+} was extracted by 1.5 N HCl and determined using spectrophotometer LKB Ultraspec II model. The absorption was measured at 510 nm.

Determination of chlorophylls

Chlorophylls were extracted from leaf tissue with acetone (80% v/v) and quantitatively measured as described by (Lichtenthaler, 1987).

Determination of amino acids

The composition of amino acids were detected by using amino acid analyzer LC 3000 Eppendorf model after acid hydrolysis of dry plant tissues (roots and leaves) according to (Ivarson and Sowden, 1968).

Enzyme extraction

For extraction of peroxidase (POD) and catalase (CAT) enzymes, the method of (Rothe, 1997) was used. Plant tissue roots and leaves were excised and homogenized using a mortar and pestle (tissue/ buffer ratio 1:4, w/v). Grinding buffer containing 250 mM sucrose, 0.1 mM DTT, 1% polyvinyl polypyrolidine (w/v), 0.1 mM EDTA, adjusted to pH 7.2.

The homogenate was filtered and centrifuged at 1000 g for 30 min. The resulting supernatant was used for the following analysis:

Determination of POD and CAT activities

POD activity was determined according to Amako ***et al.*** (1994). The increase in absorbance at 430 nm was recorded spectrophotometerically.

CAT activity was assayed spectrophotometically. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. Protein was measured in the extract as described by the method of Bradford (1976).

Native PAGE and detection of POD activity

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was done using the vertical slabs gel electrophoresis system (Hoefer SE 600 model). Enzymes extract (20 μg protein equivalent) of each sample (Roots, leaves) were loaded into each well.Gel were stained with 0-dianisidine as descried by (Amako ***et al.***, 1994).

Protein electrophoresis

In order to determine the molecular weight of the sample proteins, SDS-PAGE under denaturing condition were done as described by (Laemmli, 1970) Gel were fixed and stained with coomassie brilliant blue R. Molecular weight marker ranging from 10-70 KD (Sigma) were used.

Experiment were carried out with three replicates, and the difference between means were compared by the least significant difference (LSD) test at the levels 0.01 and 0.05 of probability.

Results

Active iron and chlorophylls concentration

When iron was omitted from the nutrient solution, leaves of both cultivars became chlorotic after 7 -9 days of plant growth. Consequently, the concentration of active Fe generally increased as iron-level in the nutrient solution increased Table 1.

Table 1: Active iron and chlorophylls concentration of two wheat cultivars grown in the presence (+Fe) or absence (-Fe) of iron in nutrient solution for 15 days

			Chlorophylls concentration mg g^{-1} F. W						
Plant cultivars	Iron treatment	AAcrive iron (p.p.m)	Chl.a	%*	Chl.b	%	Total chl.	%	Chl a/ Chl b ratio
Sids I	-Fe	14.7	0.81	89.0	0.27	93.1	1.10	90	3.0
	+Fe	19.3	0.91	100	0.29	100	1.20	100	3.1
L.S.D	0.05	1.86	0.06		0.006		0.07		
	0.01	3.09	0.10		0.01		NS		
Giza 164	-Fe	16.64	0.56	61.5	0.21	67.7	0.77	63.1	2.7
	+Fe	26.22	0.91	100	0.31	100	1.22	100	2.9
L.S.D	0.05	2.92	0.140		0.053		0.196		
	0.01	4.84	0.240		0.09		0.320		

*% relative to +Fe

Table 2: Amino acids composition (mg g^{-1} f.w) in leaves of two wheat cultivars grown in the presence (+Fe) or absence (-Fe) of iron in nutrient solution for 15 days

	Wheat cultivars (leaves)					
	Sids I			Giza 164		
Amino acids type	- Fe	+ Fe	- Fe/ + Fe	- Fe	+ Fe	- Fe/ + Fe
Aspartic acid	0.943	1.042	0.905	1.541	0.942	1.635
Threonine	0.192	0.221	0.868	0.252	0.193	1.305
Serine	0.301	0.309	0.974	0.363	0.219	1.65
Glutamic acid	0.698	0.776	0.899	1.234	0.767	1.608
Proline	0.258	0.231	1.116	0.305	0.314	0.971
Glycine	0.326	0.339	0.961	0.562	0.294	1.91
Alanine	0.436	0.441	0.988	0.725	0.894	0.810
Valine	0.236	0.335	0.704	0.124	0.204	0.608
Methionine	-	-	-	-	-	-
Isoleucine	0.214	0.261	0.819	0.409	0.201	2.03
Leucine	0.426	0.451	0.944	0.737	0.473	1.55
Tyrosine	0.161	0.163	0.987	-	0.494	-
Phenylalanine	0.449	0.530	0.847	0.338	0.405	0.834
Histidine	0.351	0.319	1.10	0.473	0.811	0.583
Lysine	0.358	0.384	0.932	0.696	0.305	2.26
Arginine	0.233	0.257	0.906	0.490	0.307	1.59
Total	5.582	6.059		8.25	6.82	

In case of Giza 164 cultivar, the results showed that, active - Fe concentration was decreased under (-Fe) by nearly 45% as compared with the other cultivar under the same treatments. There was a significant decrease in HCl extractable iron as Fe level in the nutrient solution was omitted. These differences were more evidence in Giza 164 more than Sids I. Other parameter such as chlorophylls concentrations were also quantified, the results showed that, total chlorophylls concentration in Sids I were decreased from 1.2 mg g^{-1} f.w under (+Fe) treatment to 1.1 mg g^{-1} f.w under (-Fe) treatment (about 8.3% decrease) it was slightly changed, whereas in Giza 164 cultivar, total chlorophylls concentration were decreased by about (37% decrease).

Table 3: Amino acids composition (mg g^{-1} f.w) in roots of two wheat cultivars grown in the presence (+Fe) or absence (-Fe) of iron in nutrient solution for 15 days

	Wheat cultivars (Roots)					
	Sids I			Giza 164		
Amino acids type	- Fe	+ Fe	- Fe/ + Fe	- Fe	+ Fe	- Fe/ + Fe
Aspartic acid	0.413	0.773	0.56	0.664	1.44	0.461
Threonine	-	-	-	0.135	0.214	0.631
Serine	0.085	0.209	0.406	0.197	0.279	0.706
Glutamic acid	0.709	0.906	0.782	0.497	0.813	0.611
Proline	0.259	0.309	0.838	0.214	0.241	0.887
Glycine	0.232	0.585	0.396	0.217	0.356	0.609
Alanine	0.351	0.883	0.397	0.515	0.596	0.864
Valine	0.237	0.348	0.681	0.034	0.360	0.09
Methionine	-	-	-	-	0.011	-
Isoleucine	0.268	0.351	0.763	0.119	0.283	0.420
Leucine	0.558	0.709	0.787	0.325	0.488	0.665
Tyrosine	0.179	0.336	0.532	0.114	0.095	1.2
Phenylalanine	0.001	0.425	0.002	0.002	0.235	0.008
Histidine	0.363	0.574	0.632	0.528	0.551	0.958
Lysine	0.322	0.733	0.439	0.266	0.437	0.608
Arginine	0.018	0.395	0.045	0.118	0.351	0.336
Total	3.99	7.536		3.943	6.75	

Table 4: Peroxidase and catalase specific activity in the leaves of two wheat cultivars grown in the presence (+Fe) or absence of (-Fe) iron in nutrient solution for 15 days

		Peroxidase activity		Catalase activity	
Plant cultivars	Iron treatment	E.U/mg pro./ min	%*	μmol H_2O_2 consumed/mg pro./min.	%*
Sids I	-Fe	16.30	52	89.10	40
	+Fe	31.60	100	220.60	100
L.S.D	0.05	0.45		0.58	
	0.01	0.75		0.96	
Giza 164	-Fe	24.30	61	302.70	58
	+Fe	39.70	100	525.10	100
L.S.D	0.05	0.57		0.71	
	0.01	0.96		1.19	

* Relative to +Fe

Amino acids composition

Concerning amino acids, the values of Table 2 showed the type of individual amino acids content in leaves of both wheat cultivars. Results reveald a distinct difference in the amounts and types of amino acids. In Giza 164 cultivar, the total amounts of amino acids of chlorotic leaves (iron deficient) were higher by 17% than non chlorotic one (iron sufficient). In addition, in the same cultivar under Fe stress condition it is important to note that aspartic, glutamic and leucine acids were detected in high value compared to other acids. It is also clear that there was a less differences in the amount of total amino acids in leaves of Sids I cultivar under both iron treatments.

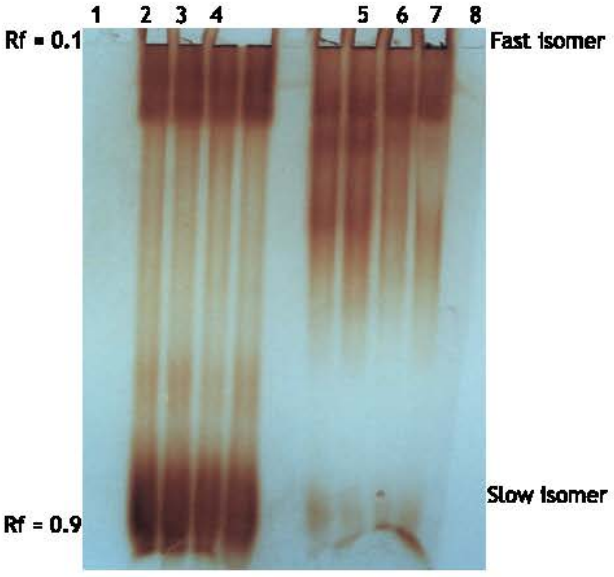

Fig. 1: Peroxidase isozyme profiles of roots and leaves of two wheat cultivars grown in the presence (+Fe) or absence (-Fe) of iron

Lane	1. Sids I (-Fe) Roots	Lane	5. Sids I (-Fe) Leaves
Lane	2. Sids I (+Fe) Roots	Lane	6. Sids I (+Fe) Leaves
Lane	3. Giza 164 (-Fe) Roots	Lane	7. Giza 164 (-Fe) Leaves
Lane	4. Giza 164 (+Fe) Roots	Lane	8. Giza 164 (+Fe) Leaves

At the same time the amino acids composition in relation to iron stress in roots tissue of both cultivars were detected (Table 3). the results showed that the amount of amino acids from roots of deficient plants (-Fe) was decreased in both cultivar when compared with the values of iron sufficient plants (+Fe).

Enzymes activity

With regard to enzymes activity the value of peroxidase and catalase specific activity indicated that, in generall the activity were decreased under deficient treatment in both cultivars (Table 4). In case of peroxidase activity the values showed that Sids I cultivar exhibited more depression than Giza 164 under Fe deficiency treatment. For example the activities were 16.3 and 24.3 Enzyme uint/mg pro./min. in Sids I and Giza 164 respectively. Also Table 4 illustrated that, under iron stress treatment catalase specific activity of Giza 164 cultivar showed a relatively higher specific activity compared with cultivar Sids I, for example the value was 302.7 and 89.1 μmol H_2O_2 consumed/mg pro./min in Giza 164 and Sids I respectively (more than 70% reduction). The obtained results revealed that Giza 164 cultivar can saved or accumulate more Fe in their leaves compared to Sids I which play a role in metalloenzyme activity.

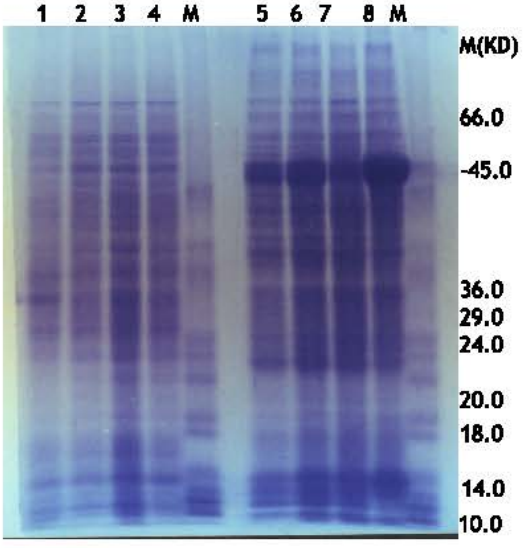

Fig. 2: SDS-PAGE protein patterns of two wheat cultivars grown in the presence (+Fe) or absence (-Fe) of iron

Lane	1. Sids I (-Fe) Roots	Lane	5. Sids I (-Fe) Leaves
Lane	2. Sids I (+Fe) Roots	Lane	6. Sids I (+Fe) Leaves
Lane	3. Giza 164 (-Fe) Roots	Lane	7. Giza 164 (-Fe) Leaves
Lane	4. Giza 164 (+Fe) Roots	Lane	8. Giza 164 (+Fe) Leaves

Peroxidase isozyme

Peroxidase isoforms which were detected in the cytoslic extract of roots and leaves in wheat cultivars grown under iron stress condition were photographed and presented in Fig. 1. The intensity of isoform bands reflected the difference pattern of individual peroxidase isoform of both cultivars. Looking at root extract under both treatments, each cultivar exhibited two bands as a total number with similar Rf values. The zymograms of leaves tissue exhibited different pattern, for example, in Sids I cultivar the numbers of bands were 4 with different Rf value under (-Fe) and (+Fe) treatment which were not appeared in the other cultivar.

Whereas Giza 164 cultivar showed only two bands with different intensity, one of them with Rf = 0.95 which not exhibited in the other cultivar. The appearance of this band might be involved with the function of peroxidase in Fe-efficiency.

Protein pattern

The protein patterns of two wheat cultivars grown at two level of iron stress were analyzed by SDS- PAGE method. The separated protein subunit were photographed and presented in Fig. 2.

High variations were detected among both cultivars either in (-Fe) or (+Fe) treatments. In general in roots tissue some new protein bands were appeared under (-Fe) which disappeared in (+Fe) treatment of both cultivars. For example in Sids I, a distinct band with Mw 37 KD was

detected (Lane 1) whereas the second cultivar showed also a new distinct band with MW 10 KD (Lane 3). In addition, several new protein bands with high MW were induced by iron stress in leaves tissue of both cultivar for example three bands with MW 90, 80 and 70 KD were detected exceptionally in Lane (7) the 90 KD band were disappeared. Also both cultivar contained one major distinct bands of MW 66 KD which exhibited in high intensity. Consequently, the disappearance of any band can be considered as a biomarker to characterize Fe- deficiency. Also the newly synthesized protein indicated that Fe stress induced related gene to produce this Fe inducible protein.

Discussion

The concentration of active iron in leaves of wheat plant is a better parameter for iron nutritional status. These results are in agreement with the results of (Lang ***et al.***, 1990) who found that extraction of Fe from fresh leaves with 1N HCL proved to be a better indicator of iron status of flowering plants, similar results were also obtained by (Ohwaki and Sugahara, 1993) who reported that the genotypic differences between sensitive and resistant cultivars of chickpea were attributed to the active iron in the leaves when grown under Fe- stress. However, (Ramirez ***et al.***, 2002) reported that the determination of leave Fe^{2+} content not suited for rice plant grown under iron deficiency. These results are in agreement with the results of (Lang ***et al.***, 1990) who found that extraction of Fe from fresh leaves with 1N HCL proved to be a better indicator of iron status of flowering plants, similar results were also obtained by (Ohwaki and Sugahara, 1993) who reported that the genotypic differences between sensitive and resistant cultivars of chickpea were attributed to the active iron in the leaves when grown under Fe-stress. However, (Ramirez ***et al.***, 2002) reported that the determination of leave Fe^{2+} content not suited for rice plant grown under iron deficiency. The results of ***(Abadia et al.***, 1991) supported the findings of the present results. They concluded that the determination of leaf chlorophyll content has been found reasonably consistent as a means to quantify Fe chlorosis providing that other possible causes of chlorophyll decrease can be excluded.

The results are also in agreement with the finding of (Reddy ***et al.***, 1993) who suggested that chlorophyll content can be distinguish between different groundnut genotypes when grown under iron deficiency.

These results are partially in agreement with those reported by (El-Gala and Amberger, 1988) and (Veliksar ***et al.***, 1997). They concluded that the increase of amino acids content in chlorotic leaves than green leaves may be due to the accumulation of amino acids in chlorotic leaves as a result of less structure of protein and nucleus content in plants. In addition, increase the structure action and continuous synthesis in health green leaves than the chlorotic plants. This result was agreement with the result of (Mori ***et al.***, 1991) who stated that in the root of Fe-deficient rice plants contents of valine, histdine and methionine were decreased compared to Fe-sufficient plant.

It is also interesting to note that the amino acids methionine was detected only in roots of Giza 164 cultivar. In this concern, (Mori and Nishizawa, 1987) reported that methionine is the dominant precursor of the chelating iron substance “phytosiderophore” in graminaceous plants.

The inhibition of peroxidase specific activity under iron stress may be due to the lack of peroxidase synthesis hence iron is essential for this process and probably blocked under iron stress (Nenova and Stoyanov, 1995). The pronounced effect of Fe-deficiency on peroxidase and catalase in the present work is in agreement with the results obtained by ***Dasgan et al.*** (2002) who demonstrated that under Fe- deficiency the activity of leaf enzymes (catalase, guaicol peroxidase and glutathione reductase) can determine Fe availability in tissues of Fe-efficiency/ Fe-deficiency resistant genotypes consequently these enzyme activities may be used as markers to determine Fe- nutritional status of tomato plants. Similar results were detected by El- Bendary ***et al.*** (1998) the authors confirmed that, the change of isoperoxidase pattern may used as a genetic markers for evaluation of iron nutritional status in maize plants. The present results are in agreement with the finding of (Suzuki ***et al.***, 1998) who stated that Fe- deficiency cause an appearance of several new protein bands in barley plant. In addition Guikema and Sherman (1984) stated that iron deficiency led to the synthesis of new proteins at 34 and 52 KD which were not present in normal cells of cyanobacterium. From the present work, it can be concluded that: these assays appear to be simple, sensitive and correlated well with Fe-status, such assays if acceptable, would be of great benefit for diagnosis of Fe-deficiency in wheat plant.

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