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Some Biochemical Variabilities in Wheat Callus: Nitrate Reductase, Ascorbate Peroxidase and Protein Electrophoretic Patterns Under Iron Stress

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ABSTRACT

Tissue culture experiment was carried out to study some biochemical changes and molecular aspects of iron deficiency stress in four bread wheat (*Triticum aestivum*) genotypes. Wheat embryos were grown for 30 days on LS media supplemented with $1x10^{-4}$ (Fe-sufficient) and 0.0 (Fe-deficient) Fe-EDTA, respectively. After 30 days from the beginning of the treatments, it was found that the genotypic difference in growth rate measurement can be related to iron stress, since callus growth rate was reduced under iron deficient treatments among wheat genotypes. Under Fe-deficiency, a marked reduction (25%) in the growth rate was observed in Sakha 69; whereas, the values of Sakha 61 genotype gave the highest growth rate (100%) under control treatment compared with other genotypes. The specific activity of ascorbate peroxidase (APX) was measured and showed a close relationship with Fe supply and thus a differential sensitivity of wheat genotypes to Fe deficiency. Significant differences were observed between genotypes in APX at 10 and 30 days; whereas, nitrate reductase (NR) showed in wheat genotypes to Fe deficiency. For example at 20 days growth, NR in all genotypes reached optimum level then gradually declined at 30 days growth. It was confirmed that cultivar differences in protein polymorphism could be revealed by electrophoretic patterns. One major protein band with high intensity was induced in all genotype of 26.6 kD in both treatments. However, differences in the protein pattern detected by SDS-PAGE between (Fe-sufficient) and (Fe-deficient) indicated that Fe deficiency led to an alteration in the pattern of proteins synthesis.

Key Words*:* Ascorbate peroxidase; Callus culture; Iron deficiency; Nitrate reductase; Protein pattern; Wheat genotypes

INTRODUCTION

The ability of plant to develop different biochemical reactions for adaptation to environmental changes is the characteristic that allows plant to optimize the use of available resources. Among the most frequent environmental stresses, iron deficiency is a particular concern for plant growth. Iron is present in the soil in low soluble form (Fe III), which is not readily available to plant growth and severely lead to yield decrease (Welch, 1995). According to Blair (1993) efficiency was defined as the ability of genotypes to acquire the nutrient from the growth media and utilize them to produce plant biomass. Accordingly the efficient genotypes are able to avoid nutrients deficiency by enhancing some biochemical reactions.

Moreover, many enzymes whose activity depends on iron are compromised or inactivated leading to severe metabolic alterations (Dasgan *et al*., 2003). The antioxidant enzymes, catalase (CAT, EC 1.11.1.6) and peroxidase (POD, EC 1.11.1.7) require iron in their active sites and they are part of the cell defense mechanism against reactive oxygen species (ROS). These highly reactive oxygen species cause severe damages to DNA, proteins and lead to cell death (Scandalios, 1993). Peroxidases that use ascorbate as a reductant (APX, EC 1.11.1.11) are specifically involved in the H_2O_2 detoxification in chloroplasts (Asada, 1992). Indeed as a consequence of iron deprivation, specific APX activity was reduced at an intracellular level as reported by Ranieri *et al*. (2001). Similarly, in iron deficient pea leaves, the content of iron was extremely low and consequently the APX activity was markedly low as reported by Iturbe-Ormaetxe *et al.* (1995). The authors also studied several biological relevants related to active oxygen species as defense mechanisms to iron deficiency. In addition, nitrate reductase (NR, EC 1.6.6.1) is the enzyme that catalyses the first step of nitrate reduction into nitrite. It is cytosolic enzyme containing flavin, haem-iron and occurs in NADH-dependent form in green tissue (Clark *et al*., 1997). Also, Nenova and Stoyanov (1995) used CAT, POX and NR enzymes activity as a biological marker for diagnosis of iron status in maize plant. The authors found a reduction in the activity of peroxidase and nitrate reductase (two enzymes containing iron) only part of this decrease was due to changes in soluble proteins synthesis.

Gene expression and enzyme activity of various proteins (like nitrate reductase) is responsible to internal and external factors (Mahboobi *et al*., 2002). A deficient supply of one element such as iron usually produces correlative changes in the rate of protein biosynthesis or breakdown as described by Suzuki *et al*. (1998). Electrophoresis as an

analytical tool provides a rough method for genome probing by exposing structural variations in enzymes or other protein genome (Cooke, 1984). Recently, Herbik *et al*. (1996) investigated the effects of Fe –deficiency on protein expression in leaves and roots of tomato plant using the electrophoresis techniques. In their study, three polypeptides were increased in roots grown under Fe deficiency stress compared to control treatments. The authors suggested that members of this group of polypeptides are involved in the response of the root to Fe-deficiency; although their functions remain to be identified. Therefore the aim of the present study was to use the APX, NR, and protein profile as biochemical markers to distinguish between efficient wheat genotypes to grow under iron stress.

MATERIALS AND METHODS

Plant materials. Bread wheat seeds were obtained from Field Crop Institute, Wheat Department. Agriculture Research Center, Giza -Egypt. Wheat genotypes (*Triticum aestivum*) used in the present study was, Sakha 61, Sakha 69, Sakha 93 and Gemaza 7.

Tissue culture. Embryogenic callus was initiated from mature embryos of wheat seeds using the procedure of Mackinnon *et al*. (1987). Embryos were allowed to grow on complete LS media (Table I) supplemented with 2.0 mg L^{-1} 2, 4-D (2, 4 dichlorophenoxy acetic acid) as described previously by Linsmair and Skoog (1965)

Iron treatments. After the third subculture, calli were transferred to fresh LS medium and grown in the presence (+Fe) of $1x10^{-4}$ M iron as Fe-EDTA or absence (-Fe) of iron. Cultures were maintained in a culture room and grown for 30 days under control conditions as follows: 6/8h light/dark regime (150 µ $mol.m^{-2}. s^{-1}$) at 25/20°C and 60/70% R.H. Ten replicates for each treatment were used: samples were taken at 10, 20 and 30 days of each subculture. The growth rate of the culture was periodically observed and the per cent of callus growth relative to a control was then calculated.

Nitrate reductase (NR) assay. The *in vitro* assay for NADH- NR enzyme was used as described by Mladenova *et al*. (1982) with slight modification. Callus tissue was homogenized with chilled mortar and pestle at a ratio of 4 mL buffer/2g fresh weight calli in the phosphate extraction buffer (0.25 M, pH 8.8)**.** Homogenates were centrifuged at 12.000 rpm for 20 min and the supernatant was used for determination of the enzyme activity. The incubation media contained 0.1 mL enzyme extract, 0.1 mL of 0.1 M KNO_3, 0.6 mL Na phosphate buffer (0.1 M; pH 7.6) and 0.2 mL NADH (1mg mL^{-1}). After 15 min of incubation at 30°C, the reaction mixture was colored by the addition of 1.0 mL 1.0% solution of sulfanilic acid in 1.5 N HCl and 1.0 mL of 0.02% of N-(1-Naphthyl)-ethylenediamine-dihydrochloride solution. The intensity of the obtained pink color was read at 540 nm against blank after 30 min incubation in dark. Activity was expressed in nmol NO_2 formed/mg protein/min. Standard calibration curve was obtained by using known standard solutions of sodium nitrite.

Ascorbate peroxidase (APX) assay. APX was determined by the method of Yu *et al*. (1998). The assay mixture (3.0 mL) contained 25 mM potassium phosphate buffer (pH 7.0), 1.0 mM EDTA, 0.1mM H_2O_2, 0.25 mM ascorbate, and 0.15 mL of the crude enzyme extract. The APX activity was measured as a decrease in absorbance at 290 nm due to oxidation of ascorbate during 1.0 min period (Σ=2.8$mM^{-1}cm^{-1}$). Protein concentration was determined as described by Bradford (1976), using bovine serum albumin as standard.

Gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was used for determining the molecular weight of proteins according to Laemmli (1970). PAGE of proteins was performed by standard cooled dual vertical slab units SE 600 (Hoefer Scientific Instruments). Aliquots (20-50 µg protein per lane) were loaded on 1.5 mm thick 12% denaturing gels and run at 4°C. The total soluble protein bands were visualized by staining the gel overnight in staining solution containing Coomassie Brilliant Blue R-250. The molecular weight (MW) in kilo Dalton of protein corresponding to each band was calculated by protein marker (Sigma).

Statistical analysis. The data were statistically analyzed as randomized complete block design (RCBD) according to Sendecor and Corchran (1967). Comparisons among means of treatments were tested for significance against LSD values at 5% level of probabilities.

RESULTS

Growth rate. Data presented in Table II and Fig. 1 showed differences in growth rate percent after 30 days growth among used genotype. Growth rate of the studied wheat genotypes were dramatically affected by the absence of iron in the culture media; whereas, under complete iron supply, the values of Sakha 61 genotype gave the highest growth rate (100%) compared with other genotypes. However, under iron deficiency the growth rate of Sakha 69 showed the lowest growth rate (25%) compared with other genotypes. This indicates that iron-deficiency compromised callus- tissue growth.

Changes in enzymatic activity in callus culture. APX, NR activities were studied in iron deficient callus tissue to see how iron limiting conditions could affect the activity of enzymes that require iron in their prosthetic groups in different wheat genotypes. Among cultivars, Sakha 61 consistently showed the highest activity at all sampling data under both treatments. In contrast, enzymatic activity of APX was decreased under deficient iron supply and the lowest activity was found in callus of Sakha 69 genotype at 30 days growth with value (5.6 µmol ascorbate oxidized/mg pro/min) as presented in Table III. Data from Table III also showed that APX activity significantly increased gradually with age under +Fe treatment in all genotypes. However, when iron was absent, APX activity in callus tissue was

Table 1. Composition of LS-media

Concentration mg/L media			
Macronutrients		**Micronutrient**	
NH_4NO_3	1650.0	$MnSO_4.H_2O$	20.58
KNO_3	1900.0	$ZnSO4.7H_2O$	8.6
$CaCl_2.2H_2O$	440.0	H_3PO_3	6.2
$Mg\ SO_4.7H_2O$	370.0	KI	0.83
KH_2PO_4	170.0	$Na_2MoO_4.2H_2O$	0.25
		$CuSO_4.5H_2O$	0.025
		$CoCl_2.6H_2O$	0.025
Vitamins (mg/L)			
Thiamine-HCl	0.04		
Myo-inisitol	100.0		
Sucrose(g/l)	20.0		
Agar Agar (g/l)	7.0		

Table II. The growth rate of wheat- callus culture grown in the presence (+Fe) or absence (-Fe) of iron supply for 30 days

Wheat genotypes	Growth rate +Fe	%	-Fe	%
Sakha 61	++++	100	++	50
Sakha 69	+++	75	+	25
Sakha 93	+++	75	++	50
Gemaza 7	+++	75	++	50

The growth rate system expressed as follows: (+): Low callus formation; (++): Some callus formation; (+++): Good callus formation; (++++): Very good callus formation

significantly reduced. No significant differences were observed between Sakha 93 and Gamaza 7 in APX at 20 days. It is interesting to note that the lowest activity of APX due to iron stress was clearly observed in Sakha 69 genotype, which was paralleled with the lowest growth rate ratio. So APX could be used as a sensitive marker for Fe-deficiency.

Significant differences between Sakha 61 and 69 genotypes when grown under control treatments were observed for NR activity as presented in Table VI. In addition, when iron was withdrawn from the culture media NR activity was depressed significantly in the same wheat genotypes when compared with the media sufficient in iron treatment. For example the magnitude of inhibition due to iron stress was observed in Sakha 69, the value was 3.9 µmol NO_2 formed/mg protein/min. compared with + Fe at 30-day growth. The same table showed that under +Fe treatment, Sakha 61 had the highest NR activity. It is interesting to note that the same genotype performed the highest value for APX activity as mentioned in Table III. In addition, NADH-NR activity in most of wheat genotypes was maximal at 30 days. Nitrate availability in the growth media would influence NRA. This is expected because NR is substrate inducible enzyme.

Iron deficiency induced proteins. To clarify the molecular basis for acclimation to Fe deprivation in callus culture, we carried out a SDS-PAGE method for water-soluble protein derived from Fe-sufficient and Fe-deficient treatments as

Fig. 1. Growth rate of callus cultures at 30 days; where 1,2,3 and 4 represent wheat genotypes (Sakha 61, Sakha 69, Sakha 93 and Gemaza 7) grown with iron; 5,6,7 and 8 the same genotypes grown without iron respectively

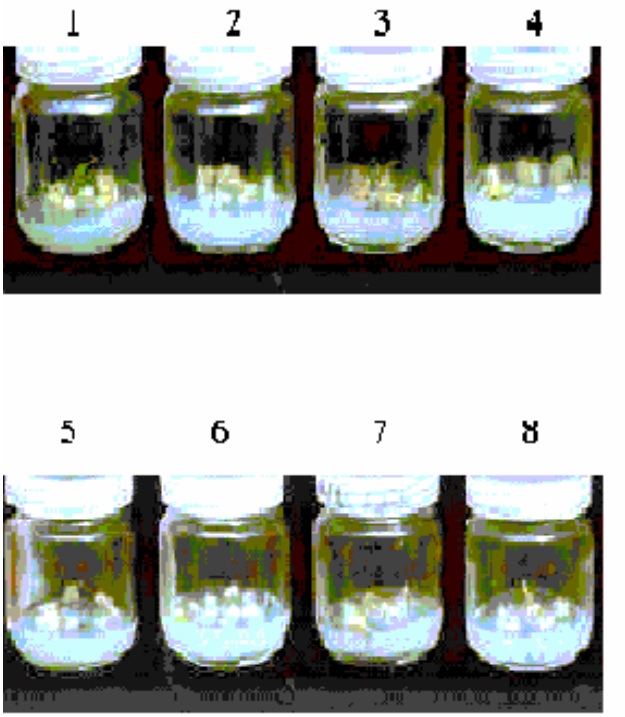
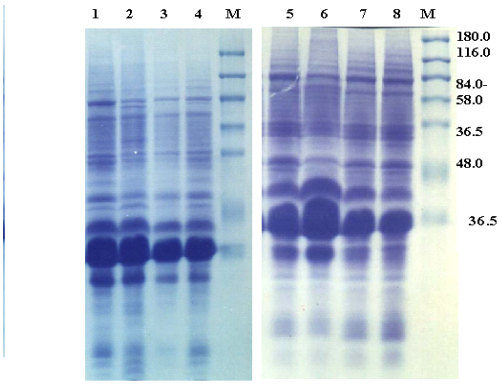


Fig. 2. Electrophoretic pattern of soluble protein from callus cultures at 30 days, where; 1,2,3 and 4 represent wheat genotypes (Sakha 61, Sakha 69, Sakha 93 and Gemaza 7) grown with iron, where; 5,6,7 and 8 the same genotypes grown without iron respectively. M, molecular weight markers in Kilo Dalton



shown in Fig. 2. One major protein band with high intensity was induced in all genotype of 26.6 kD in both treatments. All deficient genotypes showed the appearance of new polypeptides, which undetected in the sufficient ones. For example, in Sakha 61 corresponding to 36.0 kD. This alteration in appearance of new bands may be involved in

Table III. Ascorbate peroxidase (APX) activity of the wheat genotypes grown for 30 days in culture media with (+Fe) and without (-Fe) iron supply

Wheat genotypes	Ascorbate peroxidase activity (µmol ascorbate oxidized/mg protein/ min)								
	10 days			20 Days			30 days		
	+ Fe	- Fe	Mean	+ Fe	- Fe	Mean	+ Fe	- Fe	Mean
Sakha 61	21.9	17.5	19.7	28.7	19.8	24.25	31.9	12.9	22.4
Sakha 69	19.6	7.8	13.7	23.1	12.2	17.65	25.4	5.6	15.5
Sakha 93	16.8	9.6	13.2	19.3	12.8	16.05	22.6	10.2	16.4
Gemaza 7	13.7	10.3	12.0	17.3	14.6	15.95	25.1	11.2	18.5
Mean	18.0	11.3		22.1	15.85		22.25	9.97	
LSD 0.05									
Fe		0.159			0.426			0.311	
Cultivar		0.224			0.602			0.439	
Fe x Cultivar		0.685			0.682			0.424	

Table VI. Nitrate reductase (NR) activity of the wheat genotypes grown for 30 days in culture media with (+Fe) and without (-Fe) iron supply

Wheat genotypes	Nitrate reductase activity (µmol NO_2 formed/mg protein/ min)								
	10 days			20 Days			30 days		
	+ Fe	- Fe	Mean	+ Fe	- Fe	Mean	+ Fe	- Fe	Mean
Sakha 61	18.3	12.2	15.25	23.9	13.3	18.6	15.1	6.6	10.85
Sakha 69	13.6	6.6	10.1	17.7	5.1	11.4	11.6	3.9	7.75
Sakha 93	10.9	6.9	8.9	20.1	6.6	13.35	13.9	5.0	9.45
Gemaza 7	13.2	8.4	10.8	23.4	7.4	15.4	11.6	5.9	8.75
Mean	14.0	8.53		21.27	8.1		13.05	5.35	
LSD 0.05									
Fe		0.344			0.351			0.468	
Cultivar		0.487			0.326			0.662	
Fe x Cultivar		0.469			0.326			0.639	

the cellular iron requirement for growth thus enabling cells to grow under low iron condition. Therefore, the presence of this band in one genotype may be considered as a basic criterion to detect the efficient genotypes. In addition, quantitative differences between control and stress were evident for subunits of 40, 30, 20 kD in Sakha 61 genotype. Similar marked shifts of protein pattern were found in Sakha 93 genotype with two new bands of less than 20 kD.

DISCUSSION

The evaluation of wheat genotypes for resistance to iron deficiency through tissue culture technique could distinguish between Fe-efficient and Fe- inefficient genotypes. Four genotypes varying in sensitivity to iron deficiency were cultured on LS medium. Callus growth rate relative to a control medium were recorded. While genotypes differences for percent callus weight reduction were observed at the each level of iron treatments. Similar results were obtained by Graham *et al*. (1992) who used tissue culture technique to distinguish between Fe- efficient and Fe- inefficient soybean genotypes and found a reduction in callus growth weight under iron stress resulted from bicarbonate inhibition. It was known that bicarbonate inhibits the absorption and translocation of iron by the plant.

Different reactions of the enzymes to iron deficiency in the presence of a common haem pull might be explained: a) by the specific influence of iron deficiency on apoprotein synthesis and b) by the different affinity of the apoproteins to heme (Sijmons *et al*., 1985). Disturbance in the synthesis of substrate, cofactors and inhibitors is partially due to the changed photosynthesis (Nenova & Stoyanove, 1993), changes in some tissue ions concentrations (Anisimov & Alexandrova, 1981) and phytochormon content (Stoyanov & Tha, 1981) which additionally exert influence on the enzyme activities in situ. In addition, Han *et al*. (1994) used biochemical parameters to distinguish between iron efficient and in-efficient Genus *Malus* genotypes grown in water culture. One of these parameter was peroxidase activity, the efficient genotypes was able to exhibit high peroxidase activity when compared to inefficient genotypes. In this concern, Lombardi *et al*. (2003) found a reduction in the activity of catalase, superoxide dismutase and induction in gene expression in peach rootstock plantlets grown under Fe- deficiency. The authors demonstrate that *in vitro* system can be useful approach to study the biochemical alteration induced by iron deficiency. Taking into consideration the important role of nitrate reductase and peroxidase in plant metabolism and the direct and indirect effect of Fe-deficiency on their activities, it may be supposed that, the observed restricted growth of some wheat genotypes was partially due to the changes in these enzyme activities.

Total soluble protein profile showed differences between used genotypes. All four genotypes are clearly identifiable from the protein-banding pattern. SDS-PAGE of total protein profiles is, therefore, an efficient procedure for

differentiating wheat genotypes. The obtained results are in agreement with the results of Schmidt *et al*. (1997) who found an alteration in the pattern of protein synthesis in tomato roots grown under iron deficiency stress as a result of adaptive response specific to Fe-deficiency. Rengel and Hawkesford (1997) also detected new polypeptides in wheat genotypes exposed to Zinc deficiency, which may be possibly linked to a resistance mechanism.

CONCLUSION

The identification of biochemical markers or protein fingerprinting of tolerant genotypes is very important for the prediction of genotypes efficiency to iron. It could be possible to combine tolerance with other desirable traits of crop to evolve new varieties much better adapted to abiotic stress.

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