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IN VITRO BIOCHEMICAL GENETIC STUDY OF RESISTANCE TO FUSARIUM CULMORUM IN SOME WHEAT EGYPTIAN VARIETIES

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ABSTRACT

The present work aims to study the biochemical genetic background of wheat resistance to the pathogen; *Fusarium culmorum* in four Egyptian wheat (*Triticum aestivum)* varieties, i.e. Sids 1, Sids 8, Sids 9 and Giza 167 by means of tissue culture techniques. It included the study of effects for two concentrations of *F. culmorum* toxic filtrate (10% and 20%) on peroxidase isoenzymes and protein amino acids in treated calli and regenerated wheat plants. All genotypes turned to be very sensitive to the toxic filtrate, but the level of sensitivity was proven to depend upon on genotype. Gene expression of peroxidase isoenzymes varied in number and activity of isoenzymes. Treated calli revealed a markedly decrease in the concentration of some amino acids e.g. alanine, argnine and an increase in other amino acids as valine.

INTRODUCTION

Wheat is the most intensively bred species in the world. It is the second to rice in world production, which in recent years has approached 550 million metric tones per year (Young *et al*., 1990). The bread wheat (*Triticum aestivum* L.) is divided into four main categories, based on the protein content of the grain. Hard red spring (11 to 18% protein) and winter wheat (9 to 15% protein) are used primarily for bread, soft red winter (8 to 12% protein) and white wheat (8 to 11% protein) are used primarily for muffins, noodles, cakes, crackers, and cookies. Durum wheat (*Triticum durum* L.) is used primarily for macaroni and pasta. Wheat is still subject to many diseases which makes genetic engineering an appealing biotechnology for wheat improvement (Carman, 1995).

Bread wheat may suffer from a number of diseases caused by various fungi, bacteria, and viruses. Some of the pathogenic fungi can invade the seeds and produce toxic metabolites harmful for both humans and livestock. In the voluminous group of fungi attacking wheat and damaging wheat seeds, species belonging to the genus *Fusarium* became especially widespread, sometimes causing epidemics and serious health problems. On wheat, *Fusarium graminearum, F. culmorum, F. nivale* and *F. sporotrichioides* occur most frequently. From diseased plants, *F. graminearum* and *F. culmorum* could be identified as dominant species. They cause the so-called fusariose (seedling blight, foot rot, scab, or head blight). The most dangerous for health of these being *Fusarium* head blight, which directly threatens the developing seeds, especially under stress conditions (excess humidity and high temperature, unbalanced mineral supply, and lodging, etc.). The mycotoxins produced by the pathogenic *Fusarium* species are numerous and of differing chemical nature. Among them the highly toxic trichothecenes such as

deoxynivalenol or vomitoxin (DON), T-2 toxin, causing hemorrhagia, neural disorders, and damage to the immune system and the less toxic zearalenone and its derivatives, affecting animal and human reproduction (Ahmed *et al*., 1996).

The present work is an attempt to understand the biochemical genetic background of wheat resistance to *Fusarium culmorum* through *in vitro* study for the effect of the toxic filtrate on peroxidase isoenzymes gene expression and protein amino acids for four wheat (*Triticum aestivum)* Egyptian genotypes, i.e. Sids 1, Sids 8, Sids 9 and Giza 167.

MATERIALS AND METHODS

Wheat varieties

Four varieties of wheat (*Triticum aestivum)*, namely Sids 1, Sids 8, Sids 9 and Giza 167, were used in the present study. The seeds of these varieties were obtained from Nobaria Research Center, Ministry of Agriculture, Egypt.

Fungal strains

One pure culture (isolate) of *Fusarium culmorum* was used in this study. The isolate was obtained from the Department of Plant Pathology, Faculty of Agriculture, Alexandria University.

Establishment of wheat calli:

The culture medium contained the inorganic salts of Murashige and Skoog (1962), supplemented with 150 mg/L. asparagine, 0.5 mg/L thiamine, 1.0 mg/L 2 , 4 – D, 20 g/L sucrose and 7.0 g/L agar (Barakat, 1996). Supplementation of 1 mg/L 2,4-D for callus induction was used for the employed (or tested) four wheat genotypes (Sids 1, Sids 8, Sids 9 and Giza 167). Regeneration medium for the previous genotypes was the same as callus induction medium except that 2,4-D was 0.1 mg/L instead of 1mg/L.

Fusarium Culmorum Toxic Filtrate

To prepare sufficient quantities of *F. culmorum* toxin filtrate, the isolate was grown in 250 ml flasks, each contained 100 ml of potato dextrose medium (P.D), according to Shahin and Shepard method, (1979). Flasks were autoclaved, then inoculated with 5mm P.D.A. discs of *F. culmorum* isolate which was taken from margin of two weeks old cultures. The inoculated flasks were incubated at 25ºC for 4 weeks. Then, the upper layer of the fungus was removed. The filtrates were collected through Whatman No .1 filter paper, followed by filtration through sterilized bacterial filter (Seitz filter) before usage.

Treatment of callus by toxic filtrate:

Mature embryo of four varieties; Sids 1, Sids 9, Sids 8, and Giza 167 were cultured on medium. This medium was supplemented with two concentrations of sterilized toxin preparations from culture filtrate, i.e. 10% (B) and 20% (C). In addition, the non-toxic control cultures were used without the addition of sterilized toxin filtrate (12 treatments were performed, with six replications for each). Cultures were incubated at 25ºC under 16 hours illumination and 8 hours darkness. Calli of the four varieties; Sids 1, Sids 9, Sids 8 and Giza 167 (five weeks in age) were, also, transferred to culture medium which was the same as the culture medium supplemented with the

same two concentrations of sterilized toxin prepared from culture filtrate used before for callus initiation stage, i.e. 10% (B) and 20% (C) from the total volume of the medium in addition to control A and were incubated at the same conditions of temperature and light.

Electrophoresis Technique

In the present study, peroxidase isoenzymes for the tested varieties were assayed in following cases: -

1- Different pieces of callus were taken randomly (5 weeks in age). Ten plates used, each plate contained 12 samples.
2- Shoots (at the same age) were taken from different callus which formed shoots on callus induction medium. Ten plates used, each plate contained 12 samples.
3- Calli were treated by the two concentrations of toxin (B and C) and control calli (A). Three plates used, each plate contained 12 samples.
4- Shoots were treated by the two concentrations of toxins (B and C) and control shoots (A). Three plates used, each plate contained 12 samples as well.

Buffer, gel medium, staining solution and the electrophoretic procedure used were as follows:-

The Buffer:- 0.23 M Tris-citric acid pH 8.0, add 27.7 g Tris and 11 g citric acid in one liter distilled water- adjust pH at 8.

Gel Medium:- (Agar – starch – polyvinyl pyrolidine (P.V.P) gel)

1.0 g agar, 0.5 g p.v.p. and 0.3 of hydrolyzed starch were added to 100 ml 0.023 M Tris-citric acid buffer, pH 8.0 and the mixture was cooked in a boiling water bath until the solution become transparent. Gel plates were prepared by pouring the solution on glass plates 20 x 20 cm and kept at 4ºc until utilization (El–Metainy *et al,* 1977).

Procedure:- The samples (calli or shoots) were homogenized in cool mortar and the homogenate was absorbed on a 1 x 0.2 cm strips of filter paper and the next steps has been done according to (Yacout *et al.*, 1998).

Image analysis software " *phoretix*™ TotalLab " version 2003.02, (Nonlinear Dynamics Ltd, U.K), was used to locate isoenzymes bands and to determine enzyme activity in pixel units.

RESULTS AND DISCUSSION

Figure (1) shows the photograph and descriptive diagram of peroxidase isoenzymes extracted from wheat untreated calli and from *fusarium culmorum* toxic filitrate treated calli, after four weeks of treatments on regeneration medium for four genotypes, Sids 1, Sids 8, Sids 9 and Giza 167. Comparing with the control, it was noticeable that treatment B did not affect the number of bands of peroxidase isoenzymes, while the activity of isoenzymes was altered. Genotype Sids 8 showed disappearance of one band. However, it was clear that treatment (B) increased the activity of peroxidase isoenzymes especially in Giza 167 and Sids 9.

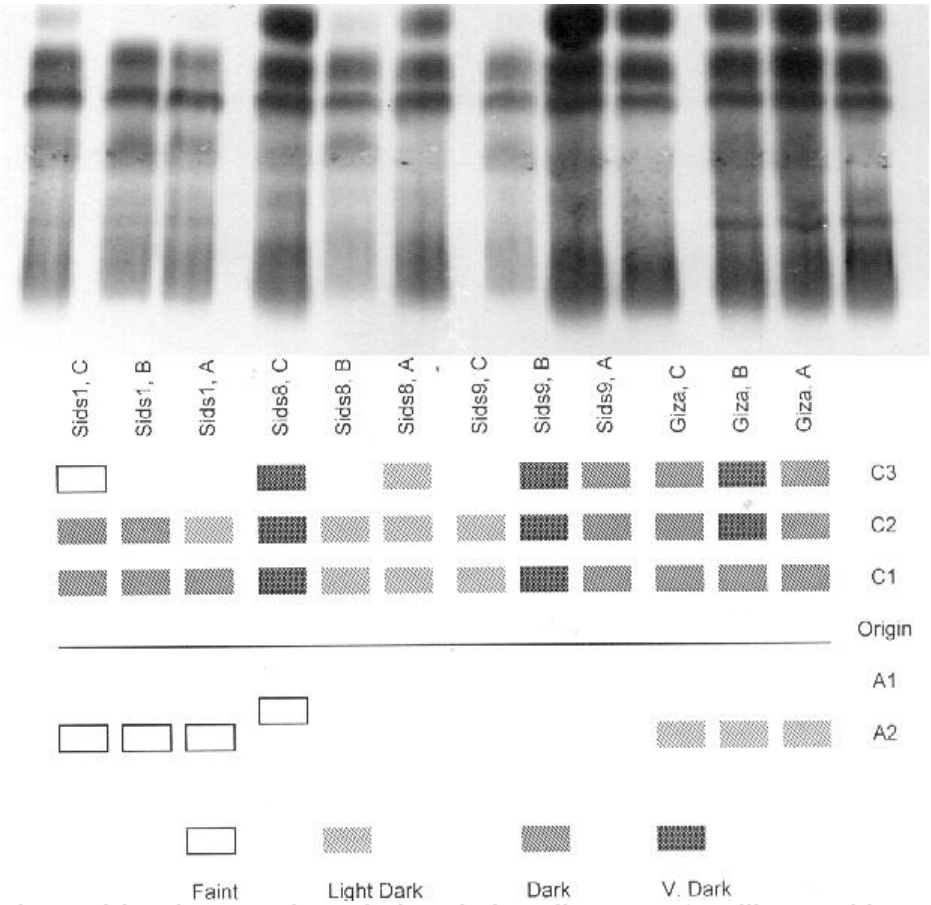

Figure (1): Photograph and descriptive diagram of calli peroxidase isoenzymes for 4 wheat genotypes after four weeks of treatment by toxic filtrate.

Where:	Faint	Light Dark	Dark	Very Dark
	100-119	120-139	140-159	160-200
Pixels				
	A=control	B=10% toxin filtrate	C=20% toxin filtrate	

Treatment (C) showed decline in peroxidase activity in Giza 167 and Sids 9, compared with treatment (B).Whereas, the samples of Sids 8 genotype showed increase in the activity of isozymes compared with the control and treatment (B) as well. Sids 1 did not show significant differences in the activity. It can be observed that treatment (C) reduced the number of bands in Sids 9 and increased it in Sids I and Sids 8. The effect of toxic filtrate by the two concentrations (B and C) on peroxidase isoenzymes of Giza 167 was slight and can be ignored, in comparison to the other genotypes. This result is in agreement with those obtained by using tissue

culture techniques in the present study which showed that the highest percentage of survival calli was observed in Giza 167.

Toxic filtrate treated regenerated leaves (treatment

B) of four genotypes showed differential responses in numbers and activity of the peroxidase isoenzymes as follows: in Giza 167, C2 declined in its activity, while C3, C4, Al and A2 bands was not observed. In Sids8, C2 declined in its activity, Cl and A2 disappeared and C3 was detected. In Sids 9, the bands did not differ from the control. In Sids 1, C2 declined in activity and C3 and A2 bands disappeared.

Peroxidase isoenzymes extracted from wheat; untreated regenerated leaves and from *F. culmorum* toxic filtrate treated regenerated leaves, are shown in Figure (2). Results suggest that, treatment (B) affected the number of bands in all genotypes, except in Sids 9. Four bands disappeared in Giza 167 and two bands in Sids 8 and two bands in Sids 1.Whereas, C3 appeared in Sids 8. All genotypes, except Sids9, showed a reduction in the activity of enzymes, but the significant effect of treatment (B) was the reduction of the band number. The bands of treatment (C) showed similarity with the control samples (A) in Sids 1 and Sids 8. The treated samples of Sids 1 differed from the control in A2 band which disappeared in treated samples. The treated samples of Sids 8 differed from the control in the activity of the enzyme in C4 band which increased in relation to the control. On the other hand, the treatment (C) of Giza 167 showed a similarity with the treatment (B), except in C4 band which existed in treatment (C) but disappeared in treatment (B). ln Sids 9, band C3 appeared in treatment (C), but did not exist in treatment (B) and the control. The treatment (C) showed an increase in the activity of the enzyme in relation to treatment (B) in all genotypes. The activity of C4 band of Giza 167 was decreased in comparison to the control, and C4 band of Sids 1 which was the same as the treatment (B) and the control. The increase in the activity of the enzymes in treatment (C) was the same as that of the control in some cases, i.e. C2 band of Giza 167 and Sids 8 and Sids 1, or this increase was higher than the treatment (B) and the control in other cases, i.e. C4 in Sids 9 and Sids 8, and C2 in Sids 9.

The reduction of the number and the activity of peroxidase isoenzymes which resulted from the treatment in the present study, is in partial agreement with that found by Sogina and Tatarinova (1987), who found that in two varieties of wheat infected by *F. culmorum* and *F. moniliforme.* The peroxidase activity in the roots rose at the onset of the infection and gradually declined as infection developed. Also, Lesney, (1990) found that the extra cellular peroxidase activity was significantly depressed by the higher concentration of the elicitor within 24 hour in the callus suspension culture. Also, Mittler *et al*., (1998) found that the viral infection induced programmed cell death in tobacco is accompanied by the suppression of cytosolic Ascorbate peroxidase possibly at the level of translation and elongation.

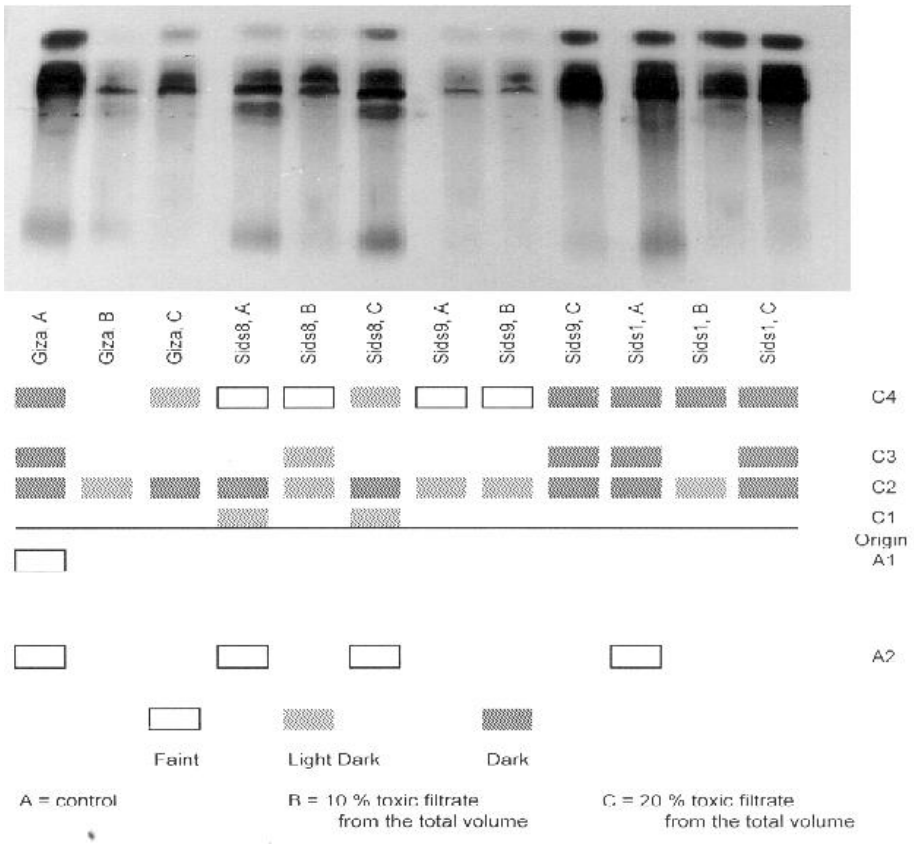

Figure (2): Photograph and descriptive diagram of peroxidase isoenzymes in regenerated leaves of four wheat genotypes after four weeks of treatment by toxic filtrate.

Where:	Faint	Light Dark	Dark
	100-119	120-139	140-159 Pixels
	A=control	B=10% toxin filtrate	C=20% toxin filtrate

On the other hand, the increase of number and activity of peroxidase isoenzymes in the present study, are in partial agreement with that found by Soliman *et al.,* (1991) who observed that in a wheat resistant variety, infection with *F. oxysporum* and *F. moniliforme* was capable to increase peroxidase activity. Also, in a susceptible variety, *F. oxysporum* caused peroxidase activity to peak earlier, inoculation with the *F. moniliforme* increased peroxidase activity. El-Abdou *et al*., (1993) indicated that at the late stages of pathogenesis, resistant lines, with highly effective resistance genes had higher activity of peroxidase isoenzymes than susceptible lines with weakly effective genes. Also, Haoqin *et al*., (1995) indicated that peroxidase activity of resistant variety increased rapidly, whereas, the susceptible variety increased slowly. Caruso, (1999) found that *F. cu!morum* infection caused

increase in peroxidase activity at the early stages of development of inoculation of wheat seedlings. Gotthardt and Grambow, (1992) found that the differences in peroxidase activity for callus suspension culture from two near-isogenic lines of *T. aestivum,* resistant and suscptible to stem rust were even more obvious, when the suspension culture was treated with elicitorThis later behavior of isoperoxidases was nearly similar to the behavior of isoperoxidase which was extracted from treated calli in the present study.

Figure (3) shows photograph and descriptive diagram of peroxidase isozymes extracted from different wheat calli (5 weeks age) which were obtained from callus induction medium (three samples of calli per one genotypes per plate used). It was clear that all bands in all samples of Giza 167 and Sids 8 had the same number and activity, there were no differences among the different samples of the genotype. Whereas, samples of Sids 9 showed differences in activity of enzymes; C4 might be low or medium in its activity and A3 and C3 might be high or medium in their activity. Finally, samples of Sids 1 showed differences in the number of anodal bands and its activity, and C4 may be high or medium in its activity. These data indicated that the calli of two genotypes; Giza 167 and Sids 8 showed high stability in peroxidase gene expression. On the other hand, *In vitro* culture disturbed gene expression for another two genotypes; Sids 9 and Sids 1.

Figure (4) ?Shows plate photograph and descriptive diagram of peroxidase isoenzymes extracted from wheat regenerated leaves with 5 weeks in age which were obtained on callus induction medium for four genotypes: Giza 167, Sids 9, Sids 8 and Sids I (three samples of regenerated leaves per one genotypes per plate used). The cathodal bands of samples in each genotype: Giza 167, Sids 8 and Sids 1 showed no differences. Whereas, the samples of Sids 9 showed differences in Cl band, which may not appear.

The anodal bands of samples in each genotype showed differences in numbers and activity of the enzyme These data indicated that the cathodal bands of regenerated calli of three genotypes; Giza 167, Sids 8 and Sids I showed high stability through tissue culture for the gene expression of peroxidase isoenzymes. Whereas, cathodal bands of regenerated calli of Sids 9 and all anodal bands of regenerated calli for all genotypes showed changes in the gene expression of peroxidase isoenzymes. This result is in agreement with that indicated by Ryan *et al.,* (1987) who reported that there is evidence that genotypes influence the total amount and expression of somaclonal variation. Also, Takvorian and Coville, (1997) suggested that *in vitro* culture could disturb the post-transcriptional regulation of gene expression. Whereas, the present data are partially in agreement with that indicated by Zhang *et al*., (1993) who reported that several regenerated potato plant showed greater resistance to *Pseudomonas solancearum* than the parents. These putative somatic variants had increased peroxidase activity.

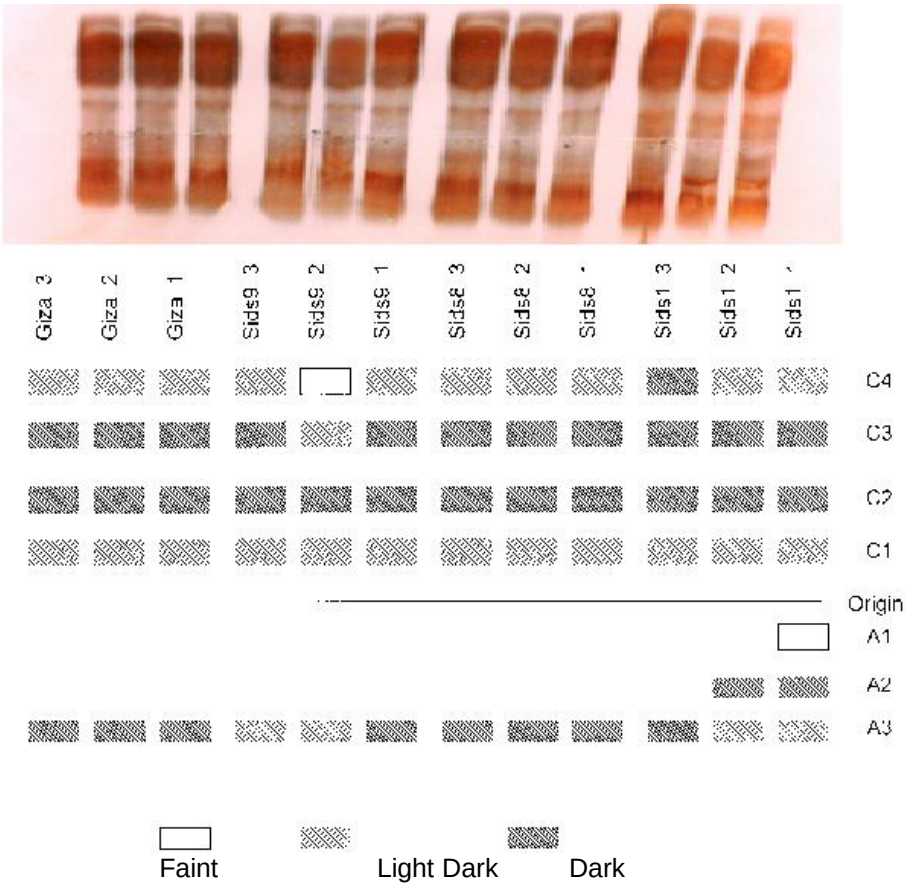

Figure (3): Photograph and descriptive diagram of peroxidase isoenzymes in different samples of calli of four wheat genotypes.

Where:	Faint	Light Dark	Dark
	100-119	120-139	140-159 Pixels
	A=control	B=10% toxin filtrate	C=20% toxin filtrate

Amino acids analysis

Tissue culture techniques in the present study showed that the highest percentage of survival calli which was obtained from toxic medium, was observed in Giza 167. On the other hand the lowest one was observed in Sids I (non-published data). Therefore, the survival calli and regenerated leaves (which transferred on toxic medium) were used to determine the effect of the two concentrations (B and C) of toxic filtrate on protein amino acids for the genotypes; Giza 167 and Sids 1 as well.

Figures (5 and 6) show the effect of the two concentrations (B and C) of toxic filtrate on protein amino acids for the survival calli of Giza 167 and Sids I. The results suggest that all amino acids concentration in treated calli

of Sids I were decreased by different degrees except, Threonine and Serine when compared to the control samples. On the other hand, most amino acids in treated calli of Giza 167 tended to increase in their concentration, or did not differ nearly from the control. These presented data agreed with those obtained from the study of tissue culture, calli of Sids I which was very sensitive to the toxic filtrate medium.

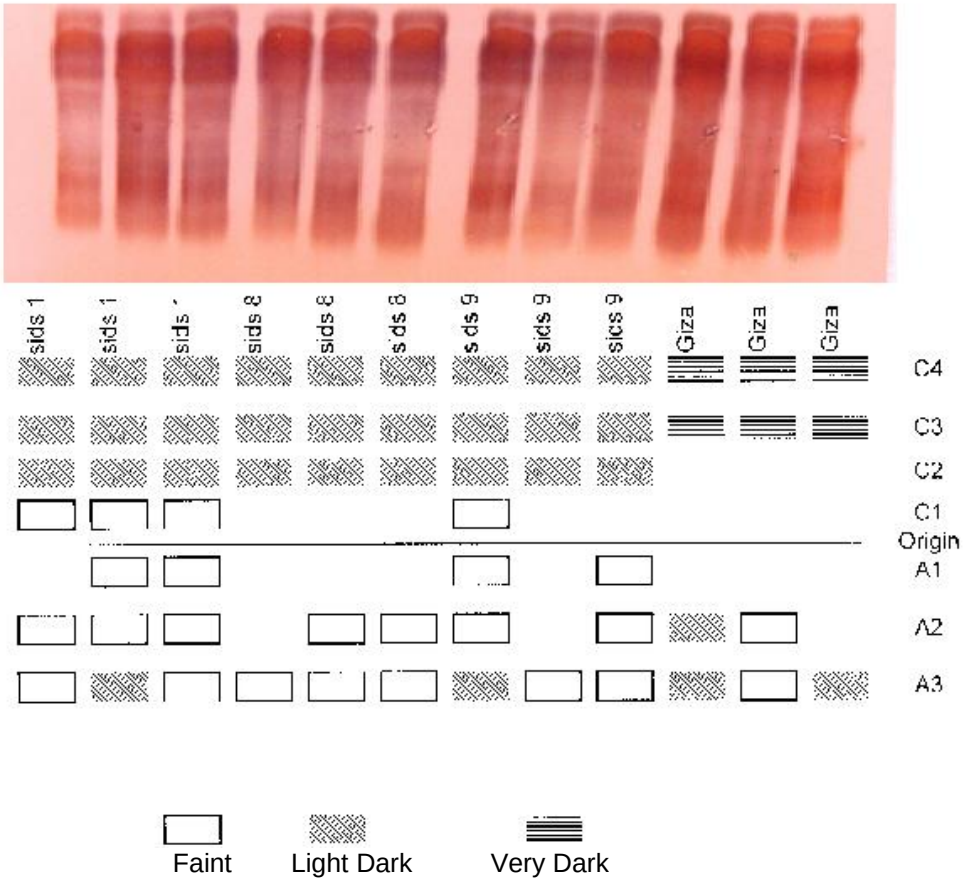

Figure (4): Photograph and descriptive diagram of peroxidase isoenzymes in different samples of regenerated leaves of four wheat genotypes.

Where:	Faint	Light Dark	Very Dark
	100-119	120-139	160-200 Pixels
	A=control	B=10% toxin filtrate	C=20% toxin filtrate

The effect of two concentrations (B) and (C) of toxic filtrate on protein amino acids for regenerated leaves (obtained from callus induction medium and transferred to toxic root induction medium for four weeks) of Giza 167 and Sidsl are shown in figures (23 and 24). It could be observed

that most amino acids in treated regenerated leaves of Giza 167 and Sids 1 tended to increase in their concentrations. On the other hand, limited number of amino acids decreased in their concentrations.

It can be concluded that, treated calli showed decreases in the concentration of some amino acids in relation to the other amino acids; Alanine, Argnine, Glutamic acid (in treatment B and C), Valine (in treatment C) for Sids 1; and Glutamic acid (in C treatment) only in Giza 167. On the other hand, treated regenerated leaves
(shoots) revealed an increase in the concentration of some amino acids in relation to the other amino acids; Valine, Asparatic acid, lsoleucine, Leucine (in treatment B and C), and Phenyl alanine(in treatmentB) for Sids I ; and Asparatic acid (in treatment B), Isoleucin, Leucine, Valine, Argnine, Phenylalanine and Lysine (in treatment C) for Giza 167. Two amino acids; Proline and Glycine decreased in their concentration in Sids I by treatment B and C, respectively.

The increase in the concentration of amino acids in treated samples in relation to the control may be attributed partially to the production of certain protein related to the defense response (DR) genes. Li *et al.,* (1999) reported that (DR) genes have been grouped into several classes on the basis of their predicted protein products. For instance, genes involved in the hypersensitive response, genes for flavonoid metabolic pathway and proline/glycine-rich protein. Also, this increase might be caused by certain proteins which are produced by the pathogen itself. This explanation agrees with that given by Kooman *et al.,* (1997) who reported that *Cladosporium fulvum* isolates expressing the avrg gene which were shown to produce a cysteinerich 28-amino acid peptide. This peptide elicits a response in tomato cultivars carrying the CF-9 resistance gene. On the other hand, the decrease in the concentration of amino acids in treated samples in relation to the control might be attributed to hydrolysis of weakened protein matrix as suggested by Somani *et al.,* (1993). Also, this decrease may be resulted from protein synthesis inhibition effects of deoxynivalenol (DON), as observed by Miller and Ewen,. (1997).

It can be concluded that the treated samples (calli or regenerated leaves) of Giza 167 and Sids 1 showed differences in the concentration of amino acids. These data agree partially with that indicated by Bajaj and Saettler, (1970) who reported that the type and concentration of amino acids of *Phaseolus vulgaris* differed in callus grown on toxin filtrate of *Pseudomonas phaseolicola* particularly the dramatic increase up to 55-fold-of ornithine in callus culture. Also, Singh *et al.,* (1989) observed that qualitative and quantitative variations in amino acids were noted in the leaf extracts of wheat after pollutant treatment.

It can be suggested that the differences in the concentration of amino acids in the present study, depend on the genotype effect and the concentration of toxic filtrate. These data agree with that indicated by Bandurska *et al.,* (1994) who reported that the vomitoxin of *F. culmorum* caused an increase of free Proline in seedlings leaves of winter wheat cultivars of 1.18-3.36 times depending on cultivar and vomitoxin concentration. Also, Miller and Ewen, (1997) observed that there were

resistance to the protein-synthesis inhibition effects of deoxynivalenol (produced by *F. graminearum*) in the head blight resistant wheat cultivar Frontana. It was suggested that due to the existence of a mutation in the peptidyle transferase. This cultivar also exhibited resistance to the membrane-damaging properties of this toxin compared to the other cultivars.

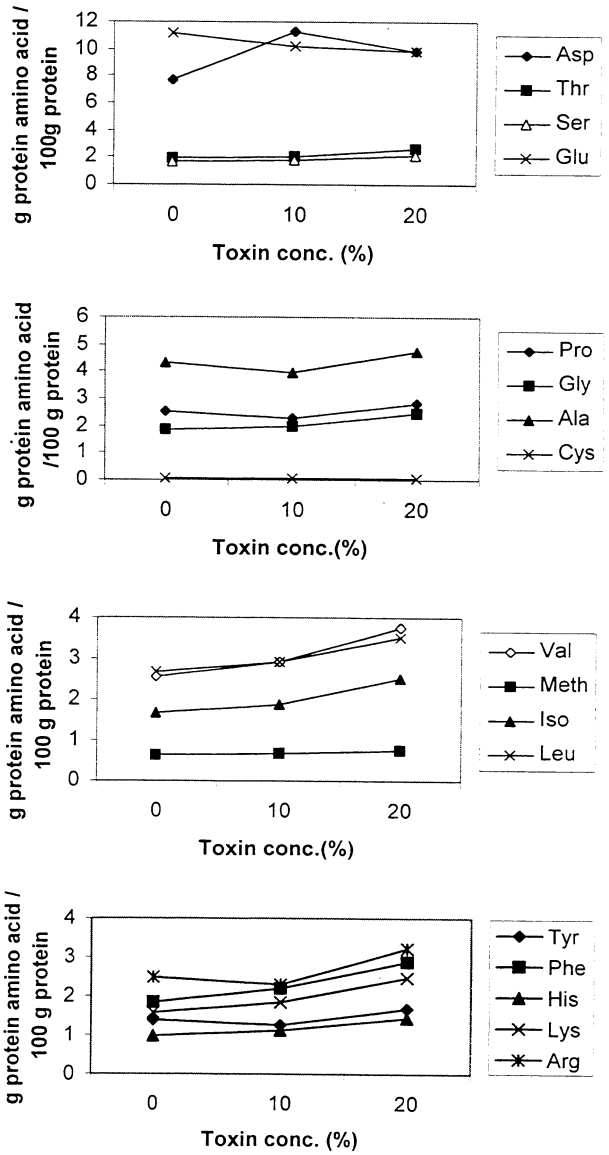

Figure (5):The effect of the increase of the concentration of toxic filtrate on protein amino acid concentration in tissue of regenerated leaves for Giza 167.

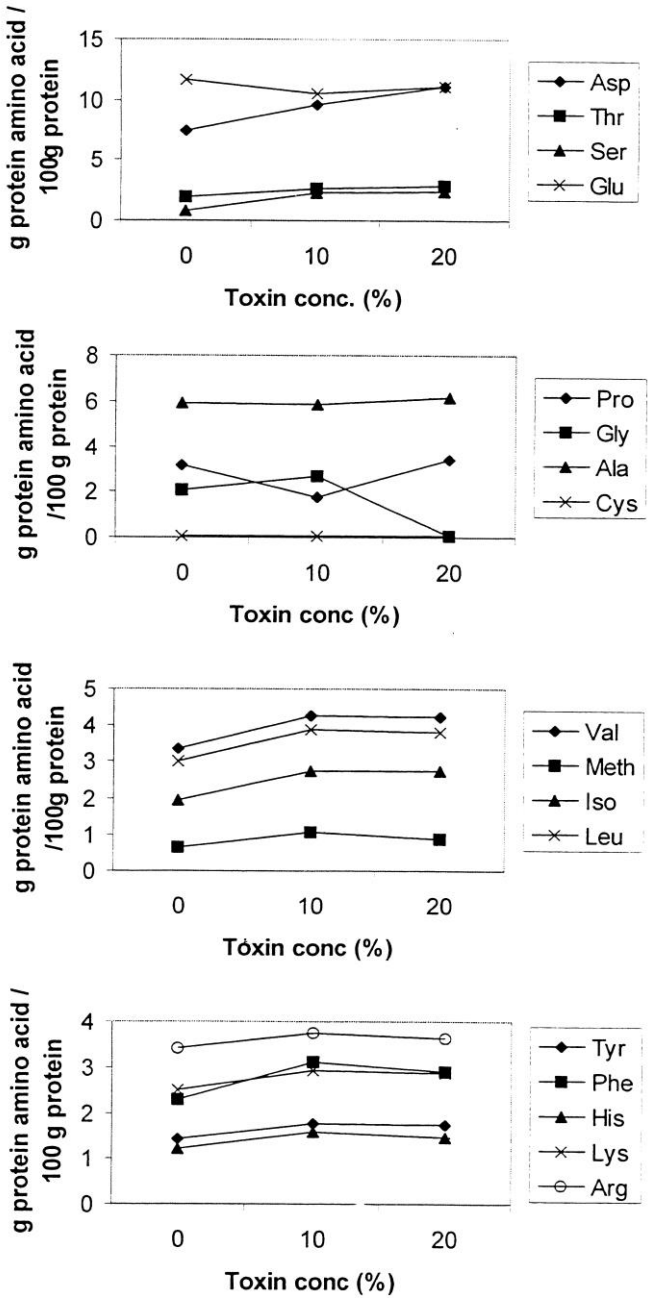

Figure (6):The effect of the increase of the concentration of toxic filtrate on protein amino acid concentration in tissue of regenerated leaves for Sids 1.

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دراسة وراثية كيموحيوية فى الأنبوب للمقاومة للفيوزاريم كولمورم فى بعض أصناف القمح المصرية

م. م. ياقوت وم. أ. السهريجى وم. م. الحداد وهـ. م. شكم
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يهدف هذا البحث الى دراسة الخلفية الوراثية الكيموحيوية لمقاومة القمح للمسبب المرضى *Fusarium culmorum* فى أربعة أصناف قمح (*Triticum aestivum*) مصرية هى سيدس١ وسيدس٨ وسيدس٩ وجيزه١٦٨ بأستخدام تقنية زراعة الانسجة. تضمن البحث دراسة يأثير تركيزين من الراشح السام للفطر (١٠% و٢٠%) على كل من مشابهات أنزيم البيروكسيدز فى كل من الكالس والنباتات الناتجة من زراعة الانسجة. أظهرت كل التراكيب الوراثية حساسية عالية لراشح الفطر السام وأختلفت تلك الحساسية تبعا لاختلاف التركيب الوراثى. كما تفاوت التعبير الجينى وتباين لمشابهات أنزيم البيروكسيدز من حيث عدد الحزم ودرجة النشاط الانزيمى. أظهر الكالس المعامل انخفاض ملحوظ فى تركيز عدد من الأحماض الامينية مثل الألانين والأرجنين وازدياد كما فى حمض الفالين.