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BIOCHEMICAL GENETIC STUDIES ON TRYPSIN INHIBITOR ACTIVITY IN A DIALLEL CROSS OF FABA BEAN (*VICIA FABA*).

BY

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ABSTRACT

The investigation was carried out at the laboratory of plant physiology, Bayreuth University, Germany on eight parents and their F_1 crosses for evaluation of trypsin inhibitor activity (TIA). Highly significant variations were noted for total protein percentage and trypsin inhibitor activity in parents and the obtained crosses at the two locations under study; Moshtohor and Nubaria. Tinova was the lowest variety for TIA while Giza 2 was the highest parent at both locations. The lowest content of Trypsin inhibitor and the highest content of protein as well as seed yield and its components were detected in the crosses; Rina Blanka X Tinova, Rina Blanka X Giza 429 and Triple white X Giza 2. The best crosses for all the studied characters especially trypsin inhibitor and protein content were Rina Blanka X Tina, Rina Blanka X Triple White and Rina Blanka X Tinova. The protein banding for trypsin inhibitor was found at 20-22 KDa for the studied parents at Nubaria location. The digested copolymerized SDS-gel electrophoresis emphasized a varied intensty of represented trypsin inhibitor bands for all parents and crosses at Nubaria location.

INTRODUCTION

The nutritive value of legume seeds is limited by the presence of toxic substances that are either indigestible or antagonistic to digestion as alkaloids (in some peoples), phytohemoagglutinins, saponins, tannins, cyanogenic factors and trypsin inhibitors (Prabhu *et al.*, 1984, Gupta, 1987, Pisulewska and Pisulewski, 2000, Berger *et al.*, 2003 and Muzquiz *et al.*, 2004). These toxic factors combine with trypsin to form an inactive complex, which reducing protein digestion. Thus, the content and type of trypsin inhibitors can be used as an important parameter in evaluating the quality of faba bean seeds. One of the important faba bean breeding aims is to produce new cultivars which superior for yield and the nutritional quality of the seeds (Polignano *et al.*, 1986 and Muntz *et al.*, 1993, Berger *et al.*, 2003). Increasing the percentage and content of seed protein with a decrease in the amount of antinutritional factors (Trypsin, Chemotrypsin and Tannin) are the main characters which can use as selection criteria in breeding programs (Muntz *et al.*, 1993, Grosjean *et al.*, 1993 and Krishnan 2001). Truchlinski and Sembratowicz (1996) investigated the concentrations of trypsin inhibitors and tannin in seeds of different varieties of legumes. They concluded that the presence of antinutritional factors in these legumes required a

pretreatment of seeds for complete or partial inactivation of these antinutritional factors before using them as food. Zimniak *et al.*, 1999 investigated 58 accessions of *Vicia faba*. Proteins of individual seeds were separated by polyacrylamide gel electrophoresis followed by staining gels for inhibitor activity against trypsin. They concluded that number of trypsin inhibitor (TI) bands varied from three in *Vicia eristalioides* and *Vicia galilaea* to 15 bands in *Vicia narbonenesis*. Filippetti and Azadegan (1994) indicated that there was no correlation between trypsin inhibitor content and protein content (r = -0.19) in seeds of a number of faba bean lines. Meanwhile, Makkar *et al.*, 1997 stated that correlation coefficient between trypsin inhibitor activity and tannins was negative (r = -0.88).

The aim of this study is to determine trypsin inhibitor and protein contents as well as their genetic relationship with yield and yield components. At the molecular level, detection of bands of trypsin inhibitor in faba bean varieties will help the breeder in breeding of faba bean programs.

MATERIALS AND METHODS

1. Plant Material

Seeds of eight parents (Rina Blanka as P_1 from Spain, Triple White as P_2, Tinova as P_3, Tina as P_4 from Germany and Giza 2 as P_5, Giza 716 as P_6, Giza 461 as P_7 and Giza 429 as P_8 from Egypt) and their F_2 seeds resulted from diallel analysis were used in this study during 2001 and 2002 at the laboratory of plant physiology, Bayreuth university, Germany.

Reagents for total protein determination:

a. Protein extraction buffer which consisted of 50 mM phosphate buffer (KH_2PO_4) at pH 7.6, 1mM EDETA and dist. water up to 100 ml.
b. BCA Reagent for protein Assay contained 1 part of BCA protein assay reagent B and 50 parts of BCA protein assay reagent A.

2. Methods

Seeds were milled to fine powder (particles of 10-20 µm in a diameter) in a Cyclotec 1093 mill (Tecator AB, Hojanas, Sweden), using a 0.2 mm mesh screen.

Total nitrogen in each defatted sample was determined by Kjeldahl procedure and the crude total protein content was calculated by using the conversion factor of (6. 25 x N).

Soluble protein extraction was achieved as the powder of seeds was mixed with acetone at room temperature for 24 h to remove fatty acids followed by filtration and rapid air drying. A sample of 0.5 g of defatted seed powder was suspended in 2ml of extraction phosphate buffer with pH 7.6. The aliquot was shaked for 4 h and then centrifuged at 7000 rpm for 15 minutes at 4°C. The resulted supernatant was centrifuged at 14000 rpm for 10 minutes at 4° C. The resulted supernatant was transferred to a new tube and then kept at -20°C for use to evaluate the soluble protein and trypsin inhibitors contents in addition to detection protease inhibitors using SDS gel electrophoresis.

The treatments used for detection of soluble protein:

Protein sample assay

2ml of BCA reagent (A+B) added to 0.1ml of protein extract sample then the mixture was incubated for 30 min at 37 °C.

Blank reagent

2ml of BCA reagent (A+B) added to 0.1ml of extraction phosphate buffer at pH 7.6 then the mixture was incubated for 30 min at 37 °C.

Standard Bovine serum albumin

For protein standard, Bovine serum albumin used as known standard protein for calibration curve as 2mg of albumin and 1 ml of extraction phosphate buffer at pH 7.6 then diluted to 100 µg /1ml, 50µg /1ml, 25µg /1ml, 10µg /1ml,5µg /1ml to 1µg /1ml at last.

Calibration linear curve

2ml of BCA reagent (A+B) added to 0.1ml from each the previous albumin dilution then the mixture was incubated for 30 min at 37 °C.

Method of detection and calculation:

The sample mixture, blank reagent, blank sample and Standard Bovine serum albumin were measured at 562 nm using a DU-650 Beckman spectrophotometer (Beckman Instr., Fullerton, Ca, USA). The measurement was carried out using the measurement of the changed ring in samples with a spectrophotometer assay then compared the reading values with the standard curve for the Bovine serum albumin in which known concentrations of protein were reacted with reagent. Soluble protein computed through using the following formula:

$(A_o - A_s - A) / 0.01\ P$ (or S), Where

A_o = absorbance of the blank reagent, A_s = absorbance of the blank sample,
A = absorbance of the sample, P = amount (g) of protein and S = amount (g) of seed sample.

3. Trypsin inhibitor activity (TIA) determination.

Trypsin inhibitor activity (TIA) was measured as trypsin inhibitor units (TIU) per gram of dry seed weight, and evaluated by using a modification of the method described by Filippetti *et al.*, (1999).

The trypsin inhibitor activity sample assay, blank reagent, blank, trypsin enzyme and trypsin inhibitor as control were measured at wave length 253 nm using a DU-650 Beckman spectrophotometer. One unite of enzyme activity is defined as the amount which releases under assay conditions, one unit of inhibitory activity is the amount of inhibitor that suppresses one unit of proteolytic activity.

4. SDS-Gel Electrophoresis of protein (SDS-PAGE):

The extracted protein samples were identified by SDS-PAGE according to the method of Jatinder et al. (1983) with minor modifications in order to detect the activity of protease inhibitors found in SDS-PAGE containing a copolymerized enzyme substrate.

Reagents:

1. Slab gel buffer: 1.5 M Tris - HCL at pH 8.8.
2. Stacking gel buffer: 0.5 M Tris - HCL at pH 6.8.
3. Running buffer: 0.025M Tris, 0.192 M glycine, 0.1% SDS (pH 8.5).
4. Washing solution: 2.5% Triton X–100.
5. Assay buffer: 0.1M glycine (pH 8.3).
6. Sample buffer: 0.3125 M Tris-HCL, 2.5% SDS, 50% glycerol, 0.02% Bromophenol blue (pH 6.8).
7. Resolving sample buffer: 80 % sample buffer + 20% β–mercaptoethanol [fresh]
8. Sample preparation: One part of protein sample : three parts from 80 % of sample buffer + 20% β–mercaptoethanol (fresh).
9. Protein marker (Sigma, MW 250-4 kDa):

Protein marker with its molecular weight for each band and its sign color was used as standard reference proteins with the following mixture: 1 % SDS, 1% β–mercaptoethanol and 0.06 M urea in a boiling water bath for 5 min prior to application to the gel according Weber and Osborn (1969).

10. Ammonium persulfate (APS 10%,Bio-Rad, Hercules, CA, USA, w/v).
11. Sodium Dodecyl Sulfate solution (SDS 10%, w/v).
12. Staining solution:

Comassie R-250 brilliant blue (0.1 %) dissolved in 10 % glacial acetic acid, 30% methanol and completed to 100 % with dist. Water and mixed well then kept at room temperature in a dark bottle.

13. Destaining solution:

10 % glacial acetic acid and 30% methanol and completed to 100 % with distilled Water and mixed well.

5. Detection of trypsin inhibitors by using SDS- PAGE :

SDS-polyacrylamide slab gels containing copolymerized substrate gelatin (0.1%) or casin (0.08%) were prepared as described by Heussen and Dowedle (1980), with minor modifications. Slab gels measuring 8.2 x 8.2 cm (thiekness 3 mm) were cast. The gel contains two gel layers (Slab gel – Stacking gel).

(1). Slab Gel (the lower layer) consisted of 2.2 ml dist. Water, 0.1 % of Gelatin, 1.5 ml of Tris – HCL and 2.2 ml of Acrylamide. The pH was adjusted to 8.8. The mixture was filtrated and mixed with 60 µl SDS (10 %), 10 µl of TEMED and 30 µl of APS (10 %). After addition of isopropanol to this mixture, polymerization started within 10 to 20 minutes. Finally isopropanol was discarded.

(2). Stacking gel layer (the upper layer) made up of 0.4 ml Acrylamide, 0.6 ml Tris (0.5M) and 0.9 ml dist. Water followed by filtration. The filtrate was mixed with 60 µl of SDS (10 %), 10 µl of TEMED and 30 µl of APS (10 %). Immediately comb was inserted.

Samples were applied by a micro syringe (25 µl). A sample volume (100µg) with three volumes of (80 % of sample buffer and 20 % of 20% β–mercaptoethanol) was applied to each well and air bubbles were avoided. Electrophoresis was carried out at 20 mA until the tracking dye was approximately 1 cm from the bottom of the gel.

Activity staining of trypsin inhibitors on SDS-PAGE gels :

The procedure of Hou and Lin (1998) was modified and applied as the follow: When the SDS-PAGE was finished, gels were cut into two parts.

One part was removed and washed with gentle stirring in 500 ml of washing solution with three consecutive changes at 40 to 45 minutes for each to remove SDS. The gels were then washed for 45 minutes in distilled water and incubated in 450 ml of assay buffer containing [14.2 µg /ml] trypsin enzyme (1645U/mg) at 37 °C for 30 minutes to 5 h depending on the concentration of samples and then washed with distilled water and stained by immersion in coomassie staining solution for 45 minutes and then destained with destaining solution. Another part was stained directly with Coomassie staining solution. Then the gels were destained in destaining solution.

Fractionation of protein extraction:

The extracted 100 ml protein was mixed with 23.2g Ammonium sulfate-$(NH_4)_2SO_2$ [40% saturation =231.5 g/L $(NH_4)_2SO_2$] and stirred in ice path at 0 °C for 30 minutes to 40% saturation and centrifuged (10000xg/ minutes) at 0° C for 20 minutes. The supernatant was mixed with 6.0 g Ammonium sulfate [10% saturation = 59.9g/L] for 50% saturation and repeated centrifuged as previous, and the sediments each times were solved in (1ml) phosphate buffer, pH 7.6 to reuse it again in gel run. These steps were continued to reach 80% saturation.

RESULTS AND DISCUSSION

Table (1) showed the contents of total protein, soluble protein and trypsin inhibitor among the eight varieties and the resulted 28 hybrids were presented in Table (1) as an indicator for seed protein quality contents at both Moshtohor and Nobaria locations.

Results indicated the quantitative content values for all genotypes as percentage for total protein, soluble protein as mg/ml and trypsin inhibitor contents (TIA) as TIU/g DW.

Electrophoretic analysis for trypsin inhibitor activity:

To support the belief that trypsin inhibitor activator stopped the work of protease enzymes, a successfully applied method for studying this approach could be achieved by using [SBTI] soybean trypsin inhibitor as an example. Therefore, the electrophoresis in polyacrylamide gels with gelatine-embedded PAGE is useful for studying the qualitative dimension to quantitative of the biological function. For detection the effect of the trypsin enzyme on proteolysis protein bands, a different concentrations of SBTI as shown in Table (2), were applied to copolymerised SDS-PAGE pattern at 45V for 4h, after the gel conduction incubated with 100ml assay buffer including 14.2 µg /ml trypsin enzyme at 37 °C for 1 h to digest the copolymerised protein (gelatine) into matrix of the SDS - polyacrylamide gel, then after washing stained and destained with coomassie.

Table (1): Percentage of total protein, Soluble protein (mg/ml) and Trypsin inhibitor activity (TIU/gDW) contents at Moshtohor and Nubaria locations.

Genotypes	% of Total protein content		Soluble protein content (mg/ml)		Trypsin inhibitor activity (TIU/gDW)	
	Moshtohor	Nubaria	Moshtohor	Nubaria	Moshtohor	Nubaria
Rina Blanka (P_1)	27.8	28.0	13.0	13.4	3154	3140
Triple White (P_2)	30.2	31.1	14.3	14.4	0917	0903
Tinova (P_3)	31.1	31.5	12.1	12.1	**0818**	**0837**
Tina (P_4)	**34.3**	**34.5**	13.5	13.7	0895	0865
Giza 2 (P_5)	29.1	29.1	14.1	13.9	3659	3531
Giza 716 (P_6)	27.4	27.5	13.0	13.1	2565	2560
Giza 461 (P_7)	31.5	31.3	11.5	11.3	2630	2930
Giza 429 (P_8)	27.4	26.1	12.6	12.1	2808	2711
P_1 X P_2	29.8	30.3	**14.6**	13.9	1730	1580
P_1 X P_3	30.0	29.8	13.8	13.5	1679	1529
P_1 X P_4	32.3	32.0	13.9	13.4	2014	1869
P_1 X P_5	30.1	29.1	**14.7**	14.0	3105	2955
P_1 X P_6	27.6	28.0	13.6	13.3	2556	2406
P_1 X P_7	32.3	30.0	12.8	12.5	2224	2074
P_1 X P_8	28.0	27.9	13.6	13.1	2681	2531
P_2 X P_3	30.8	30.5	13.7	13.1	2350	2250
P_2 X P_4	31.8	29.4	14.0	13.6	1380	1771
P_2 X P_5	29.9	26.4	12.9	12.9	1878	2175
P_2 X P_6	26.6	29.1	13.0	13.4	2202	2315
P_2 X P_7	33.0	32.7	12.9	13.5	2430	2380
P_2 X P_8	33.2	33.3	13.1	13.9	2557	2974
P_3 X P_4	33.0	32.9	13.0	13.9	2354	2324
P_3 X P_5	29.9	29.3	13.1	13.1	2703	2658
P_3 X P_6	28.8	29.0	13.1	12.9	2470	2510
P_3 X P_7	31.7	31.6	12.8	14.0	2339	2539
P_3 X P_8	30.1	29.7	13.9	12.1	2593	2488
P_4 X P_5	30.5	30.7	12.9	12.4	2062	2011
P_4 X P_6	31.0	30.8	13.9	13.6	2320	2123
P_4 X P_7	32.5	32.4	12.5	12.4	2272	2160
P_4 X P_8	30.4	30.5	13.1	13.2	2315	2360
P_5 X P_6	29.1	29.3	13.9	13.8	4039	4135
P_5 X P_7	30.3	30.7	11.3	11.5	2955	3107
P_5 X P_8	28.6	29.3	12.9	12.9	3210	3262
P_6 X P_7	29.2	29.6	12.5	12.9	2537	2635
P_6 X P_8	28.2	28.1	12.6	12.4	2773	2597
P_7 X P_8	29.8	29.8	12.1	12.6	2552	2683

Figure (1) and Table (2) revealed that all protein (gelatine) at SDS copolymerised gel digested through incubated period with trypsin enzyme, and all

the PAGE-gel appeared clearly when the gel stained and destained except, the unproteolytic bands which located in their track zone as regard to their molecular weight. The differences of their visual intensity depend on their concentrations of trypsin inhibitor contents.

Therefore, in these track zones of the unproteolytic bands, the inhibitor produced a complex with protein which prevent enzyme digestion to work and seen as in a dark blue stain.

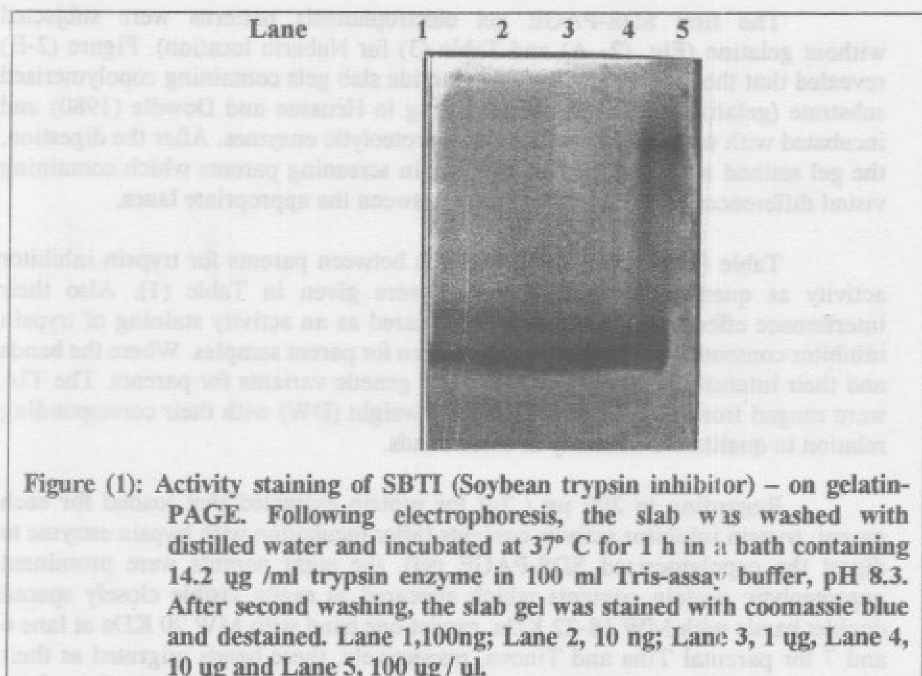


Figure (1): Activity staining of SBTI (Soybean trypsin inhibitor) – on gelatin-PAGE. Following electrophoresis, the slab was washed with distilled water and incubated at 37° C for 1 h in a bath containing 14.2 ųg /ml trypsin enzyme in 100 ml Tris-assay buffer, pH 8.3. After second washing, the slab gel was stained with coomassie blue and destained. Lane 1,100ng; Lane 2, 10 ng; Lane 3, 1 ųg, Lane 4, 10 ųg and Lane 5, 100 ųg / ųl.

Table (2): Concentration of SBTI applied to copolymerized SDS-gel.

Lane	1	2	3	4	5
Trypsin inhibitor concentration	100ng/ųl	10ng/ ųl	1ųg/ ųl	10ųg/ ųl	100ųg/ IIųl
Degrees of intensity	++	+	+++	+++-	+++++

(+) Degrees of band intensity for SDS-PAGE after enzyme incubation.

Electrophoretic analysis for protein contents:

For studying the inheritance for trypsin inhibitor content and their represented bands on electrophoresis gel, the genotypes were arranged according to their TIU/g DW contents, since the lowest genotype for TIU /g DW content was considered the best one and marked with grade number 1, the second had a higher TIU /g DW content than the first and take grade number 2, and so on as the highest entry was the last grade with number 36 (the worthiest entry).

1. Electrophoretic analysis of trypsin inhibitor for parents:

Comparison between parents through SDS-PAGE gel electrophoresis patterns at V.40 for 10 h for (200 µg/ 2µl) protein extraction of dry seed were achieved for each parent, beside trypsin inhibitor and protein marker as control. The migration of trypsin inhibitor as well as protein contents depends on their molecular weight. Narrow zones appeared as visible staining bands against coomassie brilliant blue background, Heussen and Dowdle (1980).

The first SDS-PAGE gel electrophoresis patterns were subjected without gelatine (Fig. (2- A) and Table (3) for Nubaria location). Figure (2-B) revealed that the second SDS-polyacrylamide slab gels containing copolymerised substrate (gelatine) were applied acorrding to Heussen and Dowdle (1980) and incubated with trypsin enzyme for 2 h to proteolytic enzymes. After the digestion, the gel stained and destained as previous in screening parents which containing visual differences of the inhibitor bands between the appropriate lanes.

Table (3) showed the differences between parents for trypsin inhibitor activity as quantitative content values were given in Table (1). Also their interference effect of trypsin enzyme appeared as an activity staining of trypsin inhibitor contents for SDS-PAGE gel pattern for parent samples. Where the bands and their intensity achieved the molecular genetic variants for parents. The TIA were ranged from 837 to 3531 TIU/g dry weight (DW) with their corresponding relation to qualitative intensity of these bands.

Regarding to 200 µg / 2µl for protein extracted that loaded for each parent, trypsin inhibitor activity contents (after incubation with trypsin enzyme to digest the copolymerized SDS-PAGE gel), the eight parents were prominent unproteolytic protein contents which appeared as easily visible closely spaced doublet bands with MW 18-22 KDa, except one band with MW 20 KDa at lane 6 and 7 for parental Tina and Tinova, respectively, these bands migrated as their molecular weight. Although, the visual bands varied in their intensity from faint to dark density or absent, as shown in Fig. (2-B) and Table (3) it was corresponded with TIU/g DW values and showed as quantitative contents.

As regard to Filippetti *et al.* (1999) classification for low trypsin inhibitor activity (TIA) < 900 TIU/ g DW and high content > 2000 TIU/ g DW and their putative for parental genotypes (Aabb) or (Bbaa) for low (TIA), while high parent should be (AA BB).

The results were in agreement with those obtained by Filippetti *et al.* (1999) regarding to the observation for quantitative values and qualitative intensity bands, due to the linkage between the inheritance of TIA quantitative contents and SDS-PAGE pattern as associated genetic markers.

2. Electrophoretic analysis of trypsin inhibitor activity for parents and crosses:

Protein inhibitor contents especially after incubation with trypsin enzyme convey to detect the genetic manner for inhibitor at molecular genetic level by screening the present bands after the digestion of coplymeryzed protein SDS gel.

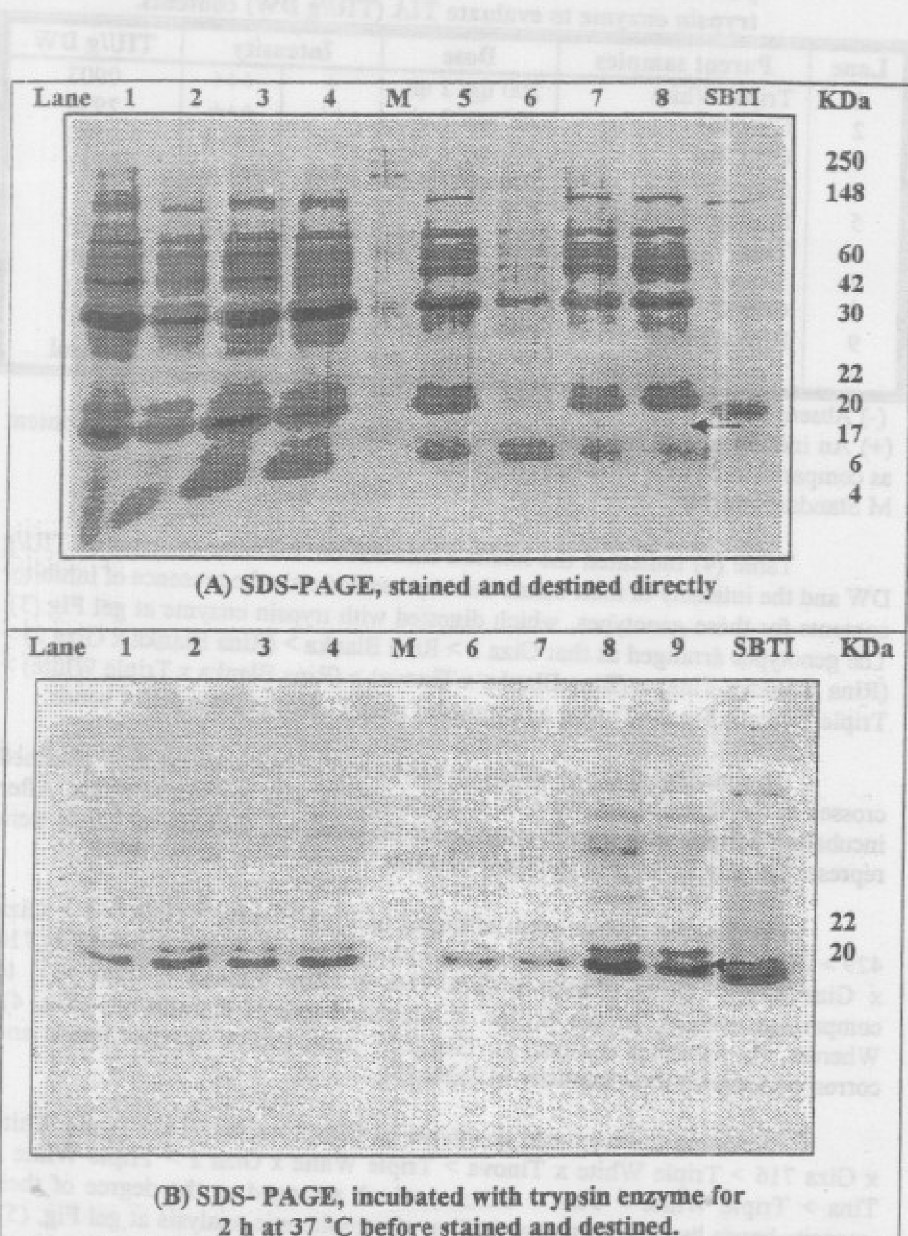


Figure (2): SDS-polyacrylamide gel electrophoresis pattern for (200 µg /2 µl) *protein extraction-dry* seed weight from eight varieties. Lane 1, 2, 3, 4, 6, 7, 8, 9 = Tripe White,Giza 461,Giza 716,Giza 429,Tina,Tinova,Giza 2, Rina Blanka, respectively, M = standard marker

Table (3): SDS-PAGE pattern of protein extracted samples for the eight parents cultivated at Nubaria location after incubation with trypsin enzyme to evaluate TIA (TIU/g DW) contents.

Lane	Parent samples	Dose	Intensity		TIU/g DW
1	Triple White	200 ųg/ 2 ųl	+	+++	0903
2	Giza 461	200 ųg/ 2 ųl	++	+++	2930
3	Giza 716	200 ųg/ 2 ųl	++	++++	2560
4	Giza 429	200 ųg/ 2 ųl	++	+++	2711
5	**Standard marker**	8 ųl			
6	Tina	200 ųg/ 2 ųl	-	++	0865
7	Tinova	200 ųg/ 2 ųl	-	++	0837
8	Giza 2	200 ųg/ 2 ųl	++++	++++	3531
9	Rina Blanka	200 ųg/ 2 ųl	+++	+++	3140
10	**Trypsin inhibitor**	15 ųg/ ųl	-	++++	control

(-) Absent band
(+) An indicator level for gradual increased intensity of trypsin inhibitor content as compared to trypsin inhibitor (control).
M Standard marker

Table (4) indicated the relation between the inhibitor contents as TIU/g DW and the intensity of their bands that represented only the presence of inhibitor contents for these genotypes, which digested with trypsin enzyme at gel Fig.(3). The genotypes arranged as that Giza 2 > Rina Blanka > (Rina Blanka x Giza 2) > (Rina Blanka x Tina) > (Rina Blanka x Tinova) > (Rina Blanka x Triple White) > Triple White > Tina > Tinova as compared with Ti for the intensity of bands.

In conclusion, the quantitative values of Ti of parents and their obtained crosses at the digested gel were in correspondance with the visual bands. After incubation periods with trypsin enzyme for the same gel, the existent bands were represented only for trypsin inhibitor contents.

Parents and crosses were arranged as Rina Blanka > Giza 461 > Giza 429 > (Rina Blanka x Giza 429) > (Giza 716 x Giza 461) > Giza 716 > (Giza 716 x Giza 429) > (Rina Blanka x Giza 716) > (Rina Blanka x Giza 461) to comparison with Ti for the degree of intensity bands at digested gel (Fig. 4). Whereas, the inhibitor contents as TIU/gDW came into as survival bands and corresponded with their intensity in Table (5).

Scanning the regulated genotypes as Giza 2 > Giza 716 > Triple White x Giza 716 > Triple White x Tinova > Triple White x Giza 2 > Triple White x Tina > Triple White > Tina > Tinova, which arranged as the degree of their intensity bands by comparison with Ti at electrophoretic analysis at gel Fig. (5). Table (6) revealed the correspondance with digestion gel. The absent bands demonstrates the lowest inhibitor content genotypes and the survival band for the highest inhibitor contents.

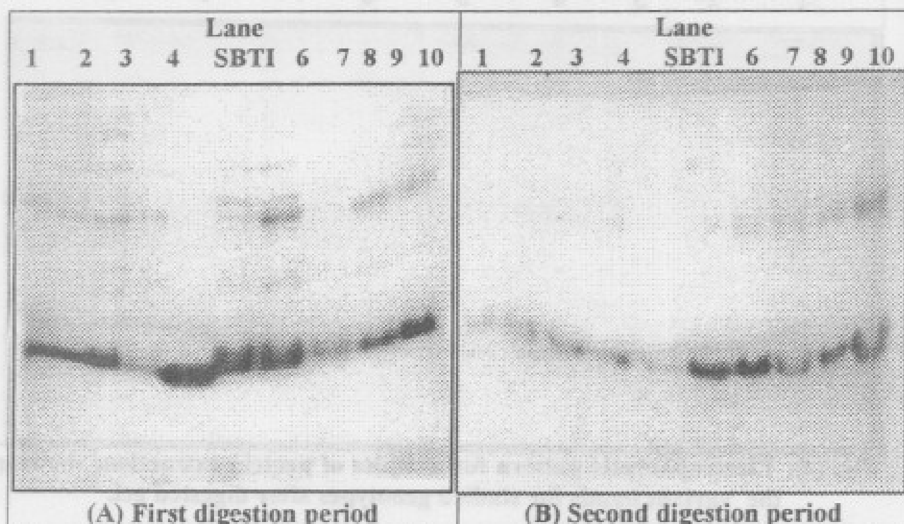


Fig . (3): Electrophoresis pattern for samples of protein extraction, showing the variant bands for studied genotypes after digested gel.

Table (4): Analysis of figure (3) for the studied genotypes with trypsin inhibitor contents in addition to the visual intensity of bands.

Lane	Genotype Samples	Dose	Degree	Intensity degree	TIU/g DW
1	Triple White	200 ųg/ ųl	3	3+	0903
2	P_1 X P_2	200 ųg/ ųl	4	4+	1529
3	P_1 X P_4	200 ųg/ ųl	7	6+	1869
4	Tinova	200 ųg/ ųl	1	1+	0837
5	**SBTI**	20 ųg/ųl			
6	Giza 2	200 ųg/ ųl	35	9+	3531
7	Rina Blanka	200 ųg/ ųl	33	8+	3140
8	Tina	200 ųg/ ųl	2	2+	0865
9	P_1 X P_3	200 ųg/ ųl	5	5+	1580
10	P_1 X P_5	200 ųg/ ųl	30	7+	2955

(+ Degree): Degree of band intensity for genotype samples on SDS gel, where (9+> 8+> 7+> .. >1+.

Degree: An indicator level for trypsin inhibitor content (TIU/g DW) for all the studied genotypes.

The analytical electrophoritc for Gel Fig. (6) exhibited the degree of intensity bands for arrangement parents and crosses Giza 2 > Triple White x Giza 429 > Giza 461 > Giza 429 > (Triple White x Giza 461) > (Tina x Giza 429) > (Tina x Giza 461) > (Tina x Giza 2) > Tina at the incubation SDS gel with trypsin enzyme, Table 7.

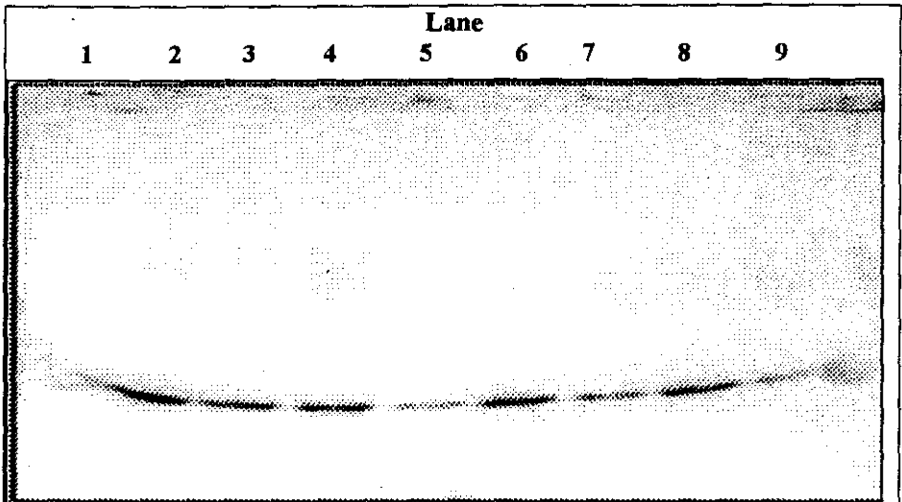


Fig. (4): Electrophoresis pattern for samples of protein extraction, showing the variant bands for studied genotypes after digested gel.

Table (5): Analysis of figure (4) for the studied genotypes with trypsin inhibitor contents in addition to the visual intensity of bands.

Lane	Genotype samples	Dose	Degree	Intensity degree	TIU/g DW
1	Rina	200 ug/ ul	33	9+	3140
2	Blanka	200 ug/ ul	29	8+	2930
3	Giza 461	200 ug/ ul	28	7+	2711
4	Giza 429	200 ug/ ul	21	3+	2531
5	P_1 X P_8	200 ug/ ul	25	6+	2635
6	P_6 X P_7	200 ug/ ul	23	4+	2560
7	Giza 716	200 ug/ ul	24	5+	2597
8	P_6 X P_8	200 ug/ ul	18	2+	2406
9	P_1 X P_6	200 ug/ ul	9	1+	2074
	P_1 X P_7				

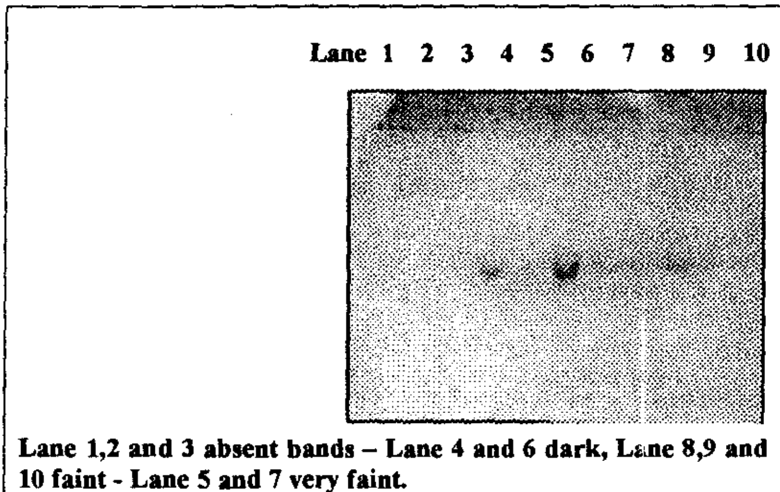
(+ Degree): Degree of band intensity for genotype samples on SDS gel, where (9+> 8+> 7+> .. >1+.

Degree: An indicator level for trypsin inhibitor content (TIU/g DW) for all the studied genotypes.

The genotypes Triple White and Tina were found to have not bands as their low contents of inhibitor, while Giza 2, Triple White x Giza 429 and Giza 461 had a sharp dark bands as their high contents of inhibitor.

Screening of the arranged genotypes Giza 2 > Giza 461 > Tinova x Giza 2 > Giza 716 > Tinova x Giza 461 > Tinova x Giza 716 > Tinova x Tina > Tina > Tinova as their intensity at proteolitc SDS Gel is given in Fig. (7). Comparison with inhibitor control after first incubation period and second incubation for the same gel showed

the visual present and absent bands. Hence at the second incubation period almost of bands gone to disappear or being very faint dependently on their own low contents of TIU/g DW unless the high contents genotypes exhibited survival bands with Giza 2 and Giza 461 as shown in Table (8). The degree of intensity bands were ranged from very dark to faint or absent at Gel Fig.(2) for the analytical digested electrophoretic pattern for the study genotypes Giza 2 x Giza 716 > Giza 2 > Giza 2 x Giza 429 > Giza 2 x Giza 461 > Giza 461 > Giza 429 > Giza 461 x Giza 429 > Giza 716 > Tinova x Giza 429 > Tinova.These intensity bands were corresponding with the recorded data at Table (9).



Lane 1,2 and 3 absent bands – Lane 4 and 6 dark, Lane 8,9 and 10 faint - Lane 5 and 7 very faint.

Fig. (5): Electrophoresis pattern for samples of protein extraction, showing the variant bands for studied genotypes after digested gel.

Table (6): Analysis of figure (5) for the studied genotypes with trypsin inhibitor contents in addition to the visual intensity of bands.

Lane	Genotype Samples	Dose	Degree	Intensity degree	TIU/g DW
1	Tinova	200 µg/ 2 µl	1	1+	0837
2	Tina	200 µg/ 2 µl	2	2+	0865
3	Triple White	200 µg/ 2 µl	3	3+	0903
4	SBTI	20 µg/ µl			
5	P_2 X P_4	200 µg/ 2 µl	6	4+	1771
6	Giza 2	200 µg/ 2 µl	35	9+	3531
7	P_2 X P_5	200 µg/ 2 µl	12	5+	2175
8	P_2 X P_3	200 µg/ 2 µl	13	6+	2250
9	Giza 716	200 µg/ 2 µl	23	8+	2560
10	P_2 X P_6	200 µg/ 2 µl	14	7+	2315

(+ Degree): Degree of band intensity for genotype samples on SDS gel, where (9+> 8+> 7+> .. >1+.

Degree: An indicator level for trypsin inhibitor content (TIU/g DW) for all the studied genotypes.

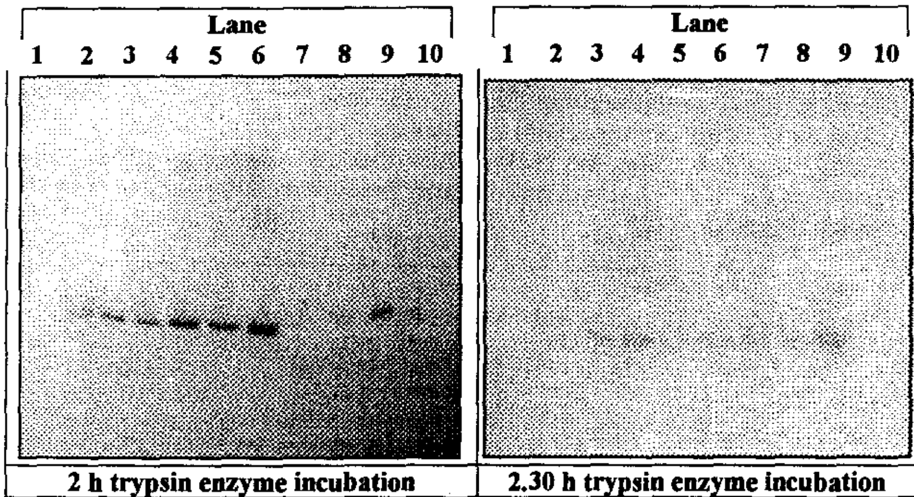


Fig. (6): Electrophoresis pattern for samples of protein extraction, showing the variant bands for studied genotypes after digested gel.

Table (7): Analysis of figure (6) for the studied genotypes with trypsin inhibitor contents in addition to the visual intensity of bands.

Lane	Genotype samples	Dose	Degree	Intensity degree	TIU/g DW
1	P_4 X P_7	200 µg/ µl	11	4+	2160
2	P_4 X P_8	200 µg/ µl	16	5+	2360
3	Giza 429	200 µg/ µl	28	7+	2711
4	P_2 X P_8	200 µg/ µl	31	9+	2974
5	Giza 461	200 µg/ µl	29	8+	2930
6	Giza 2	200 µg/ µl	35	10+	3531
7	P_4 X P_5	200 µg/ µl	8	3+	2011
8	Triple White	200 µg/ µl	3	2+	0903
9	P_2 X P_7	200 µg/ µl	17	6+	2380
10	Tina	200 µg/ µl	2	1+	0865

(+ Degree): Degree of band intensity for genotype samples on SDS gel, where (9+> 8+> 7+> .. >1+.

Degree: An indicator level for trypsin inhibitor content (TIU/g DW) for all the studied genotypes.

As general from the obtaining tables and figures for gels and screening these gels after incubated periods with trypsin enzyme, it is clear that the entry had No. 36 grade exhibited a darkest band over all the genotypes due to its highest content value 4135 TIU/g DW and the lowest content entry had 837 TIU/g DW due to controlling gene which appeared as faintest band for over all genotypes. These results agreed with those obtained by Filippetti *et al.* (1999), in case of parents having similar phenotypes (i.e. low) and the other case low (TIA) x high (TIA) the progeny crosses should had either (A-BB) or (AAB-) genotypes.

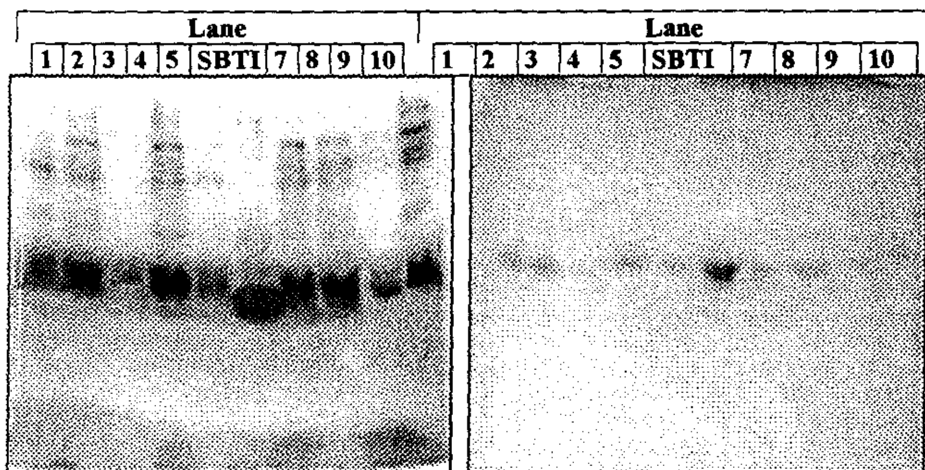


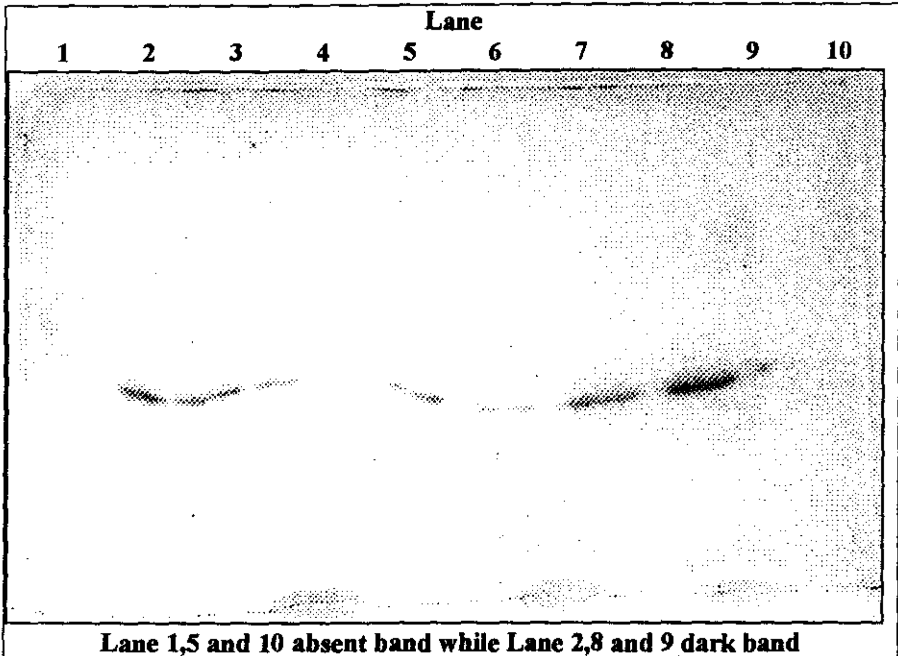
Fig. (7): Electrophoresis pattern for samples of protein extraction, showing the variant bands for studied genotypes after digested gel.

Table (8): Analysis of figure (7) for the studied genotypes with trypsin inhibitor contents in addition to the visual intensity of bands.

Lane	Genotype samples	Dose	Degree	Intensity degree	TIU/g DW
1	P_3 X P_6	200 ųg/ 2 ųl	20	4+	2510
2	Giza 716	200 ųg/ 2 ųl	23	6+	2560
3	Tinova	200 ųg/ 2 ųl	1	1+	0837
4	Giza 2	200 ųg/ 2 ųl	35	9+	3531
5	P_3 X P_4	200 ųg/ 2 ųl	15	3+	2324
6	SBTI	20 ųg/ ųl			
7	P_3 X P_7	200 ųg/ 2 ųl	22	5+	2539
8	P_3 X P_5	200 ųg/ 2 ųl	26	7+	2658
9	Tina	200 ųg/ 2 ųl	2	2+	0865
10	Giza 461	200 ųg/ 2 ųl	29	8+	2930

(+ Degree): Degree of band intensity for genotype samples on SDS gel, where (9+> 8+> 7+> .. >1+.

Degree: An indicator level for trypsin inhibitor content (TIU/g DW) for all the studied genotypes.



Lane 1,5 and 10 absent band while Lane 2,8 and 9 dark band

Fig . (8): Electrophoresis pattern for samples of protein extraction, showing the variant bands for studied genotypes after digested gel.

Table (9): Analysis of figure (8) for the studied genotypes with trypsin inhibitor contents in addition to the visual intensity of bands.

Lane	Genotype samples	Dose	Degree	Intensity degree	TIU/g DW
1	Tinova	200 ųg/ ųl	1	-	0837
2	P_5 X P_8	200 ųg/ ųl	34	8+	3262
3	P_5 X P_7	200 ųg/ ųl	32	7+	3107
4	Giza 429	200 ųg/ ųl	28	5+	2711
5	P_3 X P_8	200 ųg/ ųl	19	2+	2488
6	Giza 461	200 ųg/ ųl	29	6+	2930
7	P_7 X P_8	200 ųg/ ųl	27	4+	2683
8	Giza 2	200 ųg/ ųl	35	9+	3531
9	P_5 X P_6	200 ųg/ ųl	36	10+	4135
10	Giza 716	200 ųg/ ųl	23	3+	2560

(+ Degree): Degree of band intensity for genotype samples on SDS gel, where (9+> 8+> 7+> .. >1+.

Degree: An indicator level for trypsin inhibitor content (TIU/g DW) for all the studied genotypes.

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دراسات كيماوية حيوية وراثية على نشاط مثبط التربسين فى تهجين دائرى لبعض أصناف الفول البلدى (*Vicia faba*)

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أجريت هذه الدراسة فى معمل فسيولوجى النبات بجامعة بايرويت بألمانيا على ثمانية آباء و عشائر الجيل الثانى الناتجة منها (تهجين دائرى) و ذلك لتقييم نشاط مثبط إنزيم الهضم التربسين (TIA). أظهرت النتائج أن محتوى البروتين الكلى كنسبة مئوية و محتوى المثبط لإنزيم الهضم التربسين متباينا تباينا عالى المعنوية فى الآباء و الهجن الناتجة منها فى موقعى الزراعة ؛ مشتهر و النوبارية. كان الصنف تينوفا أقل الأصناف فى محتوى المثبط لإنزيم الهضم التربسين بينما كان الصنف جيزة ٢ هو الأعلى فى محتوى المثبط الأنزيمى فى كل من موقعى مشتهر و النوبارية. كانت الهجن رينا بلانكا × تينوفا، رينا بلانكا × جيزة ٤٢٩ و تربل وايت × جيزة ٢ (الأقل فى محتوى المثبط لإنزيم الهضم التربسين و الأعلى فى محتوى البروتين) بينما كانت الهجن رينا بلانكا × تينا ، رينا بلانكا × تربل وايت و رينا بلانكا × تينوفا هى الأفضل بالنسبة لكل الصفات تحت الدراسة متضمنة محتوى المثبط لإنزيم الهضم التربسين و محتوى البروتين. تم تحديد موقع حزم البروتين لمثبط إنزيم الهضم التربسين عند ٢٠-٢٢ كيلو دالتون بالنسبة للآباء فى موقع النوبارية و أظهر التفريد الكهربى لعينات البروتين المذاب تباينا فى كثافة حزم مثبط إنزيم الهضم التربسين لكل من الآباء و الهجن الناتجة منها فى موقع النوبارية.