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In vitro Selection of Soybean Callus Resistant to *Fusarium oxysporum* Metabolites

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Abstract: Soybean (*Glycine max L.*) callus line tolerant to 9% *Fusarium oxysporum* metabolite was selected. Initiation and maintenance of callus as well as selection process was carried out on Murashige and Skoog[1] (MS) supplemented with 5.0 mg/l Naphthalene-acetic acid (NAA) and 0.5 mg/l 6-Benzylamino purine (6-BAP). The selected line was obtained by treating the callus to increase concentration of *F. oxysporum* metabolite (3%-15%). The selected line grew better than the non-selected one at all used concentrations of metabolite. It retained its resistance after subculture for 3 months on metabolite-free medium. Fresh and dry weights increased more in the selected line than non-selected one. Free amino acids showed a significant increase in selected callus line over that of non-selected callus lines at all concentrations of *F. oxysporum* metabolite. Also, there are high accumulations of proline in all selected callus lines than non-selected callus ones, especially at concentration 9% of *F. oxysporum* metabolite at which the selection procedure occurs. Protein profile of both selected and non-selected soybean callus lines show the appearance of polypeptide bands in the selected other than non-selected ones. No quantitative differences have been obtained between both callus lines in the presence or absence of metabolites. DNA banding pattern indicated that the B-7 primers contain a high number of DNA fragment than the other primers used. Also, treating the selected soybean callus line to the 9 % metabolites which induced by *F. oxysporum* fungus was accompanied by the induction of a new unique DNA fragments of an approximate sizes of 400 bP. The better performance of selected line under metabolites stress has en attributed here its greater disease resistance adjustment in relation to non-selected line.

Key words: *In vitro* selection; disease resistance; *Fusarium oxysporum*; *Glycine max L.*

INTRODUCTION

Soybean (*Glycine max L.*) is one of the world's most important sources of oil and protein. It has the highest protein content among leguminous crops[2].

Soybean plants are subjected to attack by several fungal, bacterial and viral diseases that cause great losses in the yield. Wilt disease of soybean plant caused by *F. oxysporum* is one of the most destructive serious diseases of the crop and is a very common soilborne fungus[3].

Plant tissue culture is fundamental to our understanding of the control of plant function and the modification to meet the needs of modern agriculture, horticulture and forestry. Through tissue cultures, it is now possible to examine complex development proceses such as embryogenesis and host-pathogen interactions and to enhance charac-teristics such as salt or disease resistance, production of secondary metabolites, somatic hybridization, genetic engineering and germplasm conservation[4]. *In vitro* selection for disease resistance is advantageous for several reasons, including: (1) Cultured cells can be uniformly exposed to the selective agent, thus reducing the incidence of escape., (2) Culture system maintained in small spaces can potentially replace expensive greenhouse or field testing facilities., (3) The disease causing agent remains confined to the laboratory., (4) Generation of disease resistant plants[5].

Generally, free amino acids and protein metabolism play, an important role in determining whether a host will be resistant or susceptible to fungal infection. Shalaby[6] reported that, the free amino acids content was increased in tomato plants infected with *F. oxysporum p.v. lycopersici* than that of healthy ones. Wang *et al.*[7] reported that, cucumber when treated with pathogen–toxin culture filtrates *F. oxysporum F. SP. Cucumerinum*, the contents of soluble protein after treatment increase and the extent of increase in the resistant cultivar was larger than that in the susceptible one. Also, the contents of soluble proteins in the leaf cells were greater than that in the root cell.

Nowadays DNA fingerprint considered to be a powerful tool for detecting any new mutation may be

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caused when plant was subjected to stress. The modified polymerase chain reaction(PCR) with single primers of arbitrary nucleotide sequence and requiring no prior sequence information have proved useful in detecting intraspecific polymorphism among organisms[8,9]. This amplification technique {arbitrarily primed PCR or random amplified polymorphic DNA (RAPD)} can generate specific DNA fragments useful for genome mapping, identification of isolates, and applications in molecular ecology[10]. Koretsky[11] detected that, rootlets of soybean when infected with. *F. oxysporum* induced the expression of 1, 3-β-glucanase (PR-protein) gene. The fact that, the more resistant variety of soybean has higher 1,3-β-glucanase expression than the less resistant ones, indicate that the gene encoding these enzymes play an important role in developing the resistance against *F. oxysporum*.

Pekkarinen *et al*.[12] found that when *F. culmorum* was grown in a gluten–containing medium, form which an alkaline serine proteinase was purified, this enzyme used by the fungi to hydrdyze grain proteins and this enzyme was not inhibited by soybean trypsin or Bowman-Birk inhibitors. The antifungal protein from *Urgina indica* bulbs localized to the cell wall of the bulb, by antifungal tests, exerts fungistatic effects. It completely inhibits the germination of spores and hyphal growth of *F. oxysporum*. Deepak *et al*.[13]. Analysis of the xylem fluids of cotton *Gossypium hirsutum* infected with *F. oxysporum F. SP. Vasinfectum* and then invasion with Root-Knot nematode *Meloidogyne incognita*, showed a decrease in total protein and total carbohydrates levels and an increase in peroxidase enzyme concentration[14].

MATERIALS AND METHODS

Plant Materials: Soybean (*Glycine max L.*) seeds were surface sterilized by immersing in 70% ethanol for 30 sec., followed by soaking in NaOCl 10% for 15 min. Seeds then rinsed several times in sterilized distilled water. All operations were carried out aseptically in a laminar air flow cabinet. The seeds were aseptically transferred to glass jars conta-ining 25 ml of a solidified sterile basal Murashige and Skoog (MS) medium[1] containing 3% sucrose and 0.8% agar. The medium was adjusted to PH 5.8 before autoclaving at 121°C and 1.2 kg/cm^2. Cultures were then incubated at 27°C in darkness.

Callus Initiation and Maintenance: One week old aseptically grown seedlings were used as a source of explants. Leaf explants (5 mm long) of Soybean (*Glycine max L.*) were plated onto solidified MS medium supplemented with 5.0 mg/l NAA and 0.5 mg/l 6-BAP. Within 10 – 14 days, callus was initiated at wounded regions and extended to include most of the explants. The initiated callus was sectioned and subcultured on the same fresh medium to produce large quantities of callus tissues.

Preparation of *Fusarium Oxysporum* Metabolite: Dox liquid medium was used throughout this investigation 7 days after inocu-lation at 30 ± 1°C, biomass growth of tested *F. oxysporum* was harvested and the metabolite was filter sterilized through Seitz filter membrane (0.2 μm).

Selection Procedure: The selected soybean callus line was obtained by subculturing every 3 weeks, on sterile fresh solidifying MS medium supplemented with 5.0 mg/l NAA and 0.5 mg/l 6-BAP, with gradually increasing the concentrations of the filter sterilized *F. oxysporum* metabolite (3, 6, 9, 12 and 15 %). Cultures were then incubated at 27°C under 8 hr photoperiod of light intensity 1700 lux at the shelf surfaces.

The growth of calli were determined by weighting fresh weights of calli after three weeks of subjecting cultures to different *Fusarium* metabolite levels, then calcu-lating the rate of increase or decrease in callus fresh as compared with control (callus on metabolite free medium).

Control soybean calli were grown on metabolite free sterile MS medium. Callus was examined visually and all soft and necrotic tissues were discarded at each subcultured. The survival calli growing on 9% metabolite of *F. oxysporum* was subcul-tured on the same medium and selection procedure was then continued on MS medium supplemented with 9% metabolite since higher metabolite concentrations were found to kill almost all the cells. This type of callus a quired the name selected cell lines [S]. The whole selection process took five passages about 4 months. After three weeks, ten replicates were used for each levels of *F. oxysporum* metabolite for the measurement and determination of the physiological process of **NS** & **S** callus lines.

A known fresh weight of both S and NS callus lines were treated with five levels of *F. oxysporum* metabolites (3, 6, 9, 12 and 15%).

Two weeks later ten replicates of each level were used for determination of fresh and dry weights, free amino acids, protein, and DNA determination.

Estimation of Free Amino Acids: Free amino acids were extracted according to the proposed method of Shad *et al*.[15]. The contents (mg %) of selected and non-selected soybean callus lines were determined using a HPLC system (HP 1050) with a UV detector at 254nm loca-ted at National center of Agricultural Research. Giza, Egypt.

Electrophoretic Detection of Protein: The method of Laemmili[16] with slight modifications was adopted to use in the present study. The modification, was reducing TEMED from 30 µl to 25 µl and also APS was reduced from 1.5 ml to 1.3 ml. Molecular weight and amount of protein (protein %) of selected and non-selected soybean callus lines were calculated.

Estimation of DNA by PCR:

DNA Extraction: The method of Doyle and Doyle[17] was followed to extract and determine DNA.

PCR Amplification: Amplification reaction volumes were, 25µl, each conta-ining 1× PCR buffer with $MgCl_2$ (50 mM KCl; 10 mM Tris-HCl (pH 9.0); 2 mM $MgCl_2$ and 1% trition x-100), 200 µM each of dATP, dCTP, dGTP and dTTP, 50 PM primer, 50 ng template DNA and 1.5 unit of tag polymerase. Reaction mixtures were overlaid with 15 µl mineral oil and exposed to the following conditions: 94°C for 3 min; followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, and a final 7-min. extension at 72°C. Amplification products were visualized with DNA marker on 1.67 agarose gel with lx TBE buffer and detected by staining with an ethidium bromide solution for 30 min. Gel were then destained in deionized water for 10 min and photographed on Polaroid films under U.V light. Both protein and PCR analysis were carried out in National center of Agricultural Research. Giza, Egypt.

Statistics: Data were statistically tested using student's t-test for comparison between means of treatments.

RESULTS AND DISCUSSION

Fresh and Dry Weight: The growth criteria including fresh and dry weight of selected (S) and non-selected (NS) soybean (*Glycine max L.*) callus lines grown in MS medium supplem-ented with 5.0 mg/l NAA and 0.5 mg/l 6-BAP, with different concentrations of (0, 3, 6, 9, 12 and 15 %) *F. oxysporum* metabolite is shown in tables (1 and 2). The fresh and dry weights of (S) soybean callus line exhibited a marked increase in the growth rate (expressed as the rate of increase in the callus fresh and dry weights) on *F. oxysporum* metabolite than did (NS). Increasing the concentration of the metabolite up to 9% increased the fresh and dry weights of selected soybean callus line, and then it decreased slightly on 12% and sharply at higher concentration of *F. oxysporum* metabolite: 15%. On the other hand, a decrease in fresh and dry weights of

Table 1: Effect of different concentrations of *F. oxysporum* metabolites on fresh weight (g) of selected and non-selected soybean callus lines.

Soybean callus Lines	Fresh weight (g)		
F. metabolite conc. (%)			Selected
Non- Selected			
0.00	7.267±0.291	NS	7.200±0.153
3.00	12.183±0.169	S	10.150±0.025
6.00	13.500±0.173	S	11.050±0.029
9.00	15.133±0.219	HS	8.950±0.132
12.00	10.380±0.021	HS	5.100±0.115
15.00	6.800±0.208	S	3.933±0.176

*High significance < 0.050.
HS High significance.
S Significance.
NS Non significance.
-Each listed value is a mean of five replicates ± SE.

Table 2: Effect of different concentrations of *F. oxysporum* metabolites on dry weight (g) of selected and non-selected soybean callus lines.

Soybean callus Lines	Dry weight (g)		
F. metabolite conc. (%)			Selected
Non- Selected			
0.00	0.390±0.006	S	0.402±0.009
3.00	0.724±0.021	S	0.566±0.002
6.00	0.752±0.010	S	0.616±0.002
9.00	0.844±0.012	HS	0.499±0.007
12.00	0.578±0.002	HS	0.284±0.006
15.00	0.379±0.012	S	0.219±0.010

*High significance < 0.050.
HS High significance.
S Significance.
NS Non significance.
-Each listed value is a mean of five replicates ± SE.

(NS) soybean callus line was noticed on media containing *F. oxysporum* metabolite higher than 6%. Also, NS callus line could not survive on a higher concentration of *F. oxysporum* metabolite: 9%, 12% and 15%. Similar observation has been reported by Hadj Seyed Hadi *et al.* [18], Naseem *et al.*[19] they reported that, when tomato seed were sown in the nursery and when seedlings reached to 15 cm height, and before planting, seedling roots were inoculated by *F. solani.* The stem length became highest *F. solani* treatment and stem fresh weight increase.

Free Amino Acids: Number and concentration of free amino acids (mg%) in selected and non-selected soybean callus lines grown in MS medium supplemented with 5.0 mg/l NAA and 0.5 mg/l 6-BAP, and treated with different concentrations (0, 3, 6, 9, 12 and 15 %) of *F. oxysporum* metabolite are presented in table (3).

According to the results obtained in table (3), on a media supplemented with different concentrations (0, 3, 6, 9, 12 and 15 %) of *F. oxysporum* metabolite, the total number of free amino acids exhibited a significant

Table 3: Effect of different concentrations of *F. oxysporum* metabolite on free amino acids contents (mg%) of selected and non-selected soybean callus lines.

Fusarium metabolites conc. (%)	0.0		3		6		9		12		15	
Free amino acids contents(mg%)	Non-select	select	Non-select	select	Non-select	select	Non-select	select	Non-select	select	Non-select	select
1-Aspartic	0.00	0.00	0.00	0.00	0.009	0.000	0.00	0.00	0.00	0.00	0.00	0.00
2-Serine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3-Glutamic	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4-Glycine	61.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	57.28	0.00
5-Threonine	0.00	44.41	0.00	47.4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6-Arginine	28.31	55.57	0.00	0.00	0.00	0.00	0.00	36.04	21.14	0.00	9.93	42.24
7-Proline	0.00	0.00	59.21	30.1	78.14	65.85	86.04	23.69	48.93	32.4	2.15	1.5

8-Methionine	8.42	0.00	16.85	0.00	7.33	4.23	0.00	4.55	11.4	6.3	6.05	0.00
9-Valine	0.15	0.00	9.54	0.00	3.4	9.85	5.07	7.11	0.00	7.21	3.02	0.00
10-Tryptophane	0.82	0.00	3.09	13.32	0.00	4.44	3.59	0.00	3.97	4.54	0.00	0.00
11-Leucine	0.91	0.00	3	0.00	1.08	0.00	0.00	0.00	5.41	0.00	0.00	0.00
12-Cysteine	0.00	0.00	7.2	6.15	2.13	0.00	1.18	0.00	9.12	0.51	8.84	0.00
13-Cystine	0.00	0.00	0.82	1.13	0.00	1.47	2.15	0.00	0.03	0.92	0.00	0.00
14-Phenyl alanine	0.00	0.00	0.22	0.85	0.00	0.00	0.00	0.00	0.00	0.00	8.52	12.19
15-Alanine	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.66
16-Lysine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.82
17-Histidine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.54	0.41
18-Hydroxy proline	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
19-Tyrosine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total number of free amino acids	6	2	8	7	6	5	5	4	7	6	8	6

increase in both selected and non-selected soybean callus lines as compared with those growing on a media devoid of *Fusarium* metabolite. Also, at all concentrations of *F. oxysporum* metabolite the total number of free amino acids exhibited a significant increase in selected than non-selected soybean callus lines.

Exclusively, amino acid proline, exhibited a highly significant increase in selected (S) soybean callus lines than non-selected soybean callus lines at all concentrations of *F. oxysporum* metabolite especially, at concentration 9% where the selection procedure occurs. Similar observation has been reported by Hanna [20] observed that, the free amino acids contents were increased in both cultivars of tomato plants infected with *F. oxysporum p.v. lycopersici* than healthy ones. Also, similar results observed by Goodman *et al.* [21], Hegazy [22], Gorlach *et al.* [23], and recently Daif[24], in alfalfa a relationship between proline levels and adaptability to stress has been observed[25].

Protein Analysis: Table (4) and fig. (1) showed the protein banding patterns of non-selected soybean callus lines (lanes 2 to 7) and selected soybean callus lines (lanes 8 to 13) treated with 0, 3, 6, 9, 12 & 15 % of *F. oxysporum* metabolites respectively. Analysis of this pattern using gel pro-analyzer software indicated that protein profile of both selected and non-selected soybean callus lines showed the presence of at least 18 bands of special significance having approximate

Table 4: SDS Polyacrylamide gel electrophoresis of non-selected (lanes 2 to 7) and selected (8 to 13) callus lines treated with 0.0 , 3.0 , 6.0 , 9.0 , 12.0 & 15.0 % of *F.* metabolites respectively, lane (1) represents the molecular weight markers:-

M.WT of protein	Marker		Non - selected								Selected			
bands (KD)	Lane 1 M.wt.	Am. %	Lane 2 Am. %	Lane 3 Am. %	Lane 4 Am. %	Lane 5 Am. %	Lane 6 Am. %	Lane 7 Am. %	Lane 8 Am. %	Lane 9 Am. %	Lane 10 Am. %	Lane 11 Am. %	Lane 12 Am. %	Lane 13 Am. %
68	-	-	-	1.28	1.45	1.61	-	-	-	-	1.76	2.42	-	-
66	66	4.12	1.45	-	-	-	-	-	-	1.01	-	-	-	-
65	-	-	-	-	1.37	-	-	-	-	-	1.63	-	2.30	-
64	-	-	-	-	-	-	-	-	-	0.76	-	1.01	-	-
60	-	-	-	-	-	-	-	-	-	-	-	-	1.81	-
61	-	-	-	-	-	-	-	-	-	-	-	-	-	4.02
59	-	-	1.97	-	12.80	-	-	-	4.14	-	-	-	-	-
58	-	-	-	-	-	2.02	-	-	-	-	-	-	-	-
57	-	-	3.20	6.35	-	-	8.87	-	3.78	7.93	-	-	10.50	-
56	-	-	-	-	13.80	-	1.24	-	-	-	10.73	-	-	-
55	-	-	-	-	-	2.69	-	-	-	2.66	-	-	9.89	-
54	-	-	-	-	4.83	-	0.89	-	-	-	-	8.45	-	-
51	-	-	-	-	-	-	-	-	-	-	-	-	-	15.64
50	-	-	-	-	-	-	2.83	28.57	-	-	-	-	-	-
48	-	-	11.15	8.16	-	-	-	-	-	15.66	-	-	-	-
47	-	-	-	-	-	3.45	-	-	3.19	-	5.23	-	-	-
46	-	-	-	-	-	-	-	-	-	1.96	-	3.98	-	-
45	45	13.93	-	-	-	-	-	-	-	-	1.88	-	-	-
43	-	-	-	-	-	-	7.65	-	1.33	-	5.60	10.40	9.10	-
40	-	-	-	-	-	4.84	-	-	-	-	-	1.33	-	6.38
37	-	-	-	-	-	-	5.01	-	-	-	-	-	-	11.55
35	-	-	-	6.02	7.50	20.38	8.31	7.86	-	18.63	20.65	24.28	14.23	8.64
31	-	-	-	-	2.36	6.76	1.75	-	-	-	5.56	9.88	-	-
29	29	9.98	-	-	1.76	-	-	-	3.69	-	-	12.14	-	-
28	-	-	-	3.95	4.22	8.58	1.10	1.05	-	3.77	5.41	10.83	4.01	3.70
27	-	-	-	-	-	1.78	-	-	-	4.94	-	-	-	8.65
26	-	-	-	-	-	-	-	-	5.91	-	-	4.63	6.12	1.44
25	-	-	-	-	-	3.57	-	-	-	6.34	8.79	-	2.33	

24	-	-	-	-	-	-	-	-	1.15	-	-	-	-	3.78
23	-	-	-	10.48	-	-	8.49	-	11.60	12.06	5.45	-	7.13	-
22	-	-	-	-	13.32	15.67	-	13.28	1.06	1.73	12.68	17.911.30	9.88	2.23
21	-	-	8.10	-	9.69	10.86	-	-	9.03	11.68	15.47	-	-	-
20	20	12.45	-	-	-	8.51	-	-	-	-	-	16.64	-	-
19	-	-	-	-	-	-	12.32	20.62	-	10.50	14.82	-	13.16	11.56
18	-	-	-	-	-	2.83	-	-	-	-	-	-	-	1.38
16	-	-	-	11.74	-	-	-	-	-	4.26	6.36	16.57	5.00	2.29
15	-	-	-	-	3.47	5.13	13.45	-	-	-	-	10.9	-	-
14	14	25.69	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	18.02	-	-	-	-	-	6.49	-	-	-	-	-
12	-	-	-	-	-	27.05	-	-	-	-	-	-	18.50	-
11	-	-	-	14.46	16.87	-	14.7	-	-	15.53	18.41	27.17	-	-
10	-	-	12.38	-	-	-	-	-	-	-	-	-	-	5.74
7	-	-	3.71	4.13	5.52	7.66	-	5.11	10.47	14.60	17.64	20.80	-	-
No. of bands	-	-	8	9	14	17	13	6	12	17	17	18	14	14

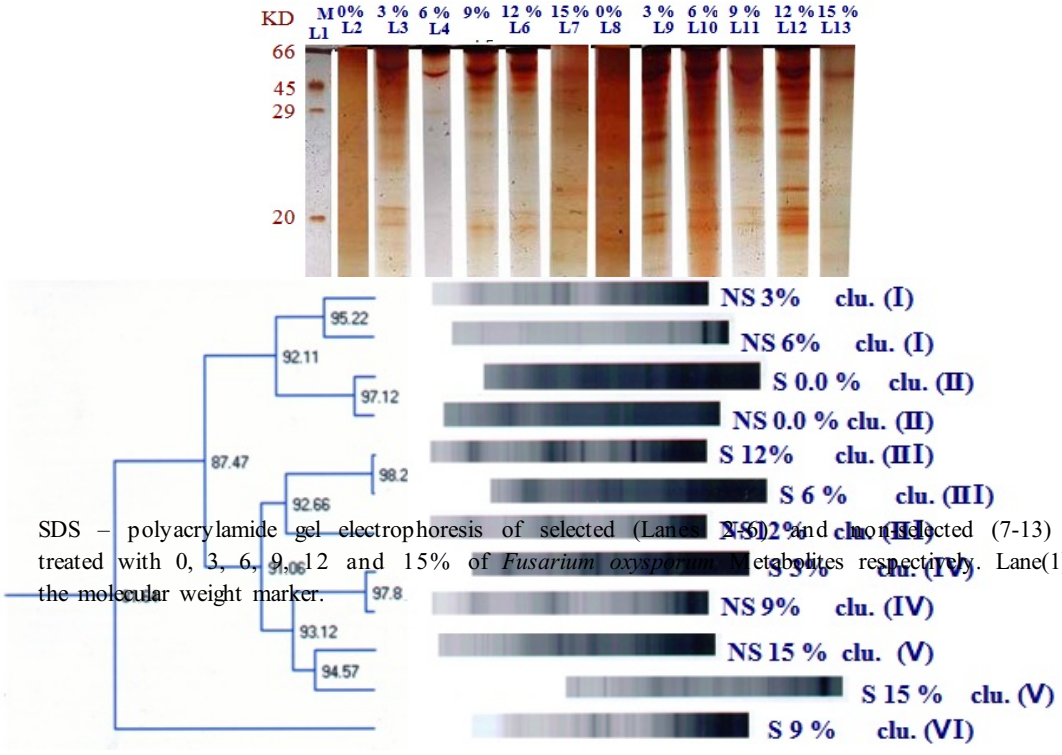

Fig. 1: SDS – polyacrylamide gel electrophoresis of selected (Lanes 2-6) and non-selected (7-13) callus lines treated with 0, 3, 6, 9, 12 and 15% of *Fusarium oxysporum* Metabolites respectively. Lane(1) represent the molecular weight marker.

Dendrogram 1: Linkage dendrogram of studied selected and non-selected soybean callus line treated with different conc. of *F.* metabolite 0, 3, 6, 9, 12 and 15% respectively based on SDS-PAGE of proteins.

molecular weights ranging from 66 to 14 KD. Also, from fig. (1), there were a marked differences concerning the amount of protein (protein %) between the selected and non-selected soybean callus lines as compared between the first 7 bands (M.wts. 66, 63,61,48,45,36 and 34 kD), of both callus lines.

Generally, proteins were fractionated to many bands in selected than non-selected callus line for each treatment. Also, bands with molecular weights 35 and 28 KD were approximately present in 83.3 % of treated non-selected and selected callus lines.

Some bands disappeared and sensitive toward increasing the concentration of fungal metabolites, as that with 57, 21 and 7 KD M.wt. in both selected and non-selected callus line that with 47, 43 and 29 KD M.wt. for selected line and 48, 59KD M.wt. for non-selected.

On the other hand, there were many characteristic properties for protein pattern of selected callus, where bands with 16 and 22 KD M.wt. were present in 83.3 % and 100 % fungal metabolites concentration respectively. Moreover bands with 24 and 26 KD M.wt. were detected in low concentration lost and then restored at high conc.

The dendrogram produced from genetic distances between selected and non-selected soybean callus lines treated with different conc. of *F.* metabolite was shown in dendrogram (1). Based on the extent of relative dissimilarity among both callus lines based on protein poly-peptides, the selected and non-selected soybean callus lines treated with different conc. of *F.* metabolite were grouped into six clusters. The grou-ping pattern and distribution of selected and non-selected soybean callus lines into different clusters was given in

Table 5: Grouping pattern of selected and non-selected soybean callus line treated with 0.0, 3, 6, 12 & 15 % of *Fusarium*

oxysporum metabo-lites respectively based on analysis of their protein band differences.

cluster	Number of callus line	callus lines falling in cluster
I	2	Non-selected callus lines treated with 3 % and 6 %.
II	2	Selected callus lines treated with 0.0 % and non-selected callus lines treated with 0.0 %.
III	3	Two selected callus lines treated with 12 % and 6 %, and one non-selected callus lines treated with 12 %.
IV	2	Selected callus lines treated with 3 % and non-selected callus lines treated with 9 %.
V	2	Non-selected callus lines treated with 15 % and selected callus lines treated with 15 %.
VI	1	Selected callus lines treated with 9 %.

table (5). Cluster III was the largest and have three callus lines treated with conc. 6 % and 12 %, where two lines from the three were selected callus lines treated with 4%, while the clusters I have two non-selected callus lines treated with 3 % and 6 %. Also, from the dendrogram must be noticed that the cluster II have two callus lines the first was selected callus lines treated with 0.0 % and the second was non-selected callus lines treated with 0.0 %. In addition to that, the cluster IV has two callus lines, the first was selected callus lines treated with 3 % and the second was non-selected callus lines treated with 9 %. Also, the cluster V has two callus lines the first was non-selected callus lines treated with 15 % and the second was selected callus lines treated with 15 %, while the clusters VI have a single selected callus line treated with conc. 9 % where this conc. was the conc. at which the selection procedure was done.

Similar results were obtained by Epple *et al.* [26], who reported that, thionins are antimicrobial proteins that are thought to be involved in plant defense. The constitutive over expression of this thionin enhances the resistances of the susceptible ecotype Columbia against attack by *F.oxysporum*. *F.oxysporum*, a plant pathogen, produced, protein under disease conditions. In addition, this evidence suggests that protein content is an important factor in determining pathogen susceptibility to proteins produced by the plant during pathogen attack specifically. Osmotin and the results show that, fungal cell wall components can increase resistance to plant defense proteins and affect virulence. Narasimhan *et al.*[27]. Also, it was established that the treatment with fungal exudates, stimulates cell growth and enhances the contents of proteins and lipids (7.6%, 13%)[11,28,29].

Variations in band intensity, high accumulation of some polypeptides and less accumulation of others, in *in vitro* cultured materials could be attributed to chromo-somal instability of calli survived under stress conditions[30].

DNA Analysis: Thirteen random primers were used in the present study to identify the mutagenic alterations among the selected and non-selected soybean callus lines. Six primers succeeded to generate DNA products table (6). Each of these six primers used for analysis of selected and non-selected soybean callus lines treated with 9% of *F. oxysporum* metabolites amplified different number of bands table (7). The B-7 primer indicated the occurrence of twelve DNA fragments in the selected and non-selected soybean callus lines, six of these DNA fragments were detected with some of other primers, these DNA fragments have an approximate size of 500, 603, 870, 1000, 1100 & 1350 bP. The other six DNA fragments were not detected with any of the other primers. These DNA fragments have an approximate sizes of 420, 510, 560, 730, 1600 & 1800 bp. {lanes 4 & 5, respectively in figure (2)}.

Table 6: The nucleotide sequence and the percentages of the G+C content of the primers used in this study.

Primer	Sequence	G + C %
G-3	3`- GAGCCCTCCA -5 `	70 %
B-7	3`- GGTGACGCAG -5 `	70 %
A-14	3`- TCTGTGCTGG -5 `	60 %
A-1	3`- CAGGCCCTTC -5 `	70 %
A-11	3`- CAATCGCCGT -5 `	60 %
B-10	3`- CTGCTGGGAC -5 `	70 %

Treatment of the selected soybean callus line to the 9 % metabolites which induced by *F. oxysporum* fungus was accompanied by the induction and presence of a new unique DNA fragments of an approximate sizes of 400 bp, these DNA fragments have not been detected with any of the other primers and also, have not been presence in the non-selected soybean callus line. Also, the changes in genomic DNA of selected and non-selected soybean callus lines was also observed with the other five primers.

Finally, from the data of this investigation and by calculation method, we showed that the dice coefficient among the selected and non-selected soybean callus lines exposed to 9 % of *F. oxysporum* metabolites was 84%**,** table (8).

Table 7: List of arbitrary primers and amplified RAPD fragments from two soybean callus lines exposed to 9% of *F. oxysporum* metabolites.

Callus lines	Primers					
	G-3	B-7	A-14	A-1	A-11	B-10
Selected	12	13	9	4	5	4
Non-selected	12	12	13	5	4	7
Total	24	25	22	9	9	11

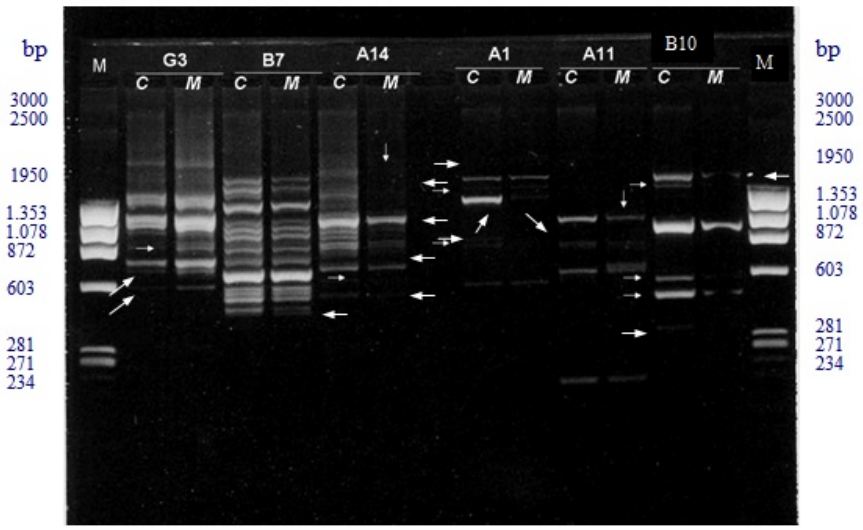

Fig. 2: RAPD profile generated by the (6) primes detecting DNA changes among non-selected }C{ & selected }M{ soybean callus lines treated with 9% of *F.* oxysporum metabolite. The 1st & last lane represents the DNA molecular size markers.

Table 8: The dice coefficient between two soybean callus

lines treated with 9 % of *F. oxysporum* metabolites.

	Non-Selected	Selected
Non-Selected	100	84
Selected	84	100
Dice Coefficient	84%	

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