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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, (1962)** (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 been 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 **Purseglove, (1979)**. 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 **Agrios, (1997)**.

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 processes such as embryogenesis and host-pathogen interactions and to enhance

characteristics such as salt or disease resistance, production of secondary metabolites, somatic hybridization, genetic engineering and germplasm conservation **Collin and Edwards, (1998)**. *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 **Brazolot et al., (1994)**.

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, (1988)** 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., (2007)** 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 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 **Welsh and McClelland.,(1990)** and **Williams et al.,(1990)**. 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 **Hadrys et al., (1992)**. **Koretsky (2001)** 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. (2002) found that when *F. culmorum* was grown in a gluten–containing medium, from which an alkaline serine proteinase was purified, this enzyme used by the fungi to hydrdye 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., (2003)**. 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 **Katsantonis et al., (2005)**.

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 containing 25 ml of a solidified sterile basal Murashige and Skoog (MS) medium **Murashige and Skoog, (1962)** containing 3% sucrose and 0.8% agar. The medium was adjusted to PH 5.8 before autoclaving at 121°C and 1.2 kg/cm². 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 inoculation 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 calculating 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 subculture 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 required 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., (2002)**. 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 located at **National center of Agricultural Research. Giza, Egypt.**

Electrophoretic detection of protein

The method of **Laemmili (1970)** 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 (1987)** was followed to extract and determine DNA.

PCR amplification. Amplification reaction volumes were, 25µl, each containing 1× PCR buffer with MgCl₂ (50 mM KCl; 10 mM Tris-HCl (pH 9.0); 2 mM MgCl₂ and 1% triton 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 1x 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 supplemented 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 (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., (2005), Naseem et al., (2005)** 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 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 (1990)** 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.*, (1967), Hegazy (1987), Gorlach *et al.*, 1995), and recently Daif (1998)}**, in alfalfa a relationship between proline levels and adaptability to stress has been observed **ILieva and Radeva, (2001)**.

Protein Analysis:

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 {Table (4) and fig. (1)}. 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 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 grouping pattern and distribution of selected and non-selected soybean callus lines into different clusters was given in 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 **Epplé et al., (1997)**, 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.*, (2003)**. Also, it was established that the treatment with fungal exudates, stimulates cell growth and enhances the contents of proteins and lipids (7.6%, 13%). **Irina *et al.*, (1999)**. **James *et al.*, (2000)**, **Liuba (2001)**. Variations in band intensity, high accumulation of some polypeptides and less accumulation of others, in *in vitro* cultured materials could be attributed to chromosomal instability of calli survived under stress conditions **Chretien *et al.*, (1992)**.

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 approximate sizes of 420, 510, 560, 730, 1600 & 1800 bp. {lanes 4 & 5, respectively in figure (2)}.

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 (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)	
<i>F. metabolite conc. (%)</i>	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)	
<i>F. metabolite conc. (%)</i>	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.

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

<i>Fusarium</i> metabolites conc. (%)	0.0		3		6		9		12		15	
	select	Non-select	select	Non-select	select	Non-select	select	Non-select	select	Non-select	select	Non-select
Free amino acids contents(mg%)												
1-Aspartic	0.00	0.00	0.00	0.00	0.009	0.00	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	47.40	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	0.00	21.14	0.00	9.93	42.24
7-Proline	0.00	0.00	59.21	30.10	78.14	65.85	86.04	23.69	48.93	32.46	2.15	1.50
8-Methionine	8.42	0.00	16.85	0.00	7.33	4.23	0.00	4.55	11.40	6.30	6.05	0.00
9-Valine	0.15	0.00	9.54	0.00	3.40	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	3.00	0.00	1.08	0.00	0.00	0.00	5.41	0.00	0.00	0.00
12-Cysteine	0.00	0.00	7.20	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-Phenylalanine	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

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 bands (KD)	Marker		Non - selected						Selected					
	Lane 1		Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13
	M.wt.	Am. %	Am. %	Am. %	Am. %	Am. %	Am. %	Am. %	Am. %	Am. %	Am. %	Am. %	Am. %	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	-	-	-	-	-
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	17.91 1.30	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	16	13	6	12	17	17	18	14	14

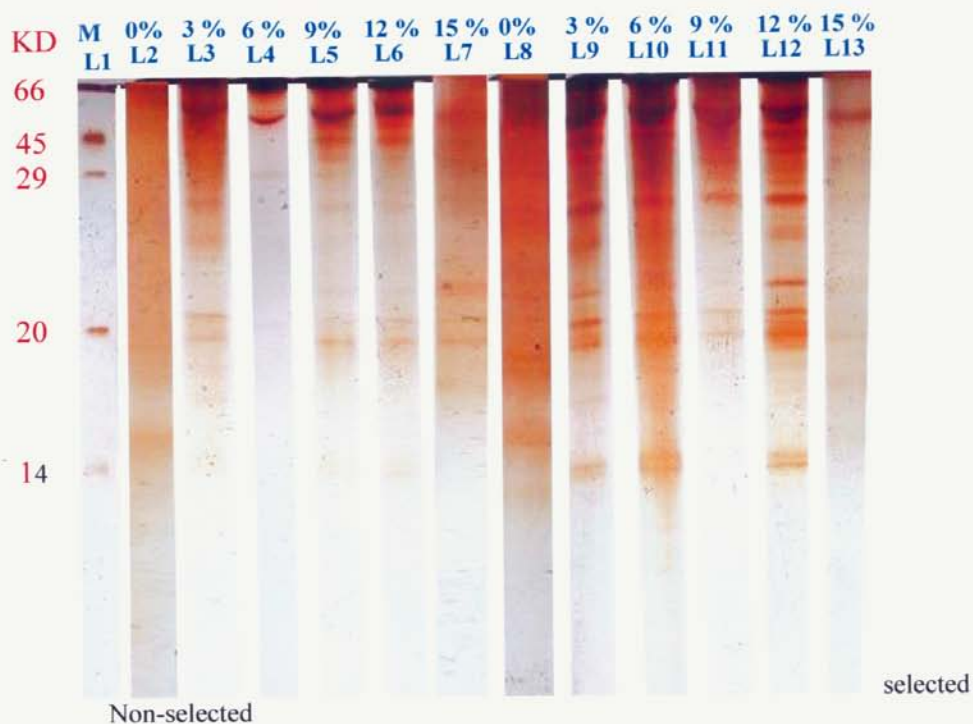
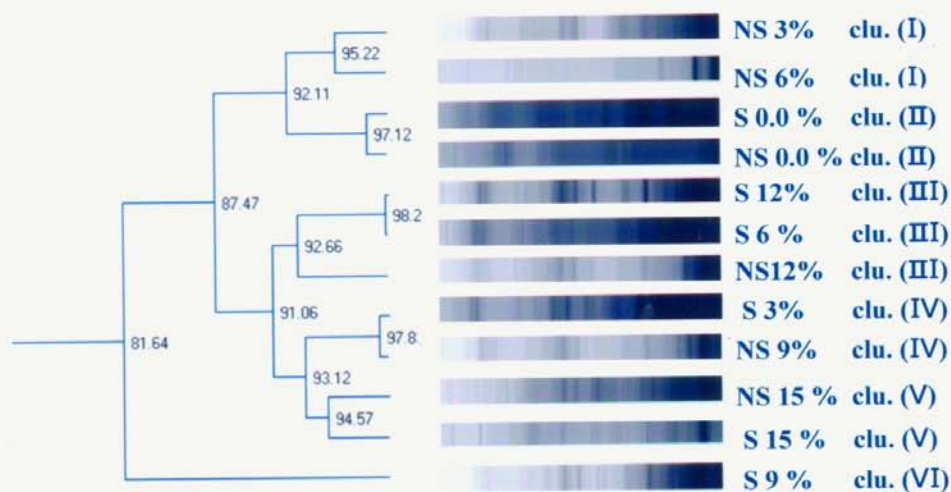


Fig. (1): SDS – polyacrylamide gel electrophoresis of selected (Lanes 2-6) and non-selected (7-13) callus lines treated with 0, 3, 6, 9, 12 and 15% of *Fusarium oxysporum* metabolites respectively. Lane(1) represent the molecular weight marker.



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.

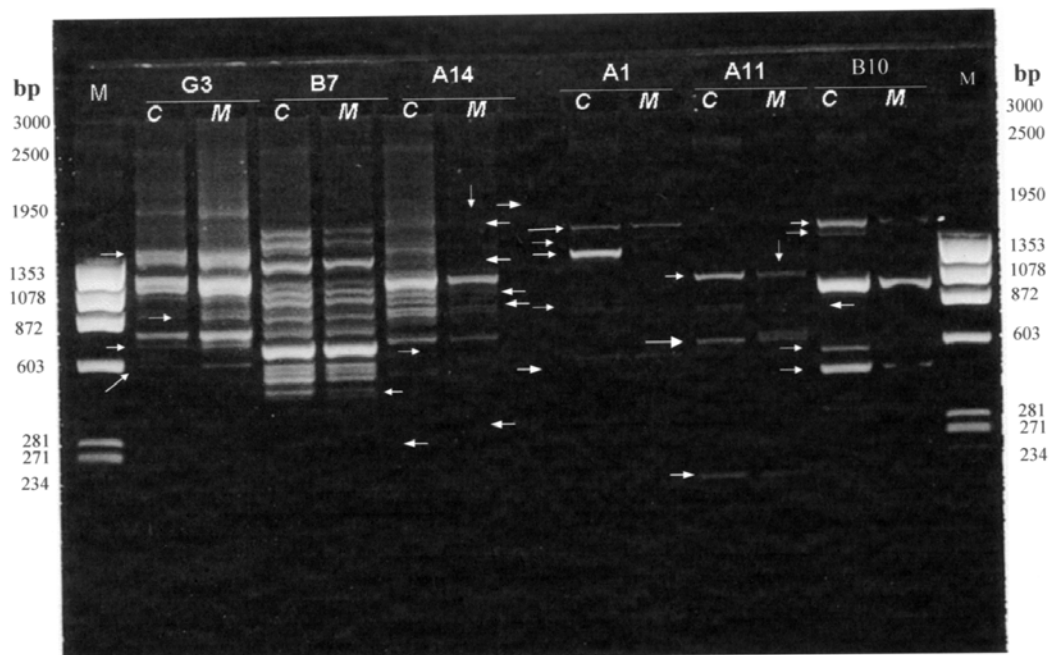


fig. (2) RAPD profile generated by the (6) primers detecting DNA changes among non-selected {C} & selected {M} soybean callus lines treated with 9% of *Fusarium oxysporum* metabolite. The first & last lane represents the DNA molecular size markers.

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

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

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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الملخص العربي

انتخاب كالوس من فول الصويا مقاوم لمرض الذبول

باستخدام مزارع الأنسجة

د. السيد هاشم ، د. حنان عبدالله ، د. ياسر حسين ، محمد أحمد حسين

قسم النبات - كلية العلوم - جامعة الزقازيق

أجريت التجارب العملية الخاصة بهذه الدراسة في معمل زراعة الأنسجة بقسم النبات - كلية العلوم - جامعة الزقازيق بهدف إستحداث كالوس من نبات فول الصويا بإستخدام أوسطة غذائية (بيئة مورايش وسكوج) المزودة ببعض الهرمونات النباتية وكانت (5 ملجم / لتر نفتالين حمض الخليك + 0.5 ملجم / لتر 6- بنزيل أمينوبيورين) ثم دراسة مقاومة الكالوس للنواتج الأيضية لفطر الفيوزاريوم أوكسيسبوريوم والتي تضاف عند تنمية هذا الكالوس على تركيزات مئوية مختلفة من النواتج الأيضية المعقمة بالمرشحات البكتيريولوجية للفطر في مزارع الأنسجة مع محاولة تكيف و تنمية هذا الكالوس على تركيزات مرتفعة من النواتج الأيضية للفطر .

وبعد الحصول على الكالوس المقاوم بإستخدام مزارع الأنسجة لعمل دراسة مقارنة للعمليات الأيضية للكالوس المقاوم والكالوس غير المقاوم بعد تنميتها على تركيزات مختلفة من النواتج الأيضية المعقمة بالمرشحات البكتيريولوجية لفطر الفيوزاريوم أوكسيسبوريوم في مزارع الأنسجة .

يمكن تلخيص النتائج التي تم الحصول عليها على النحو التالي :

أ. عند دراسة معدل نمو كل من كالوس نبات فول الصويا المقاوم للنواتج الأيضية لفطر الفيوزاريوم أوكسيسبوريوم في مزارع الأنسجة والكالوس غير المقاوم .

أظهرت النتائج أن هناك زيادة واضحة في الوزن الغض للكالوس المقاوم عنها في الكالوس غير المقاوم ، مع زيادة تركيز النواتج الأيضية لفطر الفيوزاريوم أوكسيسبوريوم في البيئة المغذية .
ب. عند دراسة مستوى الأحماض الأمينية الحرة في كل من كالوس نبات فول الصويا المقاوم للنواتج الأيضية لفطر الفيوزاريوم أوكسيسبوريوم والكالوس غير المقاوم .

أظهرت النتائج أن هناك زيادة واضحة في الأحماض الأمينية الحرة في الكالوس المقاوم عنها في الكالوس غير المقاوم وهذا عند جميع التركيزات المختلفة من الفطر ، وأيضاً تبين من التجارب أن هناك زيادة كبيرة في الحمض الأميني البرولين عند جميع التركيزات المختلفة للنواتج الأيضية للفطر ، وخصوصاً عند تركيز 9% وهذا التركيز الذي تم عنده انتخاب الكالوس المقاوم .

ت. أظهر تحليل النمط البروتيني لكل من الكالوس المقاوم والغير مقاوم باستخدام التفريد الكهربائي ELECTROPHORESIS أن كمية البروتين عند التركيز 3% ، 6% في الكالوس الغير مقاوم

أكبر من كمية البروتين في الكالوس المقاوم ، وأيضًا عند التركيزات 9% ، 12% ، 15% تكون كمية البروتين في الكالوس المقاوم أكبر من كمية البروتين في الكالوس غير المقاوم ، أما عند تركيز 0% (الوسط الغذائي الخالي من النواتج الأيضية للفطر) تكون كمية البروتين في الكالوس المقاوم أكبر من كمية البروتين في الكالوس غير المقاوم .

ثا. اتضح من نتائج تفاعل البلمرة المتسلسل باستخدام تقنية التكبير العشوائي RAPD-PCR للحمض النووي DNA باستخدام ستة من البادئات PRIMERS من ثلاثة عشر تم تجربتهم تغيرًا في الحمض النووي DNA في كل من الكالوس المقاوم والغير مقاوم نتيجة للتعرض لتركيز 9% من الناتج الأيضي لفطر الفيوزاريوم أوكسيسبوريوم ، فقد أظهر البادن B-7 أنه نتيجة تعرض الكالوس المقاوم للنواتج الأيضية لفطر الفيوزاريوم أوكسيسبوريوم إلى ظهور قطعة من الحمض النووي حجمها التقريبي 400 bp لم تظهر في الكالوس غير المقاوم . وقد ظهرت أيضًا اختلافات في الحمض النووي مع الخمس بادئات الأخرى.

CHLOROFORM INDUCED CYTOTOXICITY IN MICE HEPATOCYTES

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ABSTRACT

A plethora of biochemical changes occurs in apoptotic cells, the best characterized one is the fragmentation of chromatin into units of single or multiple nucleosomes of 180 to 200 bp or multiples of the same. The DNA laddering resolved an agarose gel electrophoresis is often regarded as the biochemical hallmark of apoptosis.

While the early changes observed during necrosis are swelling of the cytoplasm and organelles, dissolution of organelles, and the rupturing of plasma membrane there by allowing the cellular contents to leak out into the extra-cellular spaces, and it appears as smear pattern in gel electrophoresis.

In view of this, in our study male and female Swiss albino mice were treated by chloroform through oral gavage in different concentrations 130, 238, 277 and 477mg chloroform/kg mice dissolved in corn oil (10ml/kg mice), for 33 and 54 days. Treatment with chloroform with 130 and 238mg chloroform/kg mice for 54 days led to apoptosis likewise 277 and 477mg chloroform/kg mice for 33 days also led to apoptosis where as 277 and 477mg chloroform/kg mice for 54 days led to necrosis as observed in gel electrophoresis for DNA isolated from each mice at the end of each period of treatment, also polyacrylamide gel electrophoresis and different display of the expressed proteins were obvious among all the treatments for different periods.

INTRODUCTION

Chloroform induced tumor responses are secondary to cytotoxicity, most likely as a consequence of the regenerative hyperplasia which occurs following cytotoxicity in liver and kidney (*Butterworth and Bogdanffy 1999*).

(Regenerative hyperplasia) can lead to an increased probability of cancer by one or both of two alternative modes of action.

First cells that are undergoing cell division are inherently more susceptible to initiation than are slowly growing or non dividing cells. This is because DNA undergoing replication is more exposed to nucleophilic attack than DNA that is covered with histones and arranged in nucleosomes (*Ames and Gold 1991*). Also, any gene damage that occurs in a cell undergoing division has less time to be repaired before mitosis than in a slowly growing cell so a large fraction of DNA alterations could be converted into mutations.

Second, chemicals that promote cell division may convey a selective growth advantage to preexisting initiated cells in comparison with normal cells, there by facilitating clonal expansion of initiated cells are more responsive than normal cells to growth stimuli, because they are less susceptible to the toxicity of the chemical, or because they are less susceptible to endogenous regulatory signals that trigger programmed cell death apoptosis. In any case, the ratio of cell birth to cell death of initiated cells increase compared with normal cells, leading to increased likelihood that clone of initiated cells will form & survive.

EPA reviewed the strength and consistency of the association between cytotoxicity and regenerative hyperplasia and the occurrence of increased tumor frequency in exposed animals. This analysis reveled two main points:

There are numerous cases where exposure to chloroform causes an increase in cellular regeneration without any observable increase in cancer incidence. These date indicate that chloroform exposures that are adequate to cause cytotoxicity and regenerative cell proliferation do not always lead to cancer.

There are no cases in which a tumorigenic response has been observed where evidence of cell regeneration is not also observed at the same or lower dose as that which caused an increase in tumors. This consistency of evidence (i. e. cell regeneration is detected in all cases of tumorigenicity) is strong evidence supporting the conclusion that cell regeneration is a mandatory precursor for tumorigenicity (*U.S. EPA 2001*).

Programmed cell death or apoptosis occurs during development and in response to different environmental cues.

Apoptosis exhibits several distinct morphological features, including condensation of cytoplasm, fragmentation of nucleosomal DNA, condensation of nuclear chromatin, membrane blabbing, and appearance of apoptotic bodies, (*Liljeroth and Bryngelsson 2001, and Yamada et al., 2003*) changes in the nucleus provide the most important means of diagnosing apoptosis. In animal cells, the chromatin condenses and collapses into patches, the nucleus becomes fragmented and the cell parts some of which containing fragmented nucleus, bud out words to produce membrane – coated apoptotic bodies, although cytoplasm condensation occurs in animal cells, it is not indicative of apoptosis in the absence of typical nuclear changes (*Kumar, 2007*).

The global analysis of expressed cellular proteins, provides powerful tools for the detailed comparison of proteins from normal and neoplastic tissue, analysis of disease especially in fields of cancer research (*Sun et al., 2007*).

Aim of the work:

This work aimed to study the effect of chloroform as one of the by-products of chlorinated drinking water at the level of molecular biology.

The study comprised the effect of chloroform on apoptosis in the genomic DNA isolated from liver tissue of both male and female mice treated through oral gavage of chloroform.

The study was extended to study the effect of chloroform on the differential display of the expressed proteins.

MATERIALS AND METHODS

Experimental animals:

144 Swiss albino mice both sexes at 8 weeks of age were weighed and assigned to different treatment groups:

T₁ = 130 mg chloroform / kg mice.

T₂ = 238 mg chloroform / kg mice.

T₃ = 277 mg chloroform / kg mice.

T₄ = 477 mg chloroform / kg mice.

Dissolved in corn oil (10ml/kg mice). Mice receive their doses by oral gavage. The dose level of chloroform were selected so that the high dose have been previously demonstrated to be carcinogenic in mice, (NCI, 1976).

Control mice receive only water, the gavage doses were formulated daily in corn oil and taken 5 days / week for 33 , 54 days (Pereira, 1993).

After necropsy the liver organ was removed from mice at various periods after chloroform treatments, and liver was frozen in liquid nitrogen and stored at- 20 C° until use.

For DNA gel electrophoresis:

Reagents:

- a) DNA isolation kit from promega chemical Co.
- b) Ethanol 70%.
- c) Isopropyl alcohol from Adwic chemical Co.
- d) Ethidium bromide.
- e) λ Hind III/ EcoR I DNA marker from promega chemical Co.
- f) Agarose from promega chemical Co.
- g) DNA loading buffer from promega chemical Co.

Methods:

I- Isolation of DNA:

Isolation of DNA from liver tissues was carried out according to (Miller, 1988).

II– DNA gel electrophoresis

DNA was electrophoresed on 1.6% agarose gel for 6 hours at 20V. The gel was stained with 0.5µg/ml ethidium bromide to allow visualization of DNA (*Counts and Goodman, 1995*).

For differential display of expressed proteins:

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis was performed to proteins isolated from liver tissues from male and female mice treated with 130 , 238 , 277 and 477 mg chloroform / Kg mice for 33 and 54 days.

One-dimensional gel electrophoresis under denaturing conditions (i.e., in the presence of 0.1% SDS) was applied to separate proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode. The method described by *Laemmli (1970)* for the electrophoretic separation of proteins according to their molecular weights by SDS - PAGE, was applied with some modifications.

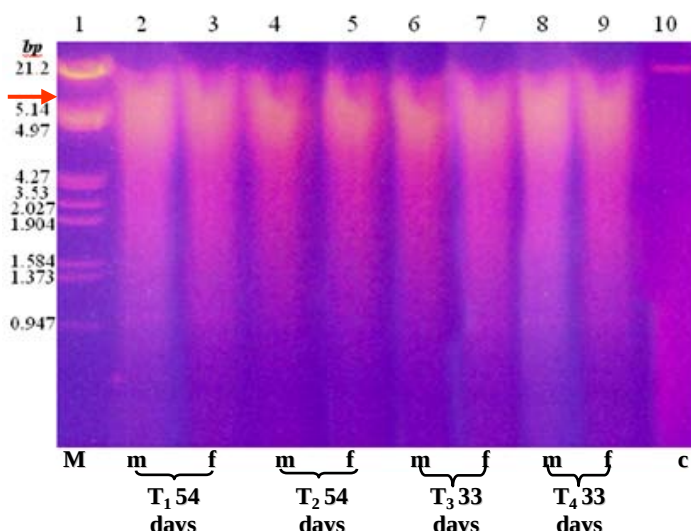
RESULTS

Agarose gel electrophoresis for genomic DNA isolated from liver of male and female mice treated with chloroform by gavage in corn oil in concentrations of 130 and 238mg chloroform/Kg mice for 54 days and concentrations of 277 and 477mg chloroform/Kg mice for 33 days induction and progression of apoptosis in fragment of DNA were obvious, the presence of such large DNA fragments is the characteristic of pre-apoptotic cells which precede inter-nucleosomal DNA cleavage as shown in (Fig. 1).

Agarose gel electrophoresis for genomic DNA isolated from liver of male and female mice treated with chloroform by gavage in corn oil in concentrations of 277 and 477mg chloroform/Kg mice for 54 days, it's obvious that there are smearing of degraded DNA which are conspicuous, this smear of oligonucleosomes termed as necrosis as shown in (Fig. 2).

Fig. (1)

Effect of chloroform gavage by concentrations of 130 and 238mg chloroform/Kg mice for 54 days and 277 and 477mg chloroform for 33 days on DNA of both male and female mice liver



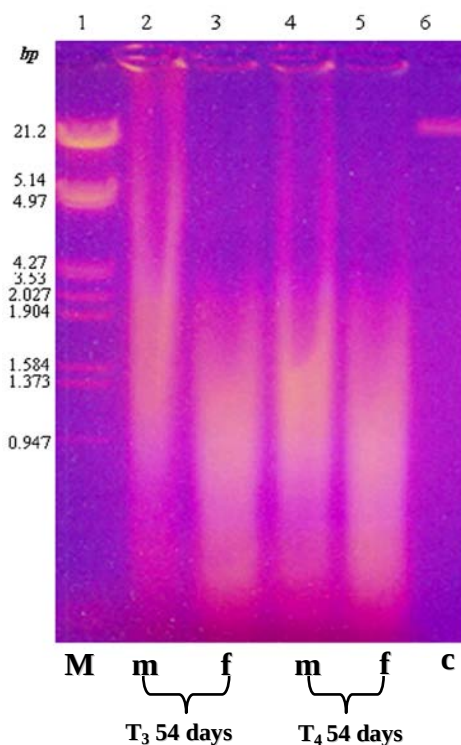


Fig. (2): Effect of chloroform gavage by concentrations of 277 and 477mg chloroform/Kg mice for 54 days on DNA of both male and female mice liver

M = λ / Hind III/ ECORI DNA marker

m = male

f = female

c = control

T₁ = 130 mg chloroform/kg mice

T₂ = 238 mg chloroform/kg mice

T₃ = 277 mg chloroform/kg mice

T₄ = 477 mg chloroform/kg mice

SDS- PAGE for the differential display of expressed proteins in the liver of male and female mice treated with 130 and 238mg chloroform/Kg mice by oral gavage in corn oil for 33 and 54 days is presented in Fig. (3). It's cleared from this figure that proteins have marked alterations where some proteins disappeared and others are appeared in comparison with control ones.

SDS- PAGE for the differential display of expressed proteins in the liver of male and female mice treated with 277 and 477mg chloroform/Kg mice by oral gavage in corn oil for 33 and 54 days is presented in Fig. (4). Alterations in different proteins expressed are more obvious due to high dose effect.

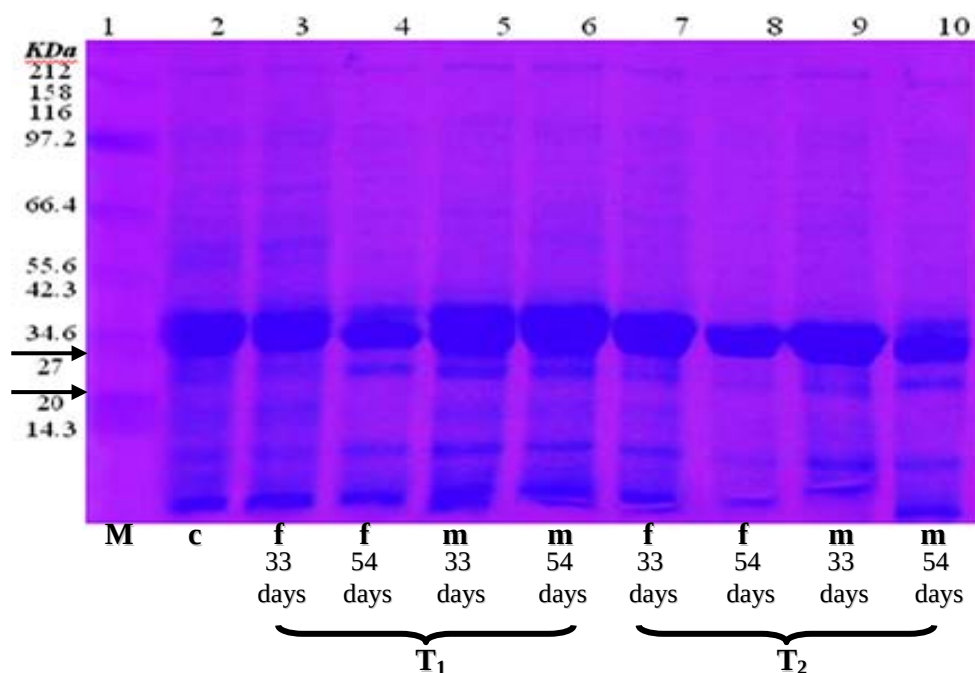


Fig. (3): Protein differential display as electrophoresed using SDS- PAGE for expressed proteins in liver tissues of male and female mice treated with 130 and 238mg chloroform/Kg mice by oral gavage in corn oil for 33 and 54 days.

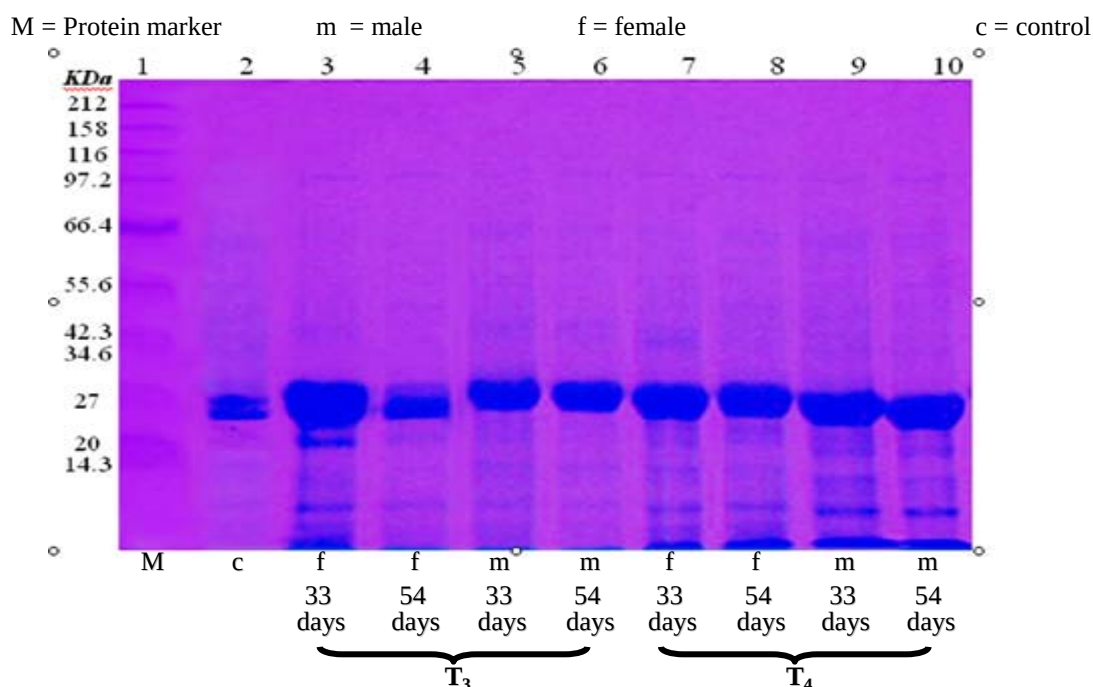


Fig. (4): Protein differential display as electrophoresed using SDS- PAGE for expressed proteins in liver tissues of male and female mice treated with 277 and 477mg chloroform/Kg mice by oral gavage in corn oil for 33 and 54 days.

DISCUSSION

Apoptosis has been best characterized by the cleavage of DNA into nucleosomal size fragments of 180- 200 bp or multiples of the same. So the progression of apoptotic DNA cleavage start as a single- strand nicks (*Gorczyca et al., 1992*) followed by formation of large DNA fragments of 50 – 200kbp and subsequently inter-nucleosomal DNA fragmentation (*Cohen et al., 1994*). But the characteristic ladder pattern is produced at a very late stage, when most of the apoptotic cell deaths occur and result in the cleavage of the inter-nucleosomal linker DNA (*Wyllie and Morris, 1982*). Contrary to this, under extreme conditions, the cells were found to show adaptive response to apoptosis and unable to regulate their own death; necrosis is therefore predominantly observed (*Cohen et al., 1992*). In the present study, the pattern of fragments originated from the endonuclease activity during apoptosis was analyzed by agarose gel electrophoresis (1.6%). DNA was extracted from hepatic tissue following treatment by chloroform gavaging in dose of 277 and 477mg chloroform for 33 and 54 days and doses 130 and 238mg chloroform for 54 days.

Induction and progression of apoptosis in hepatic cells were started by the formation of large fragments of DNA. The presence of such large DNA fragments is the characteristic of pre-apoptotic cells, which precede inter-nucleosomal DNA cleavage (*Zhen et al., 2000*).

Male and female Swiss albino mice treated with 130 and 238mg chloroform for 54 days and 277 and 477mg chloroform for 33 days respectively, a discrete band was found, the presence of such large DNA fragments is the characteristic of pre-apoptotic cells, which precede inter-nucleosomal DNA cleavage. Male and female Swiss albino mice treated with 277 and 477mg chloroform for 54 days respectively, a smear of oligonucleosomes was obvious, this smeary DNA fragmentation was suggestive of necrosis.

This is in agreement with (*Bushra et al., 2002*) who reported that butachlor as an example of non- genotoxic carcinogen led to apoptosis which appeared in the gel electrophoresis as large fragments of DNA which is characteristic of pre-apoptotic cells which under extreme conditions like high dose and long period led to necrosis which appeared in the gel electrophoresis as a smear of oligonucleosomes.

During stages of hepatocarcinogenesis , not only DNA synthesis but also apoptosis gradually increase from normal to (preneoplastic) to adenoma and carcinoma tissue .

Pre-neoplastic liver cells are more susceptible than normal hepatocytes to stimulation of cell replication and of cell death.

The rates of cell proliferation and cell loss in junction with the differentiation status of a tissue are among the factors contributing to carcinogenesis .Nongenotoxic chemicals may affect this balance by increasing proliferation through direct mitogenesis or through a regenerative response following loss of cells through cytotoxic or apoptic necrosis (*Dragan et al .,2007*) .

The present study assured that there are differential display of expressed proteins using SDS- PAGE for liver tissues of male and female mice treated with different concentrations of chloroform (130, 238, 277, 477mg/kg mice) for 33 and 54 days.

This is in agreement with *Alfonso et al., (2005)* who studied vimentin protein, they reported that this protein has four isoforms and each one suffer a different alterations in its amount in tumor cells when compared with normal ones. Resemble studies has also performed by *Stulik et al., (2001)* who reported that the presence of four isoforms of calcyclin and detected the alteration of two of them in tumors, one with increased levels and another with decreased expression.

In conclusion, our data showed that high doses of chloroform produce apoptosis in early stage and under extreme conditions the cells shows adaptive response to apoptosis and unable to regulate their own death, necrosis therefore is observed in late stage which may progress to give tumor cells and different display of the expressed proteins were observed in all treatments for different periods when compared to control ones.

We recommend that further studies should be performed to assess the possible human cancer risk of chloroform by extrapolating the results of chronic, high dose studies in rodents to estimate the potential risk in humans.

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EFFECT OF IRRIGATION WATER SOURCES ON PRODUCTIVITY AND GRAIN QUALITY OF WHEAT

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ABSTRACT

The objective of this experiment was to study the effect of irrigation water source on productivity and quality of wheat seeds. Three experiments were carried out at each of El-Salam canal, Bahr-Hadoos and Bahr El-Bakar at south Sahl El-Hossinia, El-Sharkia governorate during 2005/2006 and 2006/2007 seasons. South Sahl El-Hossinia serves a command area of 82000 feddans, irrigated from El-Salam canal. The irrigation water was from El Salam Canal (drainage water mixed with fresh water 1:1), Bahr –Hadoos (agricultural drainage water) and Bahr El- Bakar (Drainage water contaminated with sewage water). The experimental design was completely randomized block design with three replications and bread wheat cultivar Shaka 93 was used. The results indicated that wheat seed protein and microelements (Fe, Zn and Mn) were the highest using sewage water. Total carbohydrate was decreased with sewage water. Wheat grain yield was the highest when El-Salam canal water was used comparing with Bahr Hadoos and Bahr El Bakar. Plant height, spike length, No. spike lets/spike was the highest when the water from Bahr Hadoos used comparing with El-Salam canal and Bahr El-Bakar. But leaf area and 1000 - kernel weight were not affected by irrigation water sources. Rate of seed germination was decreased when irrigated by sewage water (46 - 53 %) while rate of germination ranged between (64 – 73 %) and (64 %) at El-Salam canal and Bahr Hadoos respectively for both seasons.

Results also indicated that EC of irrigation sources ranged from 1.74 - 2.16, 1.95 - 2.34 and 1.27 - 2.42 dS/m in first season for El- Salam ,Bahr Hadoos and Bahr El-Bakar, respectively and PH values ranged from 8.14 to 8.45 in first season while the values were 7.92 to 8.25 in second one. Microelements (Fe, Zn and Mn) values ranged from 1.75-1.99, 0.3 - 0.56 and 0.51- 0.71 mg/1 in the first season but the values in second season ranged from 1.85-1.99, 0.67-0.98 and 1.00-1.55 mg/1 ,with the same respectively .

Key words: Agriculture drainage water, sewage water, Nile water, wheat crops productivity.

INTRODUCTION

Wheat is the most important nutritional cereal crop in Egypt .The local production is not sufficient to meet local requirements. Egyptian production is about 7.4 million tons of wheat grain with an average production of 18.12 ardab per feddan (Anonymous, 2007). It is of great importance to increase wheat total production by

increasing wheat cultivated area with the available irrigation water from different sources in reclaimed lands. Salt –affected soil develops from a wide range of factors including soil type, field slope, drainage, irrigation system, fertilizer management, and water management practices. In Egypt, perhaps the most critical factor to reduce salt level is using high quality of irrigation water. Salinity usually affects crop yield, soil physical conditions, fertility needs, irrigation system performance and longevity, and how the irrigation water can be applied .Therefore, knowledge of irrigation water quality is critical to understand what management changes are necessary for sustainable agriculture. Egypt uses 88% of its available fresh water in the agriculture sector and it is one of the leading countries in irrigation. An area of approximately one million feddan in the Nile Delta depends on drainage water for irrigation(El-Hawary 2003) .Mostafa (2001b) reported that irrigation with Nile water gave wheat grain yield of 2.29 t/fed .compared to 2.26 and 2.21 t/fed of drainage and sewage water, respectively , while El-Saady (1991) obtained the highest straw and grain yields when wheat was irrigated by Nile water alternated with drainage water. Increasing water salinity level consistently decreased dray matter of Sakha 69wheat plant (Mostafa 2001a). In contrary , Bayoumi et al (1997) stated that water salinity increased protein content in wheat and straw. El-Kobbia and Ibrahim (1989) found that some sewage waters had an Ec of less than 1.50 ds/m could be used for irrigation purposes for most crops on most soils with little possibility of salinity problems. Mean while , Mostafa (1995) noticed that there was progressive and consistent depression in K concentration in wheat plant as a result of raising EC from 1.68 to 15.6 ds/m of using saline water . El-Sayed (2001) showed the average concentration of nitrate and ammonium nitrogen in sewage water is higher than its concentration in Nile water by four folds .This means that the concentration of available nitrogen is higher than the requirements of plants. Potassium (K) in waste water is not known to cause adverse effect on plants ,it is an essential macronutrient for plants and affect positively in soil fertility as well as crop yield and quality (FAO.1992).El-Sheikh (2000) revealed that higher N content increased activity of microorganisms in soil ,besides the increased of plant residues at rhizosphere. Selem et al (1989) and El-Skeikh(2000) found that irrigation soil with drainage water for two months or up to the plant harvest decreased available P. Lai and Lai (1990) showed that wheat uptake of K was decreased while that of Na was increased with the increase in EC and SAR.

The objective of this experiment was to study the effect of irrigation water sources on the productivity and quality of wheat grains.

MATERIALS AND METHODS

Three Experiments were carried out at each of El-Salam canal, Bahr-Hadoos and Bahr El Bakar drains at El- Sharkia governorate during 2005/2006 and 2006/2007 seasons .The Irrigation water sources were:

- 1- El- Salam canal (Agriculture drainage water mixed with fresh Nile water 1:1).
- 2- Bahr –Hadoos (agriculture drainage water).
- 3- Bahr –El-Bakar (Drainage water contaminated with sewage water).

Completely randomized block design with four replicates was used for each experiment. The bread wheat cultivar Sakha 93 was used, plot area was quarter faddan (70 m² long x 15 m² width) .Collected data were as follows:

I - Growth characters and yield components:

Flag Leaf area (cm²), plant height (cm), spike length (cm), number of spikelets / spike, number of grains / spike ,number of spikes/m² weight of kernels /spike ,1000 –Kernel weight(g) and grain yield (ardab/ fed) were estimated . All Data were statistically analyzed according to Steel and Torrie (1960)and differences among treatment means were compared using the Least Significant Differences (L.S.D) at 5% .

II- Laboratory test:

Laboratory tests were conducted at Seed Technology Research Department ARC.

1-Vigor tests:-

a- Rate of germination: Grains were incubated in moist filter paper at 25°C for 8 days.Normal seedlings were counted according to international rules of I S T A (1993) and expressed as germination percentage. Seedling vigor was assessed by measuring radical length, shoot length and its dry matter.

b- Electrical conductivity: The electrical conductivity of leachate was determined according to procedure described by A.O.S.A (1983). Four sub- samples of 50 seeds from each treatment were weighted and placed into flask with 250 ml of distilled water and held at 25°C. After 25h, the electrical conductivity of leachiest was determined using conductivity meter. The mean values were expressed in µs/cm^g.

The conductivity per gram of seed weight for each sub – sample was calculated by the following formula :

$$\frac{\text{conductivity (} \mu\text{s) for each flask}}{\text{weight (g) of seed sample}} = \mu\text{s cm g}^{-1}$$

2-Grain quality:

Total carbohydrate and crude protein in wheat seeds were determined according to A.O.A.C. (1990). Also, Macronutrients N, P, K, Micronutrients Fe, Zn and Mn, and was estimated according to Soltan Pour and Schwab (1977).

III- Soil characters:-

Soil samples were taken from each experiment at (0 – 30 cm) depth physical and chemical properties of the experimental area are shown in Table (1) Particles size distribution of the experimental soil was determined according to Piper (1950). Total soluble salts (EC), soil pH, calcium carbonate were estimated according to Jackson (1967) . Soluble cations (Ca^{+2} , Mg^{+2} , Na^{+} , and K^{+}) and anions (Cl^{-} , CO_3^{-} , HCO_3^{-} and So_4^{-}), organic matter content and available nitrogen were determined according to Black (1962) . The available K was estimated according to Soltan Pour and Schwab (1977).

Table (1) Physical-chemical properties of soil used for the current study

Experimental plot units		Depth cm	Particulars size distribution (%)				Textural classes	O.M %	CaCO ₃ %
Location	Season planting		C. sand	F. sand	Silt	Clay			
Bahr El-Bakar drain	1 st	0- 30	1.42	38.08	13.80	46.70	Clay	0.58	12.70
	2 nd	0 - 30	2.58	40.12	12.35	44.95	Clay	0.66	12.52
El-Salam canal	1 st	0- 30	1.48	43.54	13.47	41.51	Clay	0.46	8.95
	2 nd	0- 30	1.95	44.73	15.68	37.64	Clay	0.52	8.72
Bahr Hadoos drain	1 st	0-30	1.88	39.52	19.34	39.26	Clay	0.49	10.50
	2 nd	0- 30	1.92	41.32	21.9	34.86	Clay	0.57	10.09

Water irrigation samples were collected from the three sources in the two studied seasons and some of their chemical properties and macro and micronutrients -

elements through four periods during wheat planting in two successive seasons 2005/2006 and 2006 //2007 , the dates were:

- 1: Samples irrigation water taken in November.
- 2: Samples irrigation water taken in January.
- 3: Samples irrigation water taken in March.
- 4: Samples irrigation water taken in April.

Salinity of the water as expressed by EC (dS/m), as well as individual soluble cations (Ca^{+2} , Mg^{+2} , Na^{+} , and K^{+}) and anions (Cl^{-} , CO_3^{-} , HCO_3^{-} and So_4^{-}) were determined following the same methods previously mentioned for soil analysis. Soluble B was determined using the method described by Black (1962). Nitrogen in the form of ammonium nitrate was determined following the methods described by Markus et al (1982). Phosphorus, Potassium and traces were determined using the methods previously mentioned as for soil analysis.

RESULTS AND DISCUSION

Wheat Growth Characters and Yield Components:-

Data in Table (2) show that plant height was significantly affected by the sources of irrigation water, while leaf area, spike length and number of spikeelects /spike were insignificantly affected. The highest values of plant height were 91.83 and 86.16 cm at Bahr-Hadoos and Bahr El- Bakar in the first season, respectively, while plant height in Bahr El-Bakar was only the highest in the second season (91.00 cm).

Table (2). Effect of irrigation water sources on wheat growth of Sakha 93 wheat cultivar. during 2005/2006and 2006/2007 seasons.

Treatments	Leaf area (cm ²)		Plant height (cm)		Spike length (cm)		No. Spikelets/ Spike	
	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd
El-Salam canal	18.80	17.29	65.16	69.19	10.66	10.66	17.00	16.66
Bahr-Hadoos	21.13	17.53	91.83	91.00	12.00	11.00	19.00	18.33
Bahr El Bakar	19.01	17.33	86.16	79.00	11.00	11.00	18.30	17.33
L.S.D 5%	n.s	n.s	6.72	9.86	n.s	n.s	n.s	n.s
C.V	10.18	10.92	3.65	5.45	8.65	4.84	10.95	12.08

Data in Table (3) revealed that grain yield and number of spikes/m² were significantly affected by the sources of irrigation water in the two seasons. El-Salam canal irrigation water produced the highest values of grain yield (13.3 and 13.2 ardab /fed) and number of spikes /m² (273.7 and 298.3) in the two seasons respectively. These results are in the same line with that reported by Mostafa (2001b). Sources of irrigation water had significant effect on number of kernels /spike in the second season. Also using irrigation water from El- salam canal gave the highest values For number and weight of kernels/ spike followed by Bahr Hadoos and than Bahr El Bakar .However insignificant effect on 1000-kernel weight and wieght of kernal / spike was observed.

Results in Table (4) indicate that the three sources of irrigation water had significant effect on EC seed, rate of germination, shoot and radical length but insignificant effect on seedling dry weight.

Table (3) Effect of irrigation water sources on wheat grain yield and yield components during 2005-2006and 2006-2007seasons.

Treatmen ts	No. spikes/m2		No. kernels/spike		1000-kernel weight (g)		Weight of kernels (g)		Grain yield/ ardab/fed	
	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd
El-Salam- canal	273.66	298.33	39.00	44.00	37.71	35.60	0.54	0.66	13.26	13.24
Bahr – Hadoos	216.33	246.66	40.66	41.66	35.43	30.90	0.99	0.64	10.74	10.62
Bahr El- Bakar	250.00	221.66	39.33	33.33	34.40	28.47	0.79	0.55	10.00	9.61
L.S.D 5 %	29.26	48.00	n.s	9.52	n.s	n.s	n.s	ns	1.48	0.36
C.V	5.23	6.60	12.1	10.5	5.55	5.09	7.80	8.80	3.87	2.49

Table (4): Effect of irrigation water sources on seed vigor test during 2005-2006 and 2006 – 2007

Treatments	Electrical conductivity μscmg^{-1}		Rate of Germination %		shoot length (cm)		Radical Length (cm)		seedling dry weight (g)	
	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd
El-Salam canal	15.27	20.00	68.23	73.16	10.46	9.70	8.13	7.41	0.18	0.17
Bahr-Hadoos	27.59	26.20	64.63	64.2	8.60	8.50	5.70	5.43	0.17	0.11
Bahr El-Bakar	18.35	20.21	46.16	53.36	7.57	6.00	5.50	4.60	0.14	0.11
L.S.D 5%	5.02	7.22	7.08	9.50	2.80	1.42	2.43	1.29	n.s	n.s
C.V	3.34	1.03	4.23	4.59	3.93	5.59	1.66	3.60	1.11	2.84

The least value of EC values (15.3 and 20.0 μscmg^{-1}) were obtained when the water from El-salam canal was used to irrigation wheat. The irrigation water of El-salam canal surpassed the other two sources and gave the higher rate of germination (68.3 and 73.2%), shoot length (10.5 and 9.7) and radical length (8.1 and 7.4 cm), in the two seasons, respectively.

Data in Table (5) indicate that crude protein %, carbohydrate and N,P,K were significantly affected by source of irrigation water. The highest values of crude protein (12.90 and 12.60 %) were obtained from Bahr El-Bakar for two seasons, respectively. This result attributed to high nitrogen in sewage water while the least value were (10.30 and 10.63 %) at El-Salam canal. In this connection, Abed El-Kader (2007) found that nitrogen uptake wheat and protein in wheat grains were highly significant affected by the application of sewage water.

The highest total carbohydrate percentage of (%) was obtained from Bahr Hadoos (70.96 and 70.60) while the least value were for Bahr El Bakar (8.88 and 68.00), respectively for both seasons.

The element contents of different crops were affected by irrigation with different water sources. The highest values of N % were (2.23 and 2.16) at Bahr El-Bakar for both seasons respectively, while the least values were obtained by El-Salam canal (1.75 and 1.81) for the two seasons.

The highest values of P % were obtained for Bahr –Hadoos and Bahr El-Bakar (0.64, 0.63 and 0.58, 0.58) for the two seasons respectively. In this respect, Mostafa

(2001a) they revealed that the application of sewage water increased the p concentration in plant.

The highest values of K % 2.43 and 2.32 were recorded at Bahr El-Bakar for the two seasons. While K value decreased at El-Salam and Bahr Hadoos. In this connection, Rehan et al (2002) reported that K concentration in grain was significantly decreased under irrigation with drainage water.

Table (5): Effect of source irrigation water on seed quality and N, P, and K for two seasons (2005-2006 and 2006-2007) :

Treatments	Crude protein %		Carbohydrate %		N %		P %		K %	
Seasons	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd
El-Salam	10.30	10.63	69.73	70.30	1.75	1.81	0.48	0.46	1.70	1.63
Bahr Hadoos	11.60	11.50	70.96	70.60	1.99	2.02	0.64	0.63	1.80	1.76
Bahr El Bakar	12.90	12.60	68.88	68.00	2.23	2.16	0.58	0.58	2.43	2.32
L.S.D 5 %	0.34	0.27	1.05	0.39	0.07	0.09	0.06	0.07	0.25	0.36
C.V	1.31	1.03	0.46	0.25	1.71	2.08	5.08	5.09	5.71	4.55

Data in Table (6) revealed that Fe, Zn and Mn contents in wheat grains were significantly affected by source of irrigation water. The highest values of Fe, Zn and Mn were detected at Bahr El-Bakar and valued 594.33 ,91.23 and 78.23 mg/kg , in the 1st seasons and 592.33 , 96.80 and 79.43 in the 2nd with the same respectively , Mostafa (2001b) reported that Fe, Zn and Mn concentrations in seed of plants grow under soil irrigation with sewage water were greater than those irrigated with Nile water ,or drainage water, in addition long –term irrigation of soil by sewage water markedly increased the amount of Zn in plant.

Table (6): Effect of Source Irrigation Water on some microelements (Fe, Zn and Mn) contents of seed for (2005-2006 and 2006-2007) seasons.

Treatments	Fe (mg/kg)		Zn (mg/kg)		Mn (mg/kg)	
	1 st	2 nd	1 st	2 nd	1 st	2 nd
El-Salam	461.66	460.33	62.33	63.60	65.36	65.36
Bahr –Hadoos	513.33	515.33	75.43	79.93	75.83	75.13
Bahr El-Bakar	594.33	592.33	91.23	96.80	78.23	79.43
L.S.D 5 %	6.58	4.95	13.97	2.10	1.20	0.76
C.V	0.55	0.41	2.93	1.15	0.72	0.46

II- Laboratory test and characters of irrigation water sources:-

Annually, Egypt uses about 88% of its available fresh water resources. This quantity of fresh waters not enough to cover all needs of the agricultural sector. So, a situation has resulted in a national policy for the reuse of drainage water. However in 1988, an area of approximately 10⁶ feddan in the Nile Delta depends on drainage water for irrigation (EL-Hawary, 2003).

Chemical analysis of irrigation water sources:

Data presented in Table (7) reveal that the EC_{iw} values are more affected by both water sources and period of sampling, where the recorded lowest & highest EC_{iw} values, for the different irrigation water resources during the cultivation periods of both wheat crop ranged between 1.74- 2.16 ; 1.95 – 2.34 and 1.27 – 2.42 dS/m in first season and 1.85 – 1.95 ; 1.96 – 2.17 and 1.89 - 2.38 dS/m in second season, for El-Salam canal, Bahr Hadoos drain and Bahr El-Bakar drain, respectively. These values indicated that all the studied irrigation water sources are classified as moderate saline water (0.75 – 3.00 dS/m) according to, Ayers and Westcot, (1985) and Kandiah (1990). Also, the variations in EC_{iw} of different irrigation sources and periods were more related to the lateral seepage from the adjacent saline soils during the leaching process. These results are in harmony with those obtained by Shaban (2005), who found that the EC_{iw} values were increased or decreased according to the seasonal variations for the different cultivated crops.

Table (7) chemical properties in irrigation water sources during the two seasons of 2005 / 2006 and 2006 / 2007

Treatment	Season	Irrigation resources															
		El-Salam canal				Bahr Hadoos drain				Bahr El-Bakar drain				LSD. % 5 season	LSD . % 5 period	LSD . % 5 source	
		Periods of irrigation				Periods of irrigation				Periods of irrigation							
		1	2	3	4	1	2	3	4	1	2	3	4				
pH (1 : 2.5)	1 st	8.23	8.25	8.27	8.19	8.35	8.41	8.45	8.29	8.37	8.24	8.17	8.33	0.055	0.077	0.067	
	2 nd	7.92	7.95	8.04	8.00	8.19	8.25	8.28	8.22	8.15	8.21	8.25	8.23				
EC (dSm ⁻¹)	1 st	1.74	1.84	2.16	2.05	1.95	2.18	2.34	1.95	1.98	2.09	2.42	1.27	0.013	0.018	0.016	
	2 nd	1.85	1.88	1.95	1.90	1.96	2.13	2.17	2.10	1.89	1.95	2.38	2.21				
SAR	1 st	8.04	7.96	8.27	8.90	7.66	7.99	7.93	6.48	7.99	8.93	7.81	7.68	0.340	0.479	0.415	
	2 nd	7.10	6.81	6.83	6.86	7.53	7.70	7.60	7.73	7.54	7.41	9.70	9.25				
Soluble cations and anions (meq/l)																	
Ca ⁺²	1 st	3.24	3.36	4.27	3.31	4.52	4.86	5.10	4.92	3.86	4.12	5.78	5.54	0.131	0.185	0.160	
	2 nd	3.45	3.57	3.76	3.66	3.66	3.94	4.12	3.88	3.80	3.95	4.22	4.10				
Mg ⁺²	1 st	1.44	1.86	2.18	2.10	1.58	1.88	2.30	2.21	1.19	1.48	1.89	1.77	0.177	0.251	0.217	
	2 nd	2.31	2.39	2.81	2.61	2.45	2.76	2.89	2.67	1.75	1.88	1.99	1.76				
Na ⁺	1 st	12.31	12.90	14.88	14.60	13.40	14.70	15.23	12.12	12.70	14.91	15.30	14.66	0.264	0.374	0.324	
	2 nd	11.96	10.25	12.36	12.15	13.17	14.10	14.22	14.00	12.60	12.67	16.98	15.82				
K ⁺	1 st	0.20	0.24	0.28	0.26	0.32	0.38	0.41	0.39	0.31	0.37	0.40	0.38	0.010	0.012	0.011	
	2 nd	0.31	0.37	0.39	0.35	0.41	0.46	0.49	0.44	0.33	0.35	0.40	0.44				
HCO ⁻ ₃	1 st	2.17	2.25	2.36	2.33	3.42	3.61	3.87	3.70	3.33	3.57	3.80	3.70	0.160	0.227	0.196	
	2 nd	3.86	3.93	4.15	4.11	4.30	4.36	4.45	4.28	4.25	4.80	4.35	4.31				
Cl ⁻	1 st	9.61	10.20	10.41	10.35	10.77	10.98	11.35	11.22	10.35	10.78	11.22	11.11	0.239	0.338	0.293	
	2 nd	10.71	10.89	11.00	10.95	11.16	11.25	11.40	11.38	10.77	10.90	11.22	11.10				
SO ⁻ ₄	1 st	5.41	5.91	8.84	7.59	5.63	7.23	7.82	4.72	4.38	6.53	8.35	7.54	0.474	0.670	0.580	
	2 nd	3.46	3.76	4.17	3.71	4.23	5.65	5.87	5.28	3.46	3.67	8.10	6.71				

*(1) Sample taken in November. (2) Sample taken in January. (3) Sample taken in March. (4) Sample taken in April.

Soluble ions in different irrigation water sources

The effects of sampling periods on water soluble ions are presented in Table (7) and showed that , the highest values of cations Ca^{++} , Mg^{++} , Na^+ and K^+ and anions HCO_3^- , Cl^- and SO_4^{--} were recorded in month March for all water sources during the tested two season of wheat cultivation . The soluble ions could be arranged according to their amounts in first season (2005/2006) during wheat planting as shown in the following:

Bahr El-Bakar drain > Bahr Hadoos drain > El-Salam canal for Ca^{++} , Na^+ and HCO_3^- ; Bahr Hadoos drain > El-Salam canal > Bahr El-Bakar drain for Mg^{++} ; Bahr Hadoos drain > Bahr El-Bakar drain > El-Salam canal for Cl^- and El-Salam canal > El-Bakar drain > Bahr Hadoos drain for SO_4^{--} .

As for the second season (2006/2007) during wheat planting the soluble ions could be arranged according to their amounts as shown in the following : Bahr El-Bakar drain > Bahr Hadoos drain > El-Salam canal for Ca^{++} , Na^+ , HCO_3^- and SO_4^{--} . Bahr Hadoos drain > El-Salam canal > Bahr El-Bakar drain for K^+ and Mg^{++} . Bahr Hadoos drain > Bahr El-Bakar drain > El-Salam canal for Cl^- . These results are in harmony with the findings of Bauder et al (2006).

pH irrigation water:

Data presented in Table (7) show that pH values ranged between 8.19 – 8.47 for the first season and 7.92 – 8.25 for the second season at all sampling periods during cultivation of wheat crop. It is clear that pH value of the different irrigation water sources laid a range of moderate level this limitation as described by Ayers and Westcot (1985) and Bauder et al (2006) found that the normal pH range for irrigation water varied from 6.5 to 8.4. Concerning the effect of water sources on SAR values, obtained data in Table (7) reveal that water of Bahr El-Bakar drain have the highest SAR values followed by Bahr Hadoos drain water. The SAR values of the used irrigation water sources lay in the first and second categories, (i.e., 6.0 - 9.70). Thus used all irrigation water varied from are slight to moderate level indicting the possibility of their usage as described by USSl (1954) and Ayers and Westcot (1985) .

Macro elements contents in different irrigation water sources

Nitrate and ammonium – Nitrogen content:-

The concentrations of $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ in the studied different irrigation water sources at various sampling periods during wheat cultivation of the two seasons are presented in Table (8). The data show that the $\text{NH}_4\text{-N}$ content ranged between 12.34 – 18.52 ; 14.89 – 20.14 and 17.35- 21.79 for first season and 11.66 – 11.90 ; 13.49 – 13.89 and 13.32- 13.77 for second season , of El- Salam canal , Bahr Hadoos drain and Bahr El-Bakar drain, respectively . These findings are in agreement with those obtained by Shaban (2005). Whereas, the decrease of nitrate concentrations in most of the drainage water may be due to the reversible conditions that are not favorable for nitrification process as mentioned before. These limited values of nitrate were slightly in all studied irrigation water (5 – 30 mg/l) and listed in the permissible limits of irrigation water, (Ayers and Westcot, 1985).

In addition, the increases in $\text{NH}_4\text{-N}$ values in most drainage water (Bahr El-Bakar and Bahr Hadoos drains) than El-Salam canal water may be due to the un-favorable condition of nitrification. El-Sayed (2001) found that the concentrations of $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ in drainage water during whole year are considered to be higher than its concentration in Nile water by 4 fold. The reasons for the geographical differences in $\text{NH}_4\text{-N}$ content of the irrigation water resources may be primarily due to the different rates of nitrification. These results are in harmony with the findings of Shaban (2005).

Phosphor content:

Excessive phosphorus in irrigation water has not been cause a problem, and no guidelines value is given for its concentration, but evaluation of P in the reclaimed waste water might be valuable for fertilization planning. Data presented in Table (8) show that the P content in different irrigation water during two season of wheat cultivation ranged between 3.87 to 6.10 for first season and 5.20 to 6.43 for second one. The highest value was found in Bahr Hadoos drain water in March month of end season.

Table (8) Macro- Micronutrients content in irrigation water during the two seasons of 2005 / 2006 and 2006 / 2007.

Treatment (mg/l)	Season	Irrigation resources																LSD. % 5 season	LSD. % 5 period	LSD. % 5 source
		El-Salam canal				Bahr Hadoos drain				Bahr El-Bakar drain										
		Periods of irrigation*				Periods of irrigation				Periods of irrigation										
		1	2	3	4	1	2	3	4	1	2	3	4							
NO ₃ -N	1 st	7.21	8.74	8.99	7.58	8.36	9.59	11.4	9.83	10.65	12.45	12.87	11.21	0.49	0.70	0.61				
	2 nd	7.75	7.88	7.95	7.91	8.76	8.91	9.22	9.13	8.62	8.81	8.96	8.98							
NH ₄ -N	1 st	12.34	14.27	18.52	16.75	14.89	18.51	20.14	18.21	17.35	18.59	21.79	19.88	0.47	0.67	0.58				
	2 nd	11.66	11.78	11.90	11.89	13.49	13.58	13.89	13.79	13.32	13.49	13.7	13.71							
P	1 st	4.82	5.4	6.32	5.99	3.87	4.65	5.19	4.96	5.12	5.65	6.10	5.70	0.27	0.38	0.33				
	2 nd	5.21	5.29	5.31	5.28	6.31	6.38	6.43	6.40	5.45	5.48	5.55	5.50							
K	1 st	7.82	9.38	10.95	10.17	12.51	14.86	15.99	15.25	12.12	14.47	15.64	14.82	0.48	0.68	0.59				
	2 nd	12.12	14.47	15.25	13.68	16.03	17.98	19.16	17.16	12.87	13.68	15.60	17.20							
Fe	1 st	1.75	1.79	1.85	1.80	1.91	1.98	1.99	1.95	1.88	1.92	1.94	1.91	1.67	2.36	2.04				
	2 nd	1.85	1.89	1.94	1.91	1.88	1.95	1.99	1.96	1.87	1.89	1.93	1.90							
Mn	1 st	0.36	0.46	0.52	0.49	0.44	0.48	0.56	0.53	0.40	0.47	0.49	0.43	0.006	0.008	0.007				
	2 nd	0.67	0.70	0.78	0.72	0.82	0.90	0.98	0.95	0.79	0.83	0.91	0.87							
Zn	1 st	0.51	0.54	0.60	0.57	0.63	0.66	0.71	0.64	0.55	0.58	0.62	0.59	0.21	0.30	0.25				
	2 nd	1.00	1.12	1.18	1.15	1.41	1.48	1.55	1.50	1.25	1.29	1.33	1.30							

* (1) Sample taken in November. (2) Sample taken in January. (3) Sample taken in March. (4) Sample taken in April.

Potassium content in different irrigation water sources:

Potassium in wastewater is not known to cause adverse affects on plants. It is an essential macronutrient for plants and affects positively on soil fertility as well as crop yields and quality (FAO, 1992). The data in Table (8) show that K content in the different irrigation water ranged between 9.31 to 11.28 for the first season and 7.43 to 7.90 mg/l for the second season during wheat cultivation.

Micronutrient contents in irrigation water sources:

Concentrations of Fe, Mn and Zn, in all water resources used for irrigation are presented in Table (8). Data show that their values ranged between 1.75- 1.99; 0.36 – 0.56 and 0.51 – 0.71 mg/l of first season and 1.85 – 1.99; 0.67- 0.98 and 1.00 – 1.55 mg/l of second season for Fe, Mn and Zn, during wheat planting, respectively. The concentration of Fe, Mn and Zn, in all irrigation water are presented within safe or permissible limits and possible using these water sources for irrigation in the studied soils. The recommended limits for concentration of micronutrients in irrigation water are 5 – 20 mg/l for Fe, 0.2 – 10 mg /l for Mn and 2.0 – 10 mg /l for Zn, (National Academy of Engineering (1972).

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الملخص العربي

تأثير مصادر الري المختلفة على إنتاجية وجودة حبوب محصول القمح

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أقيمت ثلاث تجارب حقلية لموسمين شتويين متتالين 2005/2006 و 2006/2007 بمنطقة جنوب سهل الحسينية بمحافظة الشرقية ومساحتها حوالي 82 ألف فدان تروى بمياه ترعة السلام وبحر حادوس وبحر البقر . 1- ترعة السلام (مياه صرف زراعي مخلوطة بمياه النيل بنسبة 1:1). 2 - بحر حادوس (مياه صرف زراعي) . 3- بحر البقر(صرف صحي).

تهدف الدراسة إلى معرفة تأثير استخدام هذه المياه على إنتاجية وجودة محصول القمح لصنف سخا 93 وما تحتوي هذه المياه من ملوحة وكاتيونات وانيونات والعناصر الكبرى والصغرى . واستخدم التصميم القطاعات العشوائية في أربع مكررات وتم اخذ صفات المحصول ومكوناته بالإضافة إلى الصفات التكنولوجية الخاصة للحبوب (نسبة الإنبات - EC للبذرة - بروتين - كربوهيدرات - العناصر الكبرى وبعض العناصر الصغرى (حديد-زنك ومنجنيز). كما تم اخذ عينات مياه من كل مصدر من المصادر الثلاثة على أربعة تواريخ مختلفة من ميعاد الزراعة وتم تحليلها .

وأوضحت النتائج مايلى:

زيادة محصول الحبوب في المنطقة المروية بمياه الصرف الزراعي المخلوطة بمياه النيل بالمقارنة بالمياه الأخرى كما زادت نسبة البروتين في الحبوب المروية بمياه الصرف الصحي نتيجة لارتفاع نسبة النترات في هذه المياه كما زادت أيضا نسبة العناصر الصغرى في الحبوب المروية بمياه الصرف الصحي ولكن كانت هذه الزيادة في الحدود المسموح بها.

كما أوضحت النتائج انخفاض نسبة الإنبات في الحبوب خاصة المروية بمياه الصرف الصحي مما لة تأثير كبير على المحصول الناتج حيث كانت نسبة الإنبات 68 % ؛ 64 % ؛ 46 % في الموسم الأول بينما كانت 73 % ؛ 64 % ؛ 53 % للحبوب المروية بمياه ترعة السلام ؛ بحر حادوس ؛ بحر البقر على التوالي. مما يوصى بعدم استخدام الحبوب الناتجة كتقاوي للزراعة نتيجة لانخفاض الحيوية.

مياه الري:

تراوحت قيم الملوحة المياه الري EC ملليموز /سم 1.74 – 2.16 ؛ 1.95 – 2.34 ؛ 1.27 – 2.42 في الموسم الأول لمياه ترعة السلام؛ بحر حادوس ؛ وبحر البقر على التوالي . وقد تراوحت قيم pH في مياه الري 8.40 – 8.14 في الموسم الأول و 7.92 – 8.25 في الموسم الثاني على التوالي .

أظهرت النتائج أن قيم النترات تراوحت من حوالي 7.21 – 8.99 ؛ 8.36 – 11.4 ؛ 10.65 – 12.8 بينما تراوحت قيم الامونيوم من 12.39 – 18.52 ؛ 14.89 – 20.14 ؛ 17.35 – 21.79 لمياه ترعة السلام ؛ بحر حادوس ؛ بحر البقر على التوالي.

أوضحت النتائج اختلافا واضحا في قيم العناصر الصغرى لمياه بؤعة السلام و بحر حادوس وبحر البقر حيث تراوح تركيز عنصر الحديد من 1.75 - 1.94 ؛ 1.95 – 1.99 ؛ 1.88 – 1.94 ملليجرام /لتر ؛ بينما تراوح تركيز عنصر المنجنيز من 0.36 – 0.78 ؛ 0.44 – 0.98 ؛ 0.40 – 0.87 ملليجرام / للتر في حين راوح عنصر الزنك من 0.51 – 1.18 ؛ -0.63 - 1.55 ؛ 0.55 – 1.33 ملليجرام / للتر في مياه ترعة السلام و بحر حادوس و بحر البقر على التوالي ومن الجدير بالذكر أن هذه التركيزات مسموح بها ولم تصل إلى حد السمية أو التلوث .

SENSITIVE SPECTROPHOTOMETRIC DETERMINATION OF CARBARYL IN ITS FORMULATIONS AND ENVIRONMENTAL SAMPLES

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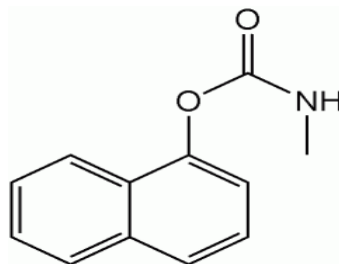
ABSTRACT

A simple, rapid, selective and sensitive spectrophotometric procedure has been established for the quantitation of carbaryl in insecticidal formulations and environmental samples. The procedure was based on acid and alkaline hydrolysis of the carbaryl pesticide, and the resultant hydrolysis product of carbaryl was coupled with diazotized 4-methylaniline (*p*-toluedine) to give an orange color product with $\lambda_{\max}$ of 476 nm, 4-nitroaniline (*p*-nitroaniline) to produce orange red colored product with $\lambda_{\max}$ of 485 nm and with aniline to form yellow colored product has a $\lambda_{\max}$ of 411 nm. The formation of colored derivatives with the coupling agents is instantaneous and stable for 24, 30, and 48 h. Under optimal conditions, Beer's law ranges were found to be 0.2–4.0 $\mu\text{g mL}^{-1}$, 0.2–3.0 $\mu\text{g mL}^{-1}$ and 0.5–5.0 $\mu\text{g mL}^{-1}$ for *p*-toluedine, *p*-nitroaniline and aniline, respectively. The molar absorptivity and Sandell's sensitivity of the color systems were calculated. The effect of the non-target species on the determination of carbaryl was studied to enhance the selectivity of the proposed methods. Performance of the proposed methods were compared statistically in terms Student's *f* and *t*-tests with the reported methods. The proposed methods have been found to be applicable to the determination of carbaryl in its formulations, tap and distilled water, grains, polluted water samples.

INTRODUCTION

Carbaryl (1-Naphthyl -N-Methyl carbamate) is a broad-spectrum pesticide in the carbamate family (**Scheme 1**), extensively used as contact and systemic insecticide on agricultural products, introduced in the year 1957. Even though it is a cholinesterase inhibitor, it has been accepted to have low mammalian toxicity. This type of concept is due to upon chemical hydrolysis and biodegradation carbaryl producing metabolites, among which 1-Naphthol is a major product which does not accumulate in the body, but it excretes in urine and faeces. Despite these merits, recent data shows that some adverse effects have also been reported. It includes, alteration of liver microsomal enzymes ¹, sub chronic neurotoxicity ², changes on immunological function in in-vitro culture ³ and the

combination of cyano-bacteria with Carbaryl and 1-Naphthol showing more toxicity when compared to cyano- bacteria alone ⁴. Carbaryl, a toxic pesticide for human systems finds a wide applicability to control a number of pests of various crops like paddy, sugar and potato. It is, therefore, inevitable to find a simple, sensitive and reliable technique to analyse the residues of these pesticides in air, water, food grains and in other related environments.



Scheme 1. Chemical Structure of Carbaryl

A detailed survey of literature reveals few methods including chromatographic ⁵⁻⁸, immunoassay ⁹⁻¹⁰ and Spectrophotometric methods ¹¹⁻²⁴ are reported using various coupling reagents in their formulations, air, water and food grains. But these reported methods are having their own demerits; example HPLC methods are very expensive due to high cost of solvents and other spectrophotometric methods are of low sensitivity and multiple steps involved.

The present paper explains a simple procedure by coupling known amounts of carbaryl pesticide with diazotised reagents, namely, 4-methylaniline (*p*-toluedine), 4-nitroaniline (*p*-nitroaniline) and aniline compounds and study their optical characteristics using spectrophotometric methods for standardisation. The qualitative and quantitative estimation of these pesticides in their formulations, fortified water (both tap and distilled water) and food grains (wheat and rice) were determined using their spectral data. This forms an additional advantageous spectrophotometric method to analyse these pesticides in various environments.

Experimental

Apparatus

All absorption spectra were made using Kontron 930 (UV-Visible) spectrophotometer (German) with a scanning speed of 200 nm/min and a band width of 2.0 nm, equipped with 10 mm matched quartz cells. Hanna pH meter (USA) was used for checking the pH values of buffer solutions.

Reagents and materials

The analytical grade sample of Carbaryl pesticide (99.9%) was obtained from Bayer company. Pesticide grade methanol and acetone were obtained from Sigma, (USA) were purified and employed for the present study. Sodium nitrite, Potassium hydroxide, Sulphamic acid, were from BDH, AnalR. Double distilled water was used throughout the experiment.

A stock solution of carbaryl ($1000 \mu\text{g mL}^{-1}$) was prepared by dissolving 100 mg of carbaryl in acetone in 100 ml calibrated flask and diluted with acetone. 5 M KOH solution was prepared in methanol. Sodium nitrate 1.0% solution in water, 2% Sulphamic acid was prepared in water.

The coupling agents for the present study were prepared as follows:

A (0.2%) solution of 4-methylaniline (*p*-toluedine), 4-nitroaniline (*p*-nitroaniline) and aniline (Merk, Germany) were prepared by dissolving (0.2 g) of *p*-toluedine, *p*-nitroaniline and aniline in 100 ml of distilled water.

The complexes of carbaryl were prepared by azo coupling reaction technique. Carbaryl samples were hydrolysed using 2% aqueous NaOH to yield naphthol. Diazonium salts of 4-methylaniline (*p*-toluedine), 4-nitroaniline (*p*-nitroaniline) and aniline were prepared using 0.3% NaNO_2 and 1N HCl for coupling reaction.

The pH of the complexes was maintained between 11 and 12. The spectra for the above complexes were recorded in the UV- Vis regions and were reported in the [Table 1](#). Beer's law was obeyed over the concentration range from 0.2 to $12 \mu\text{g mL}^{-1}$ in these complexes. This data was used for the analysis of the sample for estimation.

General procedures

An aliquot of the standard solution of carbaryl containing 2.0-140 μg of carbaryl was transferred into a series of 10 ml of calibrated flasks, 5 ml of 5M KOH solution was added to each flasks and allowed to stand for 5 min for complete hydrolysis, followed by the addition of 2ml of 0.2% 4-methylaniline (*p*-toluedine), 4-nitroaniline (*p*-nitroaniline) or aniline, 2 ml of 1.0 % sodium nitrate and 2 ml of 2% Sulphamic acid and kept aside for 2 min to complete the reaction with occasional shaking. Coloured azo dyes were formed. Dilution was made with water up to the mark and the absorbance was recorded at λ_{max} of 476 nm with *p*-toluedine, 485 nm with *p*-nitroaniline and 411 nm with aniline against the corresponding reagent blank and calibration graph was constructed.

Application for determination of carbaryl in its formulations

Carbaryl in 5 and 50% formulations were analysed using the aforesaid procedure by coupling diazotised compounds of 4-methylaniline (*p*-toluedine), 4-nitroaniline (*p*-nitroaniline) and aniline following Sastry and Vijaya [17] and Sastry et al [20] and is presented in the Table 2.

Application for determination of carbaryl in water samples

The distilled and tap water samples were fortified with the concentrations in the ranges of 0.8-4.8 and 0.4- 2.4 $\mu\text{g mL}^{-1}$ in methanol in the three methods under study which are given in the Tables 3 and 4, respectively. The fortified water samples were extracted with chloroform. The combined extracts were washed with 0.1 M potassium carbonate solution to break any emulsion formed during the extraction and dried over anhydrous sodium sulphate. Finally chloroform was evaporated to dryness on a steam bath and the residue was dissolved in methanol and the amount was determined using the procedures described earlier.

Application for determination of carbaryl in grain samples (wheat)

The grain samples (wheat) of 50 g each were taken in warming blender and blended for 5.0 min with 100 ml of chloroform. The samples were fortified with different concentrations of insecticides in methanol and blended for 3 min. Chloroform was filtered and the residue was retained. The residue was washed twice with 10 ml of chloroform and blended for 2.0 min. The chloroform extracts were evaporated on a steam bath and the

residue was dissolved in methanol and the amount was determined by the procedure outlined earlier.

RESULTS AND DISCUSSION

Spectral characteristics

The proposed method involves the formation of an orange azo dye having $\lambda_{\max}$ of 476 nm with *p*-toluidine, 485 nm with *p*-nitroaniline and 411 nm with aniline. The reagent blank had negligible absorption at this wavelength. The absorption spectrum of the coloured dye and the corresponding reagent blank are given in the Figure 1.

Optimization of the reagent concentration

Various concentration and volume ranges for all the reagents were studied in detail. It was found that 2ml of 0.2% 4-methylaniline (*p*-toluidine), 4-nitroaniline (*p*-nitroaniline) or aniline was adequate for the maximum absorbance, sodium nitrite in the range of 0.5-4.0 ml, sulfamic acid in the range of 0.5-4.0 ml and KOH 2.0- 5.0 ml were necessary to obtain a stable pink colored product with maximum colour intensity.

Quantification

Adherence to Beer's law by the coloured product of diazotized reagents with hydrolyzed carbaryl was determined by measuring the absorbance at appropriate wavelength for a set of solutions containing varying amounts of analyte and specified amounts of reagents against colorless reagent blank. Beer's law was obeyed over the hydrolyzed carbaryl range of 0.2-14 $\mu\text{g mL}^{-1}$, indicating low Sandell's sensitive values. The correlation coefficient values obtained for these three complexes were very close to unity suggesting that the absorbance depends upon the concentration of the insecticides. The values obtained for the relative standard deviation and percentage error suggest that these new procedures offer a good precision and accuracy.

The detection limit (LOD) and quantification (LOQ) for the proposed methods were calculated using the following equation ²⁵:

$$\text{LOD} = 3 \sigma / k \quad \text{and} \quad \text{LOQ} = 10 \sigma / k$$

Where σ is the standard deviation of the blank with respect to water and k is the slope of the calibration curve. Naturally, the LOQ slightly crosses the lower limit of the

Beer's law range. But LOD is well below the lower limit of the Beer's law range. The upper limit of the Beer-Lambert range is determined by a plot of absorbance against concentration at the value of $\lambda_{\max}$. Beyond this limit, the correlation results were really affected. Hence, the measurements were excluded above these limits to keep the relationship linear. The optical characteristics and precision data are given in Table 1.

Precision and Accuracy

The precision of the proposed methods was ascertained from the absorbance values of five replicates. The percentage relative standard deviation was calculated for the proposed method. The accuracy of the method was confirmed by recovery methods.

Proposed reaction mechanism

Carbaryl undergoes an alkali hydrolysis in the presence of alcoholic KOH to give α -Naphthol, which coupled with Diazotized reagents to give water soluble orange azo dye having maximum absorption at 476 nm with *p*-toluidine, 485 nm with *p*-nitroaniline and 411 nm with aniline. Tentative reaction mechanism is shown in Scheme1.

Effect of temperature and colour stability

Under the optimized condition, 5.0 min was given for complete colour development. The intensity of the colour developed was stable at the temperature range from 20°C to 70 °C and up to 48 h at room temperature.

Effect of Foreign ions

Under the diazotized condition reactions used, other pesticides and other common expedients which are present in water were studied thoroughly. It is significant to note that various common ions did not interfere even when added in excess to a known amount of hydrolyzed carbaryl. Table 2 shows the results of interference on the determination of carbaryl. An error of 2 % in the absorbance reading was considered tolerable.

Recovery of the carbaryl residues from spiked water samples

Water samples (100 ml) spiked with known amount of carbaryl ($\mu\text{g mL}^{-1}$) is added, the pH of these samples adjusted to 3-4 with 2.0M Sulphuric acid. The mixture was transferred into a separating funnel and insecticide was extracted using approximately 15 ml of Chloroform in each occasion till complete extraction. The combined extracts were washed with 2.0 ml of 0.1M potassium Carbonate to break any emulsion formed during the

extraction. The extracted insecticide was dried over anhydrous sodium sulphate. Chloroform was evaporated and residue was dissolved in methanol. Determination was carried out by above procedure and data are shown in Table 3.

Recovery of Carbaryl residues from grain samples

Grain samples such as wheat were taken of about 20g each in a warming blender and blended for 15 min with 50 ml of chloroform. These samples were fortified with carbaryl in methanol and again blended for about 5 min. Chloroform was filtered and evaporated on water bath. Thus obtained residue was dissolved in methanol and estimation of carbaryl was carried out by proposed method and results are presented in the Table 4.

Comparison of Other Spectrophotometric methods with proposed method

The data presented in the Table 5 suggests the present method is very sensitive and facile when compare with other officially reported method.

CONCLUSION

The proposed spectrophotometric method is simple to perform, cheaper and sensitive when compared to many other existing methods which are shown in Table 5. The sensitivity, simplicity, temperature independence and stability of the colored product are the advantages of this method. This method can be used to detect $\leq 0.107 \mu\text{g mL}^{-1}$ and quantitatively detect $\leq 0.357 \mu\text{g mL}^{-1}$ of carbaryl and does not involve extraction step and the use of carcinogenic solvents. The proposed method does not entail any stringent experimental variables which affect the reliability of the results. From the recovery studies it comes to know that this method can serve as an alternative method for determining carbaryl residues in various environmental samples.

Table 1. Analytical parameters and optical characteristics of the proposed methods with Carbaryl.

Parameters	<i>p</i> -toluedine	<i>p</i> -nitroaniline	aniline
$\lambda_{\max}$, (nm)	476	485	411
Stability (h)	24	30	48
Beer's law limits ($\mu\text{g mL}^{-1}$)	0.2-4.0	0.2-3.0	0.5-5.0
Ringbom Limits ($\mu\text{g mL}^{-1}$)	0.5-3.7	0.7-2.8	0.7-4.7
Molar absorptivity $\times 10^5$ ($\text{L mol}^{-1} \text{ cm}^{-1}$)	1.645	2.01	1.169
Sandell sensitivity (ng cm^{-2})	1.22	1.00	1.72
Regression equation ^a			
Slope (b)	0.0817	0.0982	0.0644
Intercept (a)	0.0094	0.0034	-0.0098
Correlation coefficient (<i>r</i>)	0.9997	0.9994	0.9995
Detection limits ($\mu\text{g mL}^{-1}$)	0.032	0.045	0.107
Quantification limit ($\mu\text{g mL}^{-1}$)	0.107	0.15	0.357
RSD ^b %	0.856	0.697	1.15

^a $A = a + b C$, where C is the concentration in $\mu\text{g mL}^{-1}$.^b Average of six determinations,

Table 2. Effect of excipients on the determination of carbaryl

Excipients	Amount of Excipients added ($\mu\text{g mL}^{-1}$)	Mean recovery in %
Parathion	5000	99.0
Malathion	3000	99.60
Chloride	200	100.05
Lead (Pb^{+2})	400	99.15
Ca^{2+}	200	99.40
Mg^{2+}	250	99.70
SO_4^{2-}	220	99.10
NO_3^-	150	99.75
CO_3^{2-}	230	99.90

Table 3. Recovery of carbaryl residue from spiked water samples

Water samples	Carbaryl added $\mu\text{g mL}^{-1}$	<i>p</i> -toluedine		<i>p</i> -nitroaniline		Aniline	
		Carbaryl found $\mu\text{g mL}^{-1}$	Mean Recovery in % and $\pm \text{SD}^b$	Carbaryl found $\mu\text{g mL}^{-1}$	Mean Recovery in % and $\pm \text{SD}^b$	Carbaryl found $\mu\text{g mL}^{-1}$	Mean Recovery in % and $\pm \text{SD}^b$
Distilled water (1)	1.5	1.437	95.83	1.433	95.55	1.444	96.25
Distilled water (2)	2.5	2.42	96.80	2.427	97.08	2.428	97.10
River water (1)	1.0	0.9495	94.95	0.9645	96.45	0.9745	97.45
River water (2)	2.0	1.95	97.50	1.941	97.05	1.903	95.15
Tap water (1)	1.5	1.45	96.67	1.439	95.90	1.423	94.90
Tap water (2)	3.0	2.837	94.57	2.873	95.75	2.841	94.70

^b n = 5

Table 4. Recovery of Carbaryl residues from wheat grain samples

Samples	Carbaryl added $\mu\text{g mL}^{-1}$	<i>p</i> -toluedine	<i>p</i> -nitroaniline	Aniline
		Mean Recovery in % $\pm$ SD ^b	Mean Recovery in % $\pm$ SD ^b	Mean Recovery in % $\pm$ SD ^b
1	0.5	95.12	95.75	98.85
2	1.0	97.91	94.00	96.82
3	1.5	96.00	95.00	94.90
4	2.0	96.67	96.50	95.80
5	2.5	97.50	95.41	97.25
6	3.0	97.01	96.87	96.55
Average	-	96.70	95.58	96.70

^b n = 5

Table 5. Comparison of Other Spectrophotometric methods with proposed method

Methods	λ_{max} , nm	Beer's law range $\mu\text{g mL}^{-1}$	Molar absorptivity. $\text{l mol}^{-1} \text{cm}^{-1}$	Remarks	Reference
P-Dimethylphenylene Diamine dihydrochloride	600	0.7-8.0	1.41×10^4	Reagent itself toxic	(15)
P- Aminophenol	600	0.8-10	1.33×10^4	Not much sensitive	(15)
P – Aminobenzoic Acid	470	0.1- 0.8	2.00×10^4	Extraction is necessary Dye stability up to 24 h	(16)
2- Amino 4- Nitrophenol	520	0.4-10	3.018×10^4	Moderately sensitive Dye stable up to 24h	(19)
2,4 - dimethoxy aniline	505	0.4-10	3.42×10^4	Moderately sensitive Dye stable up to 10h	(19)
4-4'-diaminodiphenylsulfone	540	0.06-2.0	7.014×10^4	Highly sensitive Dye stable up to 48 h	(23)
<i>p</i> -toluedine	476	0.2-4.0	1.645×10^5	Highly sensitive Dye stable up to 26 h	Present methods
<i>p</i> -nitroaniline	485	0.2-3.0	2.01×10^5	Highly sensitive Dye stable up to 30 h	
Aniline	411	0.5-5.0	1.169×10^5	Highly sensitive Dye stable up to 48 h	

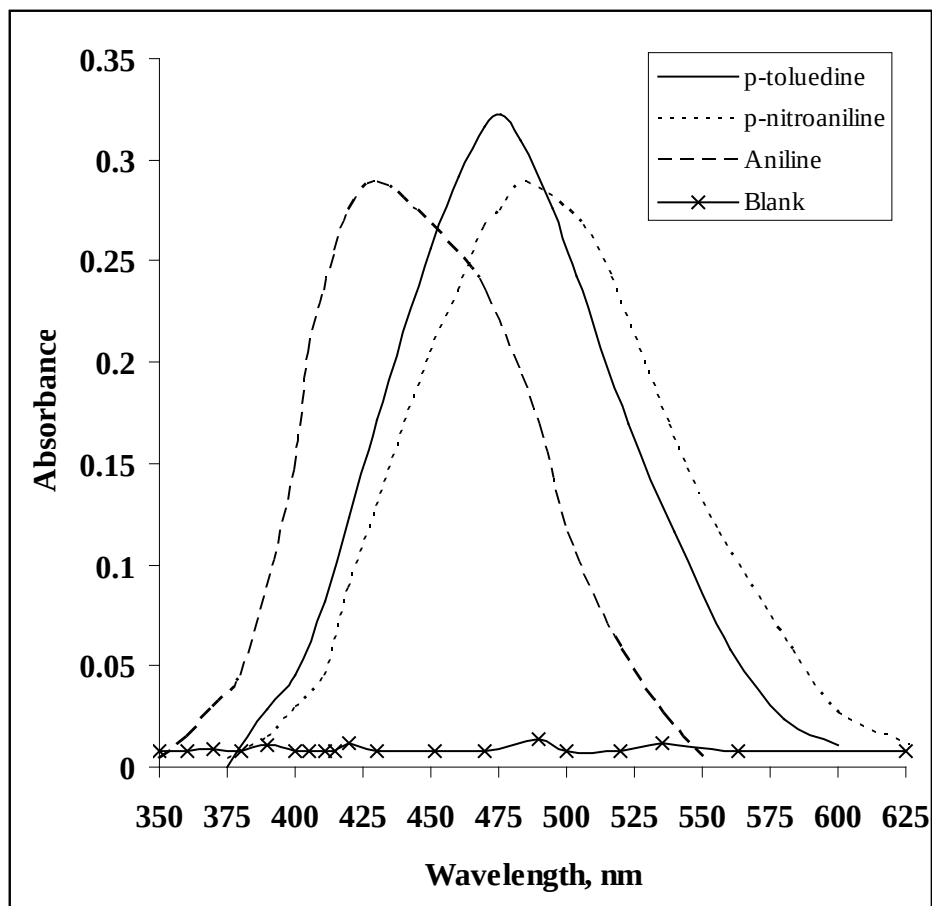
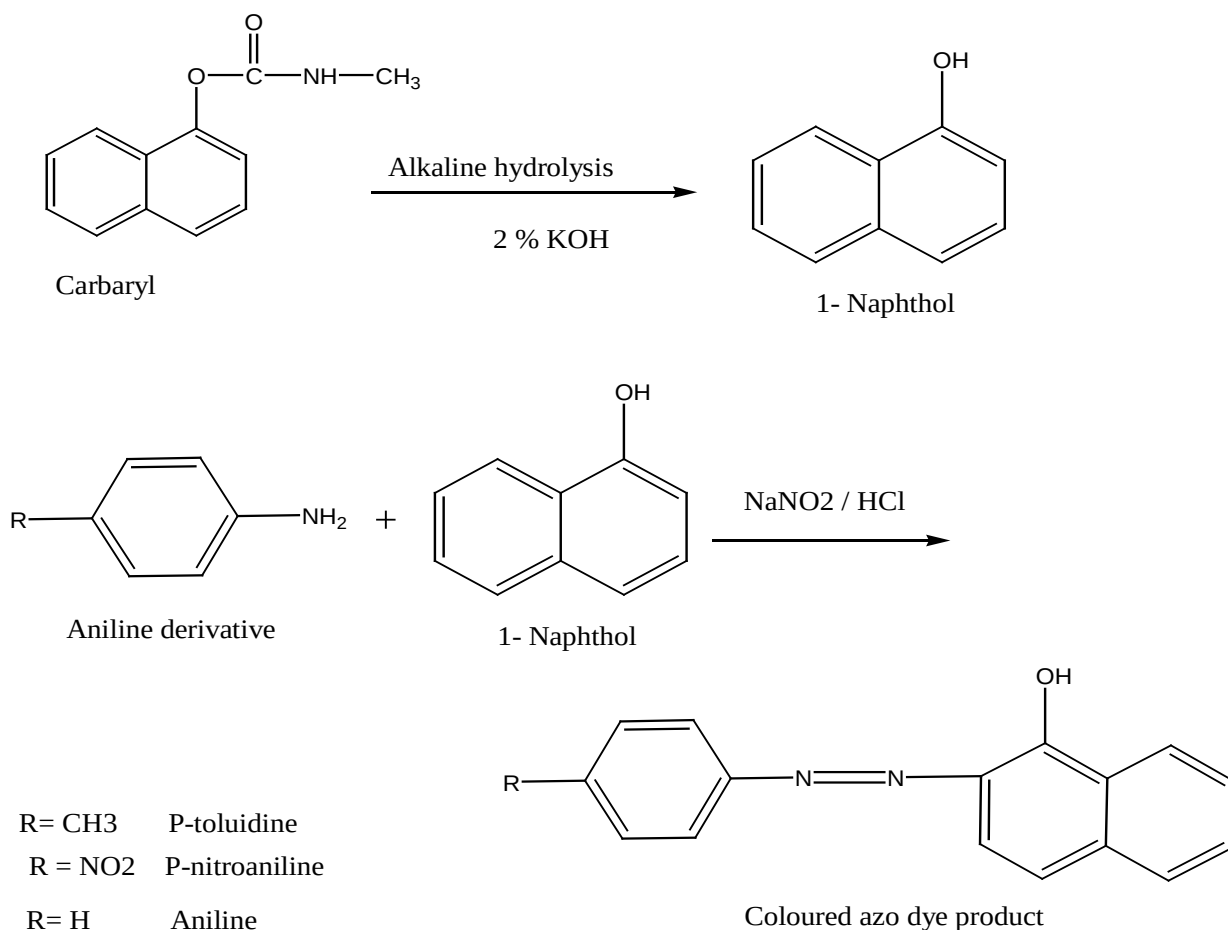


Figure 1: Absorption spectra of the reaction Product of Hydrolyzed Carbaryl and Reagent blank



Scheme II. Colored products of the interactions of carbaryl with the reagents

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SENSITIVITY OF *STAPHYLOCOCCUS AUREUS*, SP.13 TO SEPARATE AND COMBINED ACTION OF CEPHRADIN, AMPICILLIN AND *NIGELLA SATIVA* OIL

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ABSTRACT

Three different clinical specimens were tested through this research; stool (st.), urine (ur.) and sputum(sp.) these samples which five *Salmonella typhi*, 18 *Escherichia coli* and 15 *Staphylococcus aureus* were isolated obtained from Laboratory of Public Hospitals of Zagazig University . All pathogenic strains were tested for their susceptibility to 9 different antibiotics for each strain and 5 natural oil using disc agar and well agar diffusion methods respectively. Both *S. typhi* ,st.5 and *Staph. aureus* ,sp.13 strains were resistant to Cephradine (Ce) and sensitive to Ampicillin (Am) . MICs of Ce against *S. typhi*, st.5 and *Staph. aureus* , sp.13 were 900 and 1000 ug/ml. respectively , while MICs of (Am) against each strains were 500 and 600 ug/ml respectively . The isolates were tested for their susceptibility to 5 natural oils of *Thymus vulgaris* L., *Matricaria recutita*, *Nigella sativa*, *Allium sativum* and *Citrus limonum* .All tested Gram –ve pathogenic isolates *S. typhi* and *E. coli* were resistant to tested oils, while the Gram +ve *Staph. aureus* isolates were susceptible. MBCs of *Nigella sativa* oil against *Staph. aureus* sp.13 was achieved at 40% .The lethal concentrations of combination treatments against *Staph. aureus* sp.13 were 825 ug/ml Ce +20% *N. sativa* oil and 525 ug/ml Am + 4% *N. sativa* oil . Moreover 37°C, 48hrs at 7 pH were the optimum growth conditions, affecting the susceptibility of *Staph. aureus* sp.13 for separate and combined Ce , Am and *N. sativa* oil treatments. Regarding to plasmid profile analysis, *Staph. aureus* sp.13 was plasmidless for separate and combined action of Ce ,Am and *N. sativa* oil.

INTRODUCTION

Salmonella and *Campylobacter* spp. were the most important causes of bacterial gastroenteritis in humans (Tauxe, 2002) .While cases of hemorrhagic colitis (HC) and hemolytic-uremic syndrome(HUS)were due to infections with enterohemorrhagic *Escherichia coli* (EHEC). While numerous outbreaks had been attributed to EHEC strains of serotype O157:H7, other serotype also played an important role in human disease (Goldwater and Bettelheim ,1998) *Staph. aureus* is widely distributed in nature and is commonly found in the nose, throat, hair and skin of humans and animals . Foods including bakery products containing custards or cream, ham, poultry products, milk and milk products and eggs had frequently been reported to be involved in outbreaks of Staphylococcal intoxication (Hirooka et al., 1987; Umoth et al., 1988; Bergdoll, 1989 and Adesiyun et al., 1992). The production of enterotoxin by *Staph aureus* had been reported to be influenced by various environmental factors such as pH , temperature , and food composition (Jay, 1992)

The character of biological action of antibiotics depended mainly on their chemical nature, concentration, the particular species of organism, the microstructure of its cells, conditions under which the microorganism functions and on other factor (Walker 1998).

There were four major sites in the bacterial cell that were sufficiently different from the human cell that they serve as the basis for the action of clinically effective drugs: cell wall, ribosomes, nucleic acids and cell membrane (**Levinson and Jawetz, 2000**).

Antibacterial susceptibility testing may be performed reliably by either dilution or diffusion methods. Dilution methods were used to determine the MIC of an antimicrobial agent required to kill or inhibit a microorganism. Disk diffusion method allowed categorization of bacterial isolates as susceptible, resistant or intermediate to a variety of antibiotics (**Jorgensen, et al. 1999**).

Resistance of bacteria to antibiotic may be due to direct restriction from physical or chemical characteristics of the environment that either the antibiotic altered the normal physiological response of the organism to the agent (**Murray, et al. 1999**).

Staphylococci had a record of developing resistance quickly and successfully to antimicrobial agents. (**Kloos, 1998**). There were probably three types of β -lactamases in *Staph. aureus*, they were usually inducible and occasionally constitutive, they are usually coded for by plasmids which carry other important genes such as those determining resistance to heavy metals, to erythromycin and other antibiotics. A few staphylococcal strains had reported to harbor chromosomal genes for penicillinase production (**Waldrogl, 1995**).

Different volatile oils were used as antimicrobial agent and antioxidants in foods. **Sivropoulou et al. (1995)**, **Ela et al. (1996)** and **Matasyoh et al., (2006)** reported, the antimicrobial activity of different essential oils to many pathogenic microorganisms.

The antimicrobial activity of *Nigella sativa* oil against *Staph. aureus* was reported by many workers. **El-Kamali et al. (1998)** and **Morsi (2000)** studied the antibacterial activity of this oil against 16 Gram negative and 6 Gram positive bacteria. While **Tariq et al (2005)** studied the effect of *Nigella sativa* oil against multidrug resistant strains of *P. auregenosa*.

The combination of antimicrobial agents were considered to be synergistic if the combination effect was great than the effect either agent alone or greater than the sum of the effect of the individual agents (**Cappelletty and Rybak 1996**). **Zaid (2001)** studied the combination action of anise oil and amoxacillin against *P. auregenosa*, **Abascal and Yarnell (2002)** combined different concentrations of *Camellia sinensis* oil with oxacillin against *Staph. aureus*. While **El-Bouhy (2005)** showed the inhibitory activity of thyme oil alone and combined with ciprofloxacin and methicillin against methicillin resistant *Staph. aureus* (MRSA). Different growth factors as pH, temp., incubation periods may affect on the combination treatment of oils and antibiotics (**Russell et al. 1992 and El-Aidy 2007**).

A total of 22 *Staphylococcus* spp. isolates recovered from clinical sources were studied for antibiotic and heavy metal resistance patterns and plasmid profiles. Plasmid content and profile studies showed that, the tested isolates carried plasmids ranging from 2.224 to 20.650 kb in size. Fifty percent of studied strains harbored plasmids and association between occurrence of plasmids and resistance to antibiotics and heavy metals was observed (**Aysel & Özgür 2003**).

MATERIALS AND METHODS

Isolation of pathogens clinical specimens

Salmonella typhi

Isolated from stool samples on Triple sugar iron (Baron and Finegold, 1990), tetrathionate broth (Monica, 1985), selenite broth (Monica, 1985) and salmonella Shigella agar (Monica, 1985).

Escherichia coli

Isolated from urine samples on MacConkey's agar (Sonnenwirth, 1980).

Staphylococcus aureus Isolated from sputum samples on blood agar and Nutrient agar (Monica, 1985).

Identification of isolated bacteria

The clinical isolates were subjected to patterns of identification according to (Holt *et al.*, 1994 and Murray *et al.*, 1999)

Antibiotic susceptibility test disk diffusion agar method:-

Twelve antibiotics; Vancomycin (VA), Ampicillin (Am), Penicillin(P), Cefotaxim(CTX), Cefradine(Ce), Gentamycin(GM), Chloramphenicol(C), Trimethoprim-(SXT), Tetracycline(TE), Nalidixic acid(NA), Erythromycin(E), Octamycin(OT) and Nitrofurantoin(F) were selected for carrying out the antimicrobial susceptibility test. The antibiotic disks used in this research were purchased from Oxoid. Antibiotic susceptibility test for all isolates was done by disk diffusion technique using commercially available antibiotic disk as recommended by (Bauer *et al.*, 1966)

Determination of Minimum Inhibitory Concentration (MICs) and (MBCs) of selected antibiotics:-

The minimum inhibitory concentration MIC was defined as the lowest concentration of antibiotic that inhibited a visible growth in liquid media according to the antibiotic susceptibility test, MICs of some antibiotics were determined for certain resistant and sensitive bacterial isolates. The minimum bacteriocidal concentration (MBC) was defined as the lowest concentration at which no growth occurs on solid media, were determined for both tested antibiotic and natural volatile oils, (Jacobs *et al.*, 1992).

Natural Oil susceptibility of bacterial isolates:

Well diffusion technique:-

Five plant volatile oils were selected for carrying out the antimicrobial susceptibility test. Different concentrations of natural oils emulsions, were prepared and spotted separately in each well.

V- Combination treatment:-

The effect of combination treatment between selected antibiotics and natural oil on the growth of the tested organisms was carried in this experiment.

Different concentrations of antibiotics were supplemented together to tubes each contained 5 ml nutrient broth medium. These concentrations were 0, 70, 175, 350 and 525 ug/ml of antibiotic Am, 0, 110, 275, 550 and 825 ug/ml of Ce in addition to 0, 4, 10, 20 and 30 % of emulsifying *Nigella sativa* oil

Effect of some cultural condition on the susceptibility of *Staph aureus* to treatment by antibiotics, oil and antibiotic / oil mixture:-

Effect of different concentrations of antibiotics, oil and antibiotic / oil mixtures were on the growth of *Staph. aureus* (sp.13) studied at different temperatures 15, 25, 37, 45 °C for 24 h. different incubation periods 6, 18, 24, 48, 72 hrs. and different pH values 4.6, 5, 5.6, 7, 7.5, 8. (at 37°C for 24h).

Plasmid profile analysis:

The technique of plasmid profile (plasmid finger print) was carried according to (Tompkins, 1985).

EXPERIMENTAL RESULTS

Collection, isolation and purification of certain pathogens from different patients: -

Five *Salmonella typhi* had been isolated from 50 stool samples suffering diarrhea. 18 *E. coli* had been isolated from 50 different cases suffering from pus in urine and 15 *Staph. aureus* had been isolated from sputums of 50 different patients. The clinical isolates were subjected to patterns of identification according to Holt *et al.*, (1994) & Murray *et al.*, (1999).

-Susceptibility of tested pathogenic isolates to different antibiotics:-

The antibiotic susceptibility of 5 strains of *S. typhi*, 18 strains of *E. coli* and 15 strains of *Staph. aureus*, against 9 different antibiotic discs for each strain were summarized as following :-

All isolates of *S. typhi* were completely resistant (100%) to NA, TE, CE and VA, while they were sensitive (100%) to AM, CTX, SXT and C. On the other hand, all isolates of *E. coli* were completely resistant (100%) to AM, SXT and CTX, while they were highly sensitive to NA, GM, and F. Moreover the results explained that, all isolates were resistance to P, OT and TE (100%). In the contrary all isolates were sensitive to SXT and GM. The response of isolates was varied with the antibiotics, AM, CE, VA and E.

Susceptibility of tested pathogenic isolates to different oils:-

The experimental results showed that, all the tested isolates of both *S. typhi* (5 isolates) and *E. coli* (18 isolates) were completely resistant to all tested oils; there were no inhibition zones.

Also all the tested isolates (15) of *Staph aureus* were resistant to oils of *Matricaria recutita*, *Thymus vulgaris* L., and *Allium sativum*, while they were sensitive to *Nigella sativa* and *Citrus limonum* (Risso) oils. *Staph. aureus* (sp.13) was sensitive to *Nigella sativa* oil so, it was selected for further investigations.

MICs of antibiotics and MBCs of *Nigella sativa* oil against *S. typhi* (st.5) and *Staph aureus* (sp.13): -

The results in table (1) revealed that, MICs of cephradine against *S. typhi* and *Staph. aureus* were 900 and 1000 µg/ml respectively, while MICs of ampicillin against both strains were 500 and 600 µg/ml for respectively hence it was more sensitive to ampicillin than cephradine.

On the other hand, the MBCs of cephradine were detected; 1000 and 1100 µg/ml for *S. typhi* st5 and *Staph. aureus* sp13 respectively, which were higher than ampicillin; 600 and 700 µg/ml for both tested strains respectively. The MBC of *Nigella sativa* oil emulsion for *Staph. aureus* was at concentration 40%.

Table (1) MICs of antibiotics and MBCs of *Nigella sativa* oil against *S. typhi* (st.5) and *Staph aureus* (sp.13)

Treatments		<i>S. typhi</i> st.5		<i>Staph. aureus</i> sp.13	
		MICs	MBCs	MICs	MBCs
Antibiotics	Cephradine (Ce)	900 µg/ ml	-	1000 µg/ ml	-
	Ampicillin (Am)	500 µg/ ml	-	600 µg/ ml	-
Natural oil	<i>Nigella sativa</i> oil	-	-	40%	-

Combined treatments of different concentrations of Ce or Am and *Nigella sativa* oil emulsion on *Staph. aureus* sp13: -

The results in tables (2,3) indicated that, the lethal concentrations of combination treatments were 825 µg/ml of Ce combined with 20% *Nigella sativa* oil and 525 µg/ml. of Am combined with 4% *Nigella sativa* oil against *Staph. aureus* sp. 13 strain.

Table (2): Effect of treatment of different concentrations of Cephadrin and *Nigella sativa* oil emulsion on *Staph. aureus*(sp13)

Conc. Of CE ug/ml	Conc. Of oil%	No. of cells/ml	Log N
0	0	60×10^6	7.78
110	4	50×10^3	4.70
	10	20×10^2	3.30
	20	10×10^2	3.00
	30	0.3×10	1.48
275	4	25×10^2	3.40
	10	18×10^2	3.26
	20	8.0×10^2	2.90
	30	2.0×10	1.30
550	4	2.0×10^2	2.30
	10	1.8×10^2	2.26
	20	1.5×10^2	2.18
	30	1.1×10	1.04
825	4	0.6×10^2	1.78
	10	0.3×10^2	1.48
	20	0	--
	30	0	--

Table (3): Effect of treatment of different concentrations of Ampicillin and *Nigella sativa* oil emulsion on *Staph.aureus* (sp.13).

Conc. Of AM ug/ml	Conc. Of oil%	No. of cells/ml	Log N
0	0	60×10^6	7.78
70	4	50×10^4	5.70
	10	21×10^2	3.32
	20	9.0×10^2	2.95
	30	3.8×10	1.58
175	4	60×10^2	3.78
	10	19×10^2	3.28
	20	7.6×10^2	2.88
	30	3.2×10	1.51
350	4	20×10^2	3.30
	10	15×10^2	3.18
	20	5.0×10^2	2.70
	30	2.1×10	1.32
525	4	0	--
	10	0	--
	20	0	--
	30	0	--

Some cultural conditions affecting the sensitivity of “*Staph. aureus* sp. 13” separated and combined treated with Ce ,Am and *Nigella sativa* oil : -

- Effect of different incubation temperatures:-

The present results in fig. (from 1 to 5) revealed that for all combined treatments of Ce,Am and oil the optimum incubation temperature was 37°C for growth of *Staph. aureus* sp.13 but it decreased by increasing the concentration of Ce, Am ,oil and combined treatment.

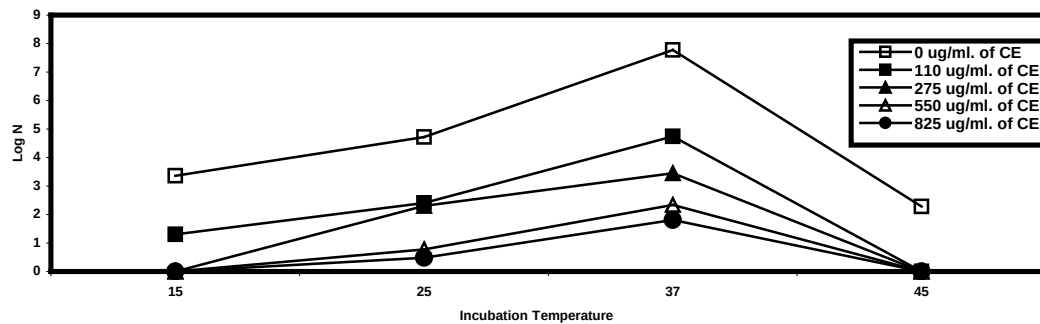


Fig. (1) Effect of different concentration of Cephradine for *Staph. aureus* at different incubation temperature

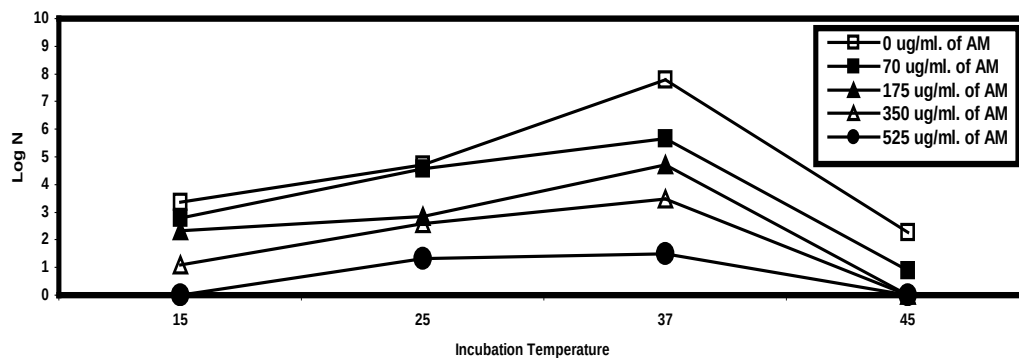


Fig. (2) Effect of different concentration of Ampicillin (ug/ml.) for *Staph. aureus* sp13 at different incubation temperature

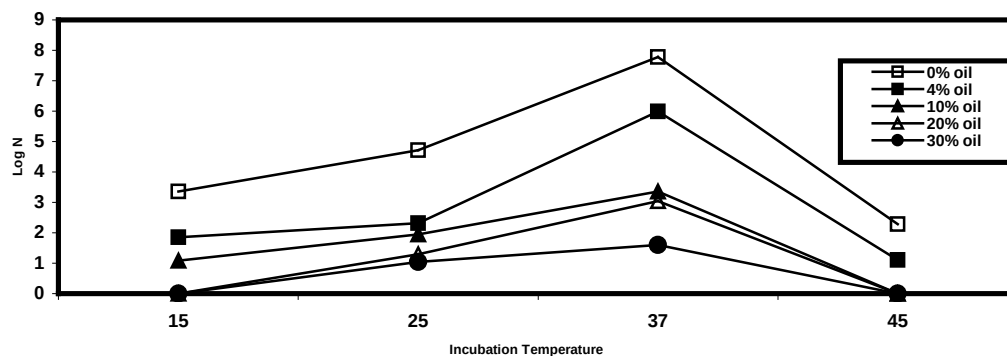
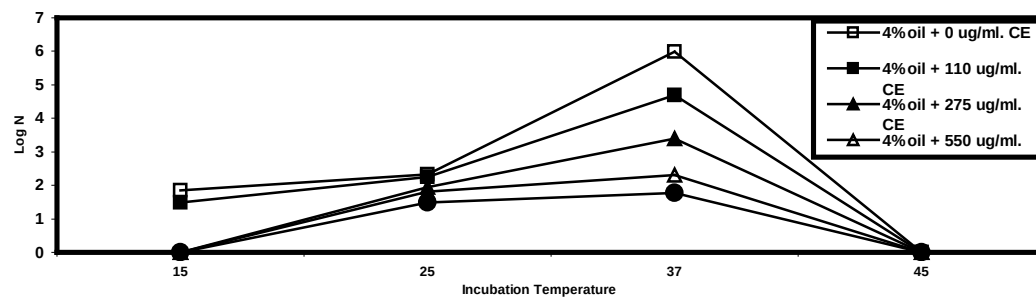
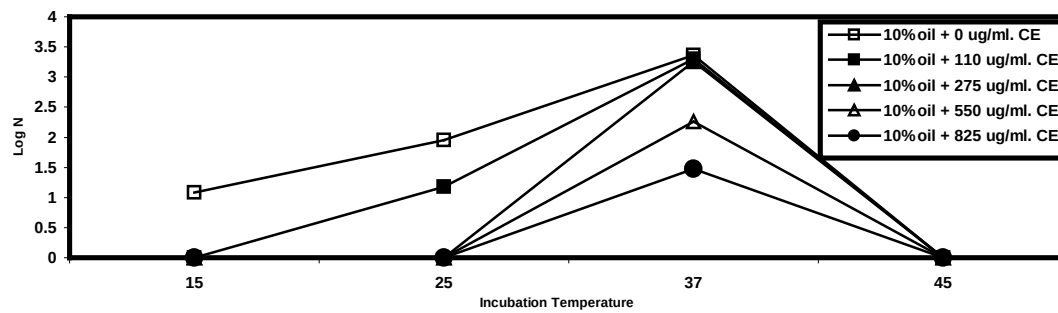


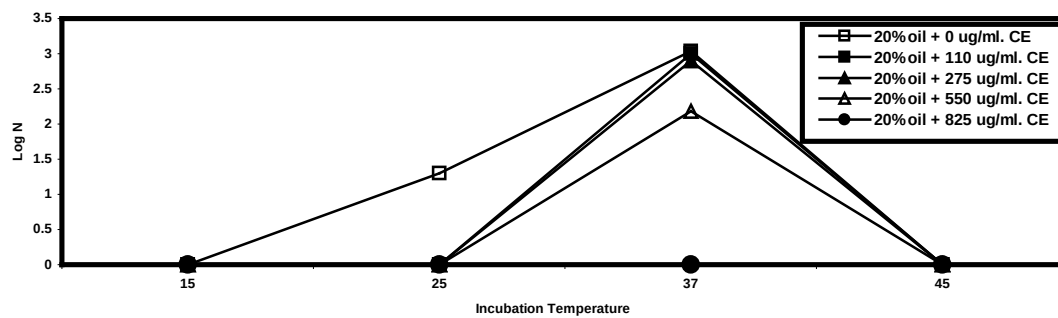
Fig. (3) Effect of different concentration of *Nigella sativa* for *Staph. aureus* at different incubation temperature



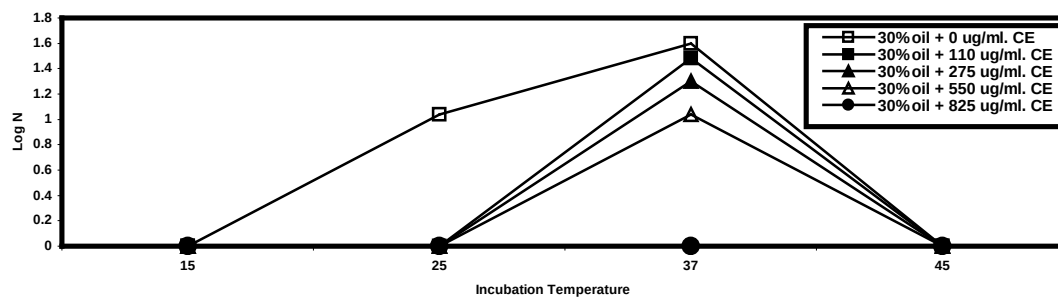
a



b

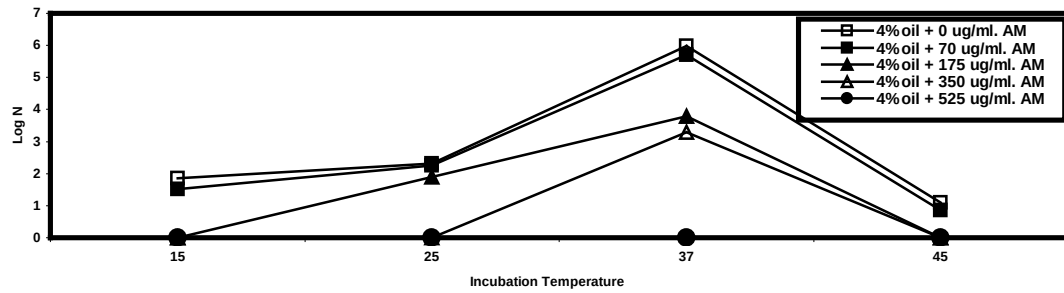


c

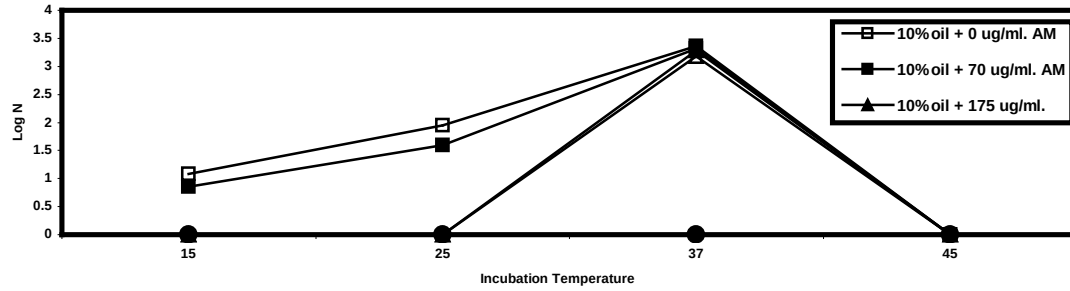


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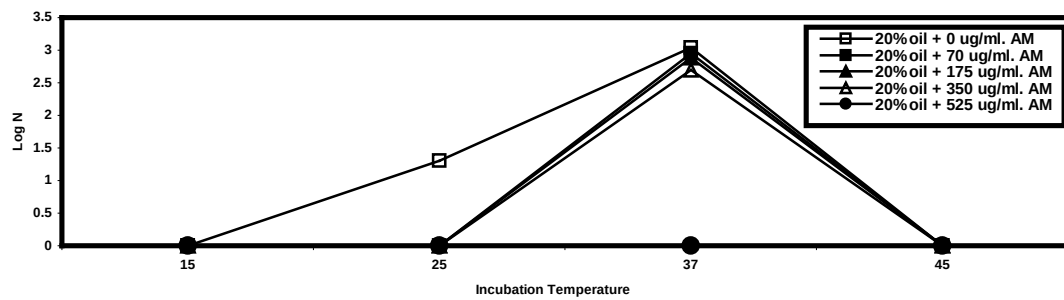
Fig. (4)(a,b,c and d) Effect of different concentration of CE ug/ml. and *Nigella sativa* % on the sensitivity of *Staph. aureus* sp13 at different incubation temperature °C



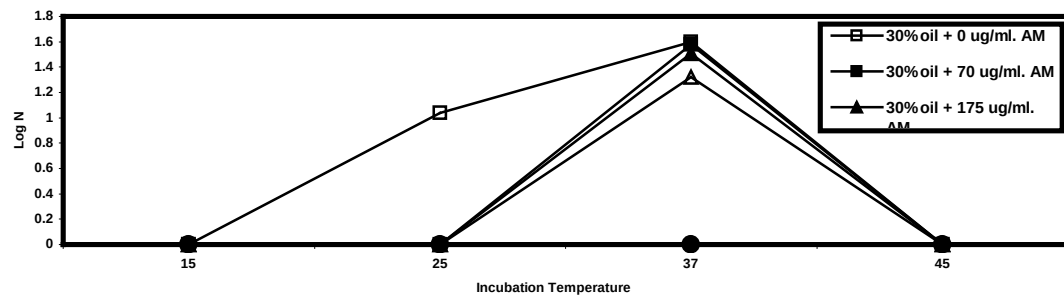
a



b



c



d

Fig. (5) (a,b,c and d)Effect of different concentration of AM and *Nigella sativa* % on the sensivity of *Staph. aureus* sp13 at different incubation temperature°C

- Effect of different incubation periods:-

The results in figs. (from 6 to 10)indicated that, the number of cells increased gradually by increasing incubation period (hrs.) and the maximum count was achieved after 48 hrs. for all treatments, and decreased by increasing

the concentration of Ce, Am , *Nigella sativa* and also the combination treatment.

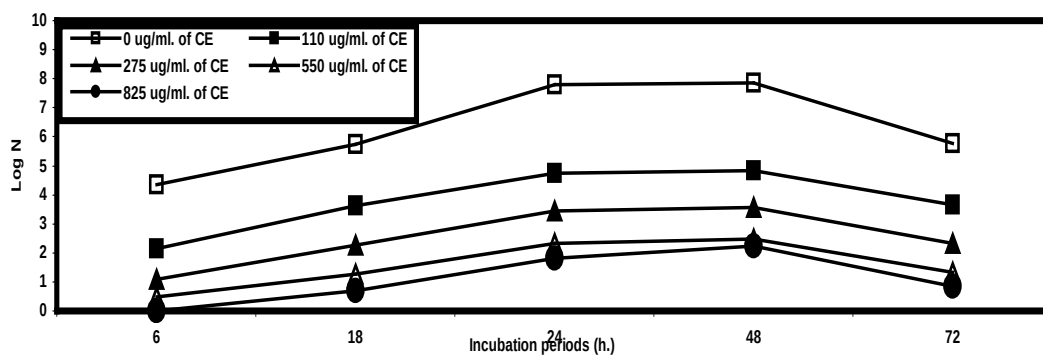


Fig. (6) Effect of different concentration of CE ug/ml. on the sensitivity of *Staph. aureus* at different incubation periods

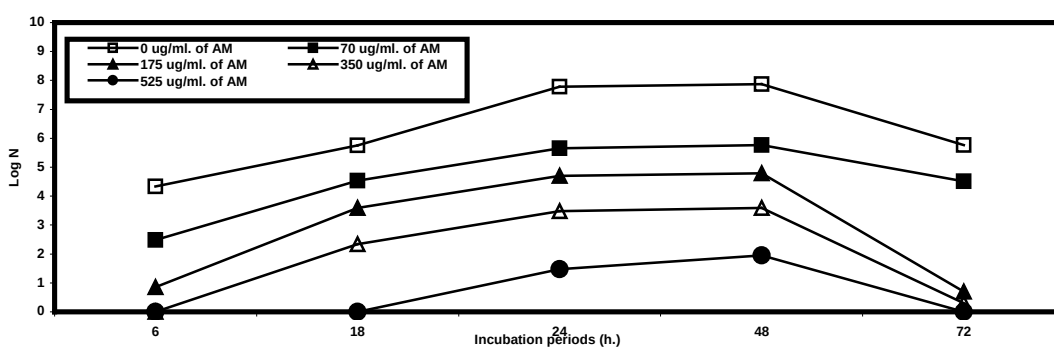


Fig. (7) Effect of different concentration of AM ug/ml on the sensitivity of *Staph. aureus* at different incubation periods

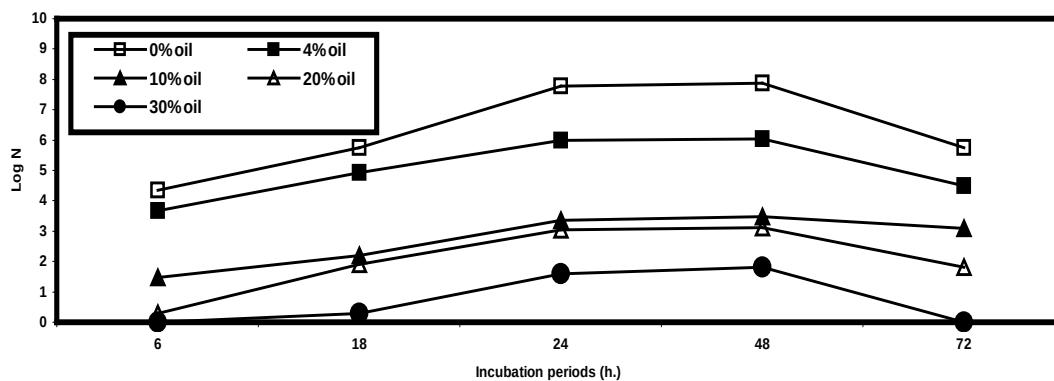
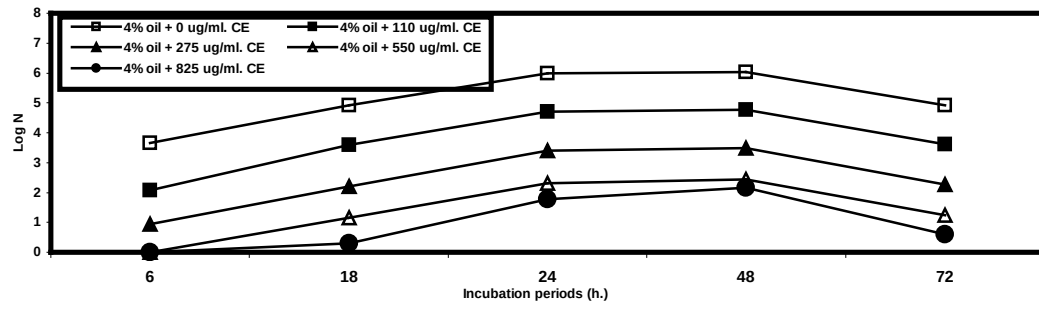
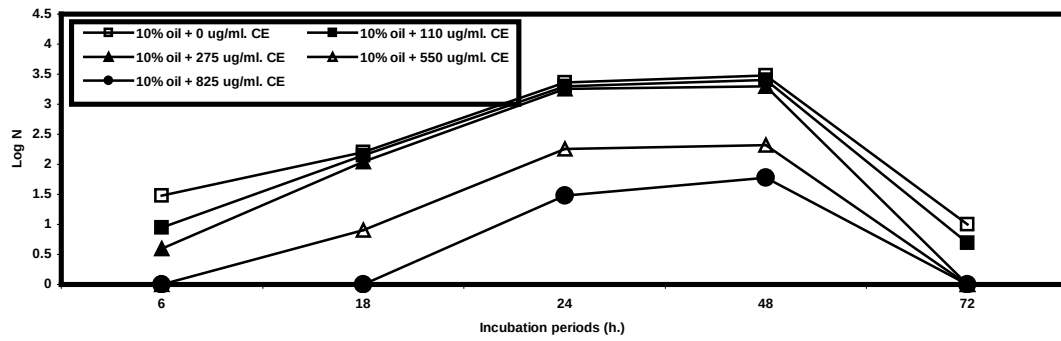


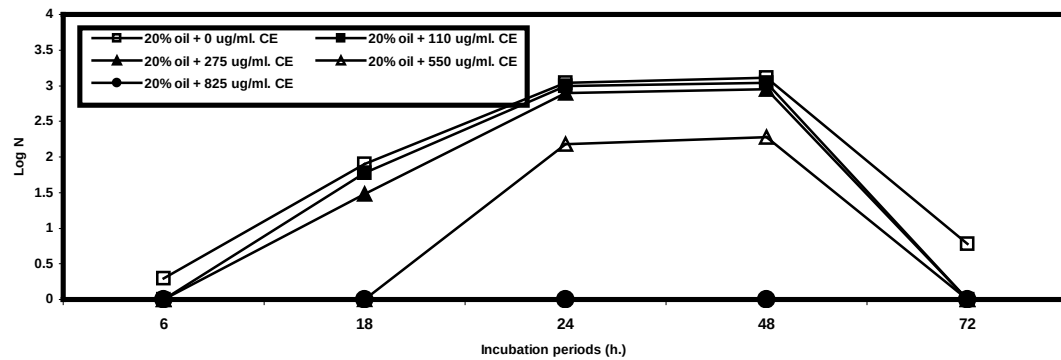
Fig. (8) Effect of different concentration of *Nigella sativa* % on the sensitivity of *Staph. aureus* sp13 at different incubation periods



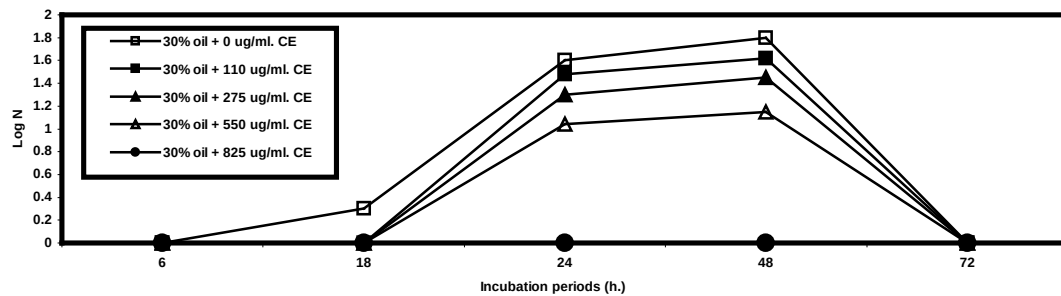
a



b



c



d

Fig (9)(a,b,c and d) Effect of different concentration of CE ug/ml. and *Nigella sativa* % on the sensitivity of *Staph. aureus* sp13 at different incubation periods

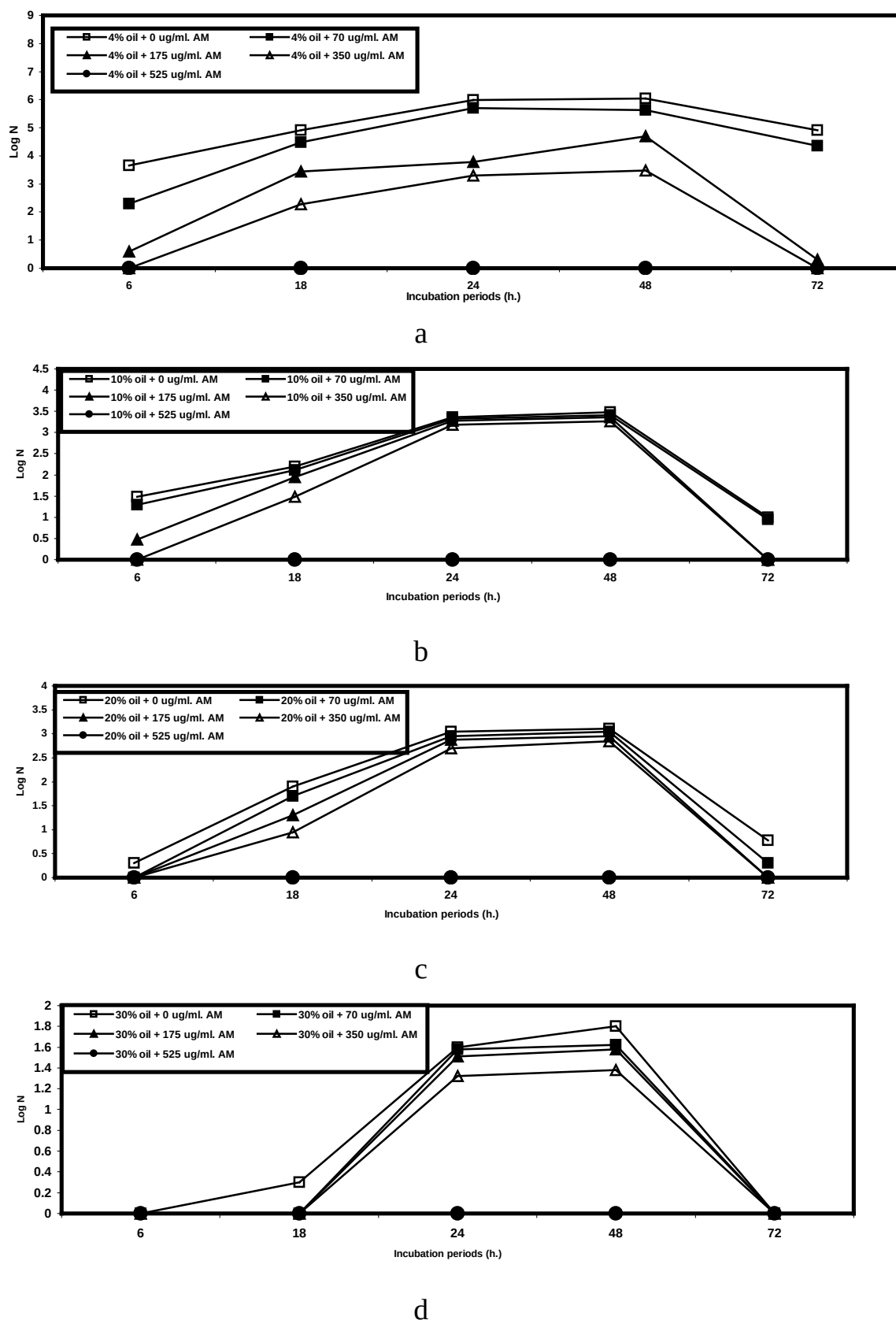


Fig. (10) (a,b,c and d)Effect of the combined action of both AM and *Nigella sativa* on the sensitivity of *Staph. aureus* sp13 at different incubation periods

- Effect of different pH value: -

The results in figs From (11 to 15) revealed that ,the growth of tested

bacteria favor both the neutral pH 7 and also the alkaline side (7.5 , 8 pH) while its growth was weak at the acidic side pH 4.6 , 5 and 5.6 which also act synergistically with Ce , Am , *Nigella sativa* oil and combination treatment concentrations.

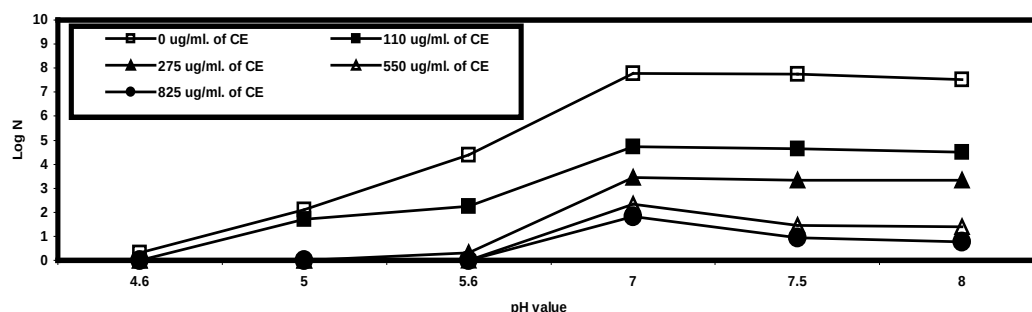


Fig. (11) Effect of different concentrations of CE for *Staph. aureus* sp13 at different pH values

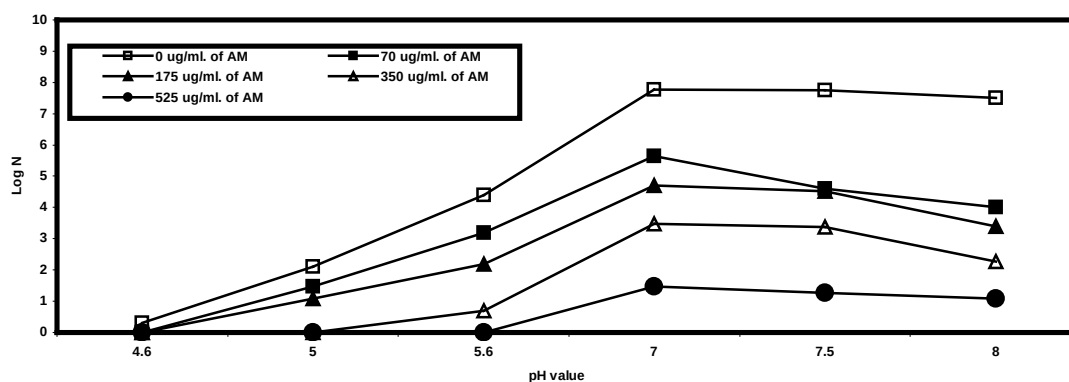


Fig. (12) Effect of different concentrations of AM for *Staph. aureus* sp13 at different pH values

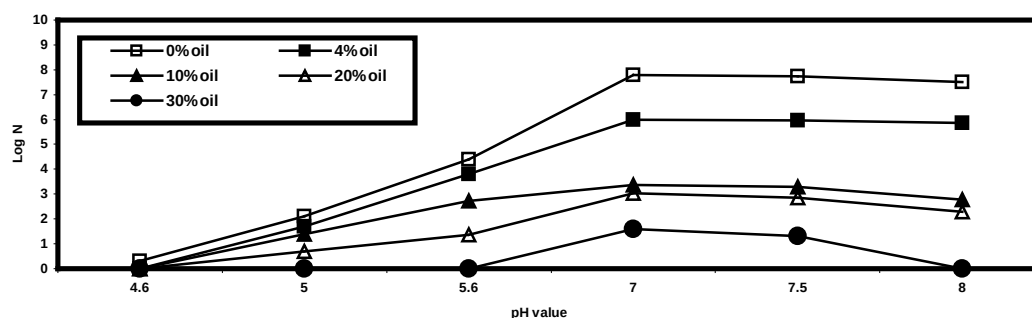
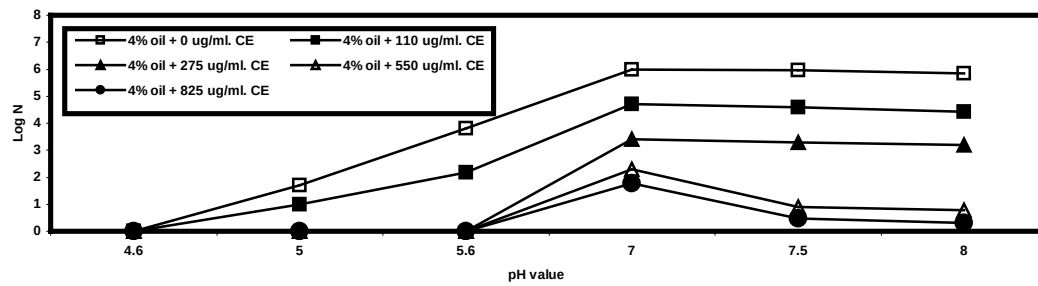
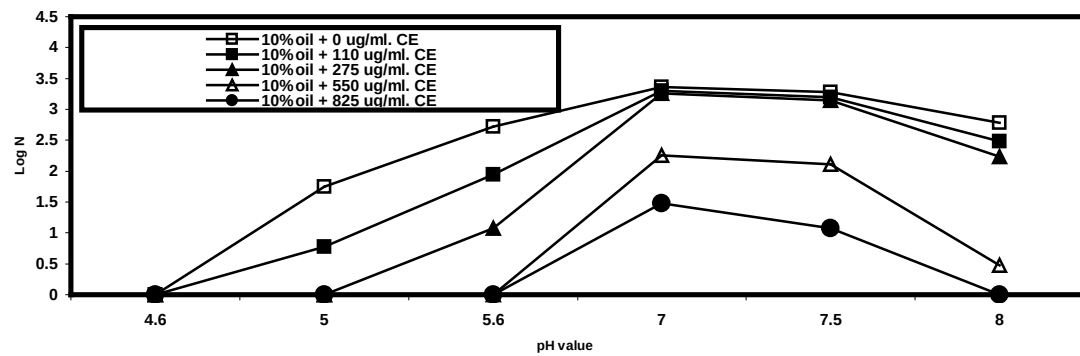


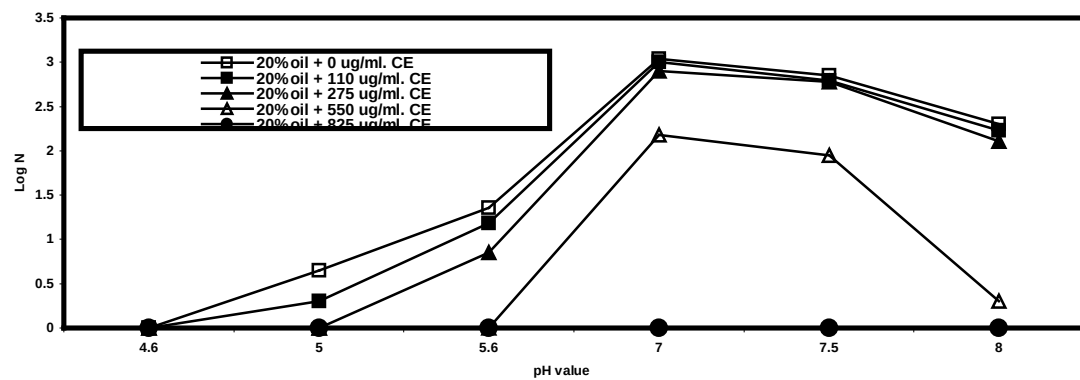
Fig. (13) Effect of different concentrations of *Nigella sativa* for *Staph. aureus* sp13 at different pH values



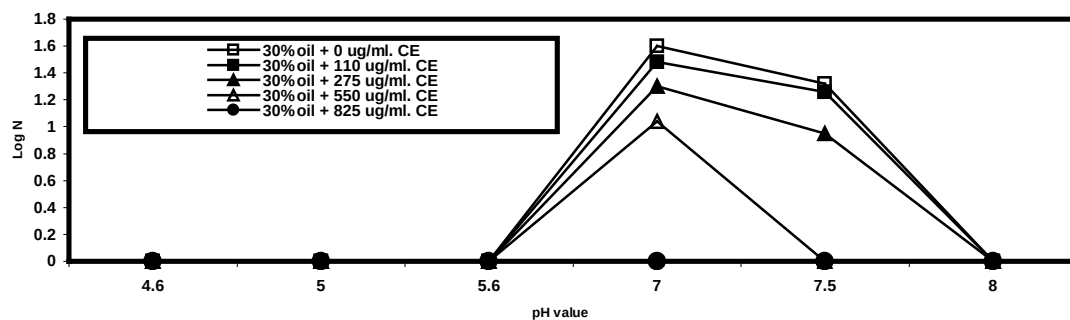
a



b

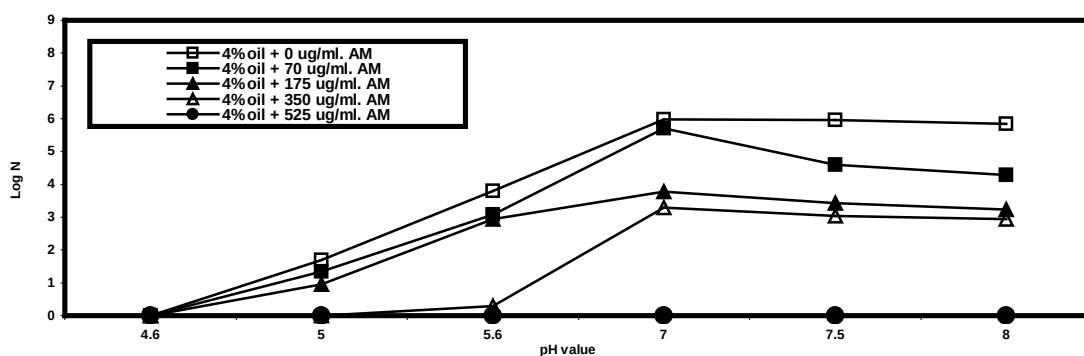


c

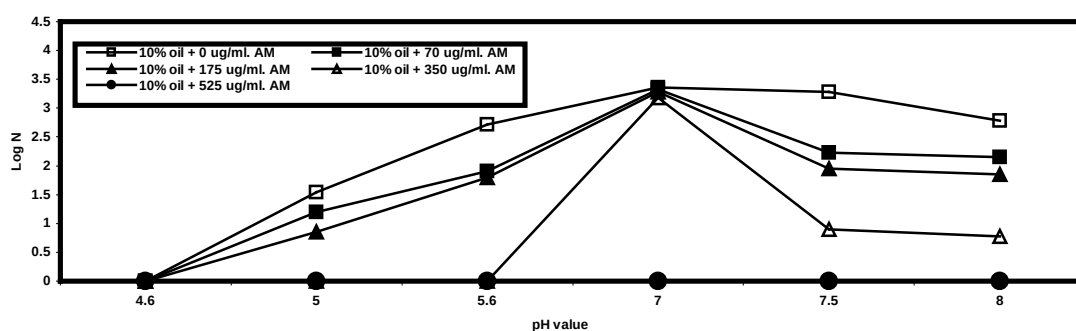


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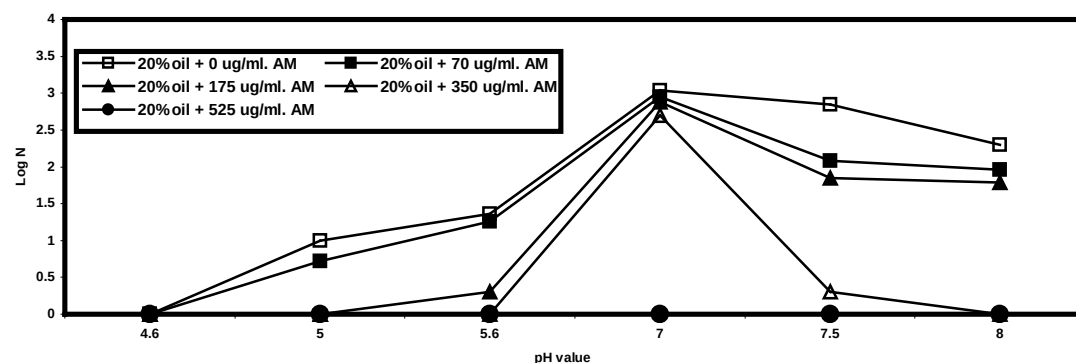
Fig. (14)(a,b,c and d)Effect of the combination action of both CE and oil of *Nigella sativa* on the sensitivity of *Staph. aureus* sp13 at different pH values



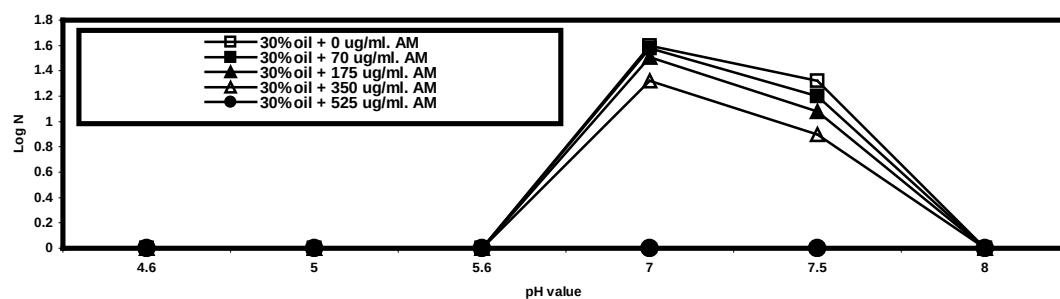
a



b



c



d

Fig. (15) (a ,b ,c and d)Effect of the combination action of both AM and oil of *Nigella sativa* on the sensitivity of *Staph. aureus* sp13 at different pH values

- Plasmid profile analysis :

The results revealed that the absence of plasmid in all specimens by Gel electrophoresis using λ Hind III DNA marker. Then, the response of the organism to antibiotic, oil or mixture treatments may be due to chromosomal DNA and not plasmid DNA. **Photo no. (1)**

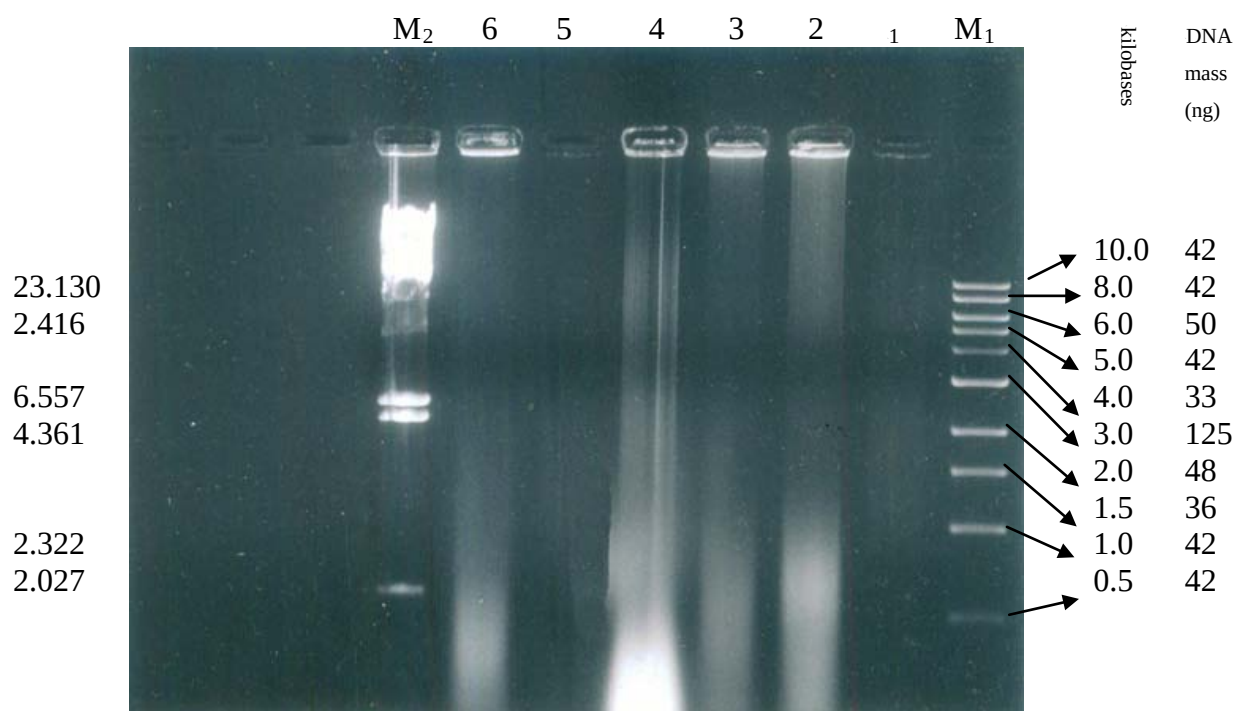


Photo No. 1 Plasmid profile analysis of Staph. aureus (sp.13)

M1: 0.5 ug of Kb DNA Ladder visualized by ethidium bromide staining on a 0.8% TAE agarose gel .

Lane 1 : Control

Lane 2 : 550 ug/ml. of CE

Lane 3 : 350 ug/ml of AM

Lane 4 : 20% of Nigella sativa oil

Lane 5 : 550 ug/ml. of CE + 20% of Nigella sativa oil

Lane 6 : 350 ug/ml of AM + 20% of Nigella sativa oil

M2 : Lambda DNA –Hind III Digest visualized by ethidium bromide staining on 1.0% agarose gel .

DISCUSSION

Some clinical *Staph. aureus* isolates were differ in a wide range of phenotypic properties, (Proctor *et al*, 1995) *Staph. aureus* was well documented as human opportunistic pathogen and was one of the most frequently identified pathogens in clinical laboratories (Merlion *et al* , 1996)

Some bacteria were fairly uniform in their response to antimicrobial agents. Certain organisms isolated from blood, urine, sputum or cerebrospinal fluid were differ for their susceptibility to different antibiotics(**Walker, 1998**)

In this research 150 specimens from stool, urine and sputum were collected from 150 patients in Zagazig Hospitals. Different bacteria were isolated from these specimens. All bacterial isolates were screened for their susceptibility to certain antibiotics (VA, Am, P, CTX, Ce, GM, SXT, TE, NA, E, OT and F) using disc diffusion technique as recommended by **Bauer et al. (1966)**

The results revealed that all isolates were completely resistant (100%) to NA, TE, CE and VA, while four isolates were resistance to F, and only one isolate was intermediate to the same antibiotic. On the other hand, all *Salmonella* isolates were completely sensitive (100%) to AM, CTX, SXT and C

This results were in agreement with **Spika et al. (1987)** and **Terakado et al. (1975)**, they reported that a high sensitivity had been expressed by *S.typhi* isolates against cefatoxime and chloramphenical. Also **Waffa et al.(1998)** reported the resistance to ampicillin achieved 39% **Reina et al. (1993)** and **Mounoz et al.(1993)** reported 34% and 32% resistance rates were attained respectively.

Moreover the antibiotic susceptibility test for *E. coli* isolates indicated that all the tested isolates (18) were resistance to NA, GM, and F, while they were highly sensitive to TE, E and P. On the other hand the isolates were varied from sensitive, intermediate and resistant to AM, SXT and CTX.

In this relation **Bryan (1982)** reported the resistance of *E. coli* towards nalidixic acid. While **Mostafa, (2005)** found that, 100% of *E. coli* isolates were sensitive to nalidixic acid this proportions was comparable to that (99%) by **DeBoy et al. (1980)** and (100%) by **Thoren (1980)**. **David et al.(2000)** reported that, 44 out of 48 diarrhegenic *E.coli* isolates (92%) were sensitive to nalidixic acid.

In this connection **Marian (2004)** found that, the antibiotic susceptibility pattern of isolated *staph. aureus* showed that vancomycin was the most active antibiotic followed by erythromycin, imipenem and chloraphenicol with susceptibility 96.2%, 90.3%, 89.6% and 89.6% respectively. Also **Bagherwal and Shukal (1997)** reported that *Staph. spp.* *Streptococcus spp.* and *E. coli* strain isolated from milk were resistant to ampicillin, cloxacillin, streptomycin, oxytetracycline and erythromycin but sensitive to gentamycin and pefloxacin and pefloxacin.

Considered what an antibiotic must do to exert its effect; it must recognize a suitable target, reach that target, achieve a sufficient concentration, and maintain that concentration for an adequate period of time **Walker (1998)**. There were four main types of antibiotics, each of which attacked a different target: 1- antibiotics that affect the cell envelope, 2- antibiotics that inhibit protein synthesis. 3- antibiotics that affect nucleic acid synthesis and structure and 4- antimetabolite antibiotics. In general, antibiotics of the first and third categories were cidal, those of the second category may be static or cidal and those of the fourth category were static.

Resistance to antimicrobial agents may mediated by agents that were encoded on the host cell chromosomes, plasmids or transposons, which were capable of integrating into the plasmid and / or into the chromosome (**Tenover and McGowan, 1996**) Some bacteria produced enzyme that inactive an antibiotic, by change permeability to the antibiotic or the target of the antibiotic may be altered.(**Walker , 1998**).

More over , MICs of Ce and Am against the selected isolates of *Staph. aureus* (sp.13) cultivated in liquid media were examined , MICs values were determined visually as previously described by **Eagle and Musselman (1948)** . They reported that, MIC was denoted as the lowest concentration of drug, which prevent the visible growth after 18 – 24 hrs. incubation.

The MIC of cephradine 900 and 1000 µg/ml for *S. typhi* (st.5) and *Staph. aureus* (sp.13) respectively, were more than MICs of ampicillin 500 and 600 µg/ml for *S. typhi* and *Staph. aureus* respectively. The MBCs of cephradine 1000 and 1100 µg/ml for *S. typhi* and *Staph. aureus* respectively, were more than MBCs of ampicillin 600 and 700 µg/ml for *S. typhi* and *Staph. aureus* respectively, hence it was more sensitive to ampicillin than cephradine. In this relation, **Low et al. (2002)** reported that, MIC of ciprofloxacin susceptible *Staph. aureus* were 0.015 and 2 mg/ml. and MIC at which 90% of organisms was inhibited, was 0.03 and 4 mg/ml. of ciprofloxacin. Also **Rand and Houck (2004)** found that, MIC of Ampicillin against 18 strains of methicillin resistant *Staph. aureus* was 128 mg/ml. Also they added that, 16 of 18 strains were resistant at oxacillin concentration of 256 mg/ml. while all strains were susceptible to daptomycin (the MIC was 1 µg/ml.). Moreover **Mättö, et al (2007)** determined the MIC for tetracycline was ≥ 16 µg/ml against *Bifidobacteria*.

On the other hand ,the antimicrobial activity of five essential volatile oils (*Thymus vulgaris* L., *Matricaria recutita*, *Nigella sativa*, *Allium sativum*, *Citrus limonum* (Risso)) were examined against all tested pathogenic bacteria, five *S. typhi*, 18 *E. coli* and 15 *Staph. aureus* strains were tested using agar well diffusion method.

In an effort to discover new bioactive drugs, many research groups screen plant extracts to detect secondary metabolites with biological activities (**Alves et al., 2000**).

The results indicated that, all isolates of both *S. typhi* and *E. coli* were completely resistant to all tested oils. Also all isolates of *Staph. aureus* were resistant to *Thymus vulgaris* L. , *Matricaria recutita* , and *Allium sativum*, while they were sensitive to *Nigella sativa* and *Citrus limonum* (Risso) . Moreover the results indicated that, increasing the concentration of tested oils achieved an increase in the diameter of inhibition zone of *Staph. aureus* isolates.

Our studies were in agreement with **Ashraf (2005)** who found that the antibacterial activity of oils increased with increasing the concentration of tested oils. Also **Toama et al. (1974)** recorded the antimicrobial activity of the volatile oil of *Nigella sativa* and Linnaeus seeds. The antimicrobial principle had been isolated, identified as thymohydroquinone, and found to be active against gram-positive bacteria and yeasts.

Regarding to determination of the minimum bacteriocidal concentration, it was found that MBC of *Nigella sativa* oil emulsion for *Staph. aureus* was achieved at concentration 40%. In this correlation, **Chowdhury et al.(1998)** reported that the activity of volatile oil of *Nigella sativa* seeds was exhibited against 8 multiple drug resistant strains of *Shigella flexneri*.

Different crude extracts of *Nigella Sativa* were tested for its antimicrobial effectiveness against different bacterial isolates. These isolates comprised 16 gram negative and 6 gram positive bacteria representatives. They showed multiple resistance against antibiotics, specially the gram negative ones. Crude extracts of *Nigella Sativa* showed a promising effect against some of the tested organisms. The most effective extracts were the crude alkaloid and water extracts. Gram negative isolates were affected more than gram positive ones (**Morsi, 2000**).

Through these research combination treatments of different concentrations of each cefradin and ampicillin with *Nigella sativa* oil against *Staph. aureus* were examined. It was found that, the lethal concentration (MBCs) of combination treatments for *Staph. aureus* were (825 µg/ml of CE combined with 10% *Nigella sativa* oil also 525 µg/ml. of AM combined with 4% *Nigella sativa* oil. The combination of antimicrobial agents were considered to be synergistic if the combination effect was greater than the effect either agent alone or greater than the sum of the effect of the individual agents (**Cappelletty and Rybak , 1996**). This was in agreement with **Mostafa (2005)** who reported that the combination between oil and antibiotic was more active than each individual alone.

Rosato et al.(2007) reported that ,the combination of Norfloxacin (antibiotic) with either *Pelargonium graveolens* essential oil or with some of the main components of this latter, in the treatment of infections caused by some bacterial species was likely to reduce the minimizing the side effects of the antibiotic.

It was found that, generally, the optimum incubation temperature, incubation period and pH value of *Staph. aureus* for all treatments were attained at 37°C, for 48 hrs at pH 7 respectively. Also the results indicated that, MBCs for Ce, Am were more than 825 and 525 µg/ml. respectively at all optimum conditions, while MBCs of *Nigella sativa* was more than 20% and 4% respectively at the same condition.

In this relation, **Lanciotti et al.(2002)** stated that, the location of the growth/no-growth boundaries for *S. enteritidis* and *Staph. aureus* were strongly affected, at the same ethanol concentration, by temperature, pH and A_w . Among the considered species; *Staph. aureus* was endowed with the highest sensitivity to low pH values. **Weinrick et al.(2004)** reported that during Staphylococcal growth in glucose – supplemented medium, the pH of a culture starting near neutrality typically decreased by about 2 units due to the fermentation of glucose.

Many of the genes encoding extracellular virulence factors were affected, as were genes involved in regulation of virulence factor gene expression, transport of sugars and peptides, intermediary metabolism, and pH homeostasis. Key results were verified by gene fusion and northern blot hybridization analyses. The results point to, but do not define, possible regulatory pathways by which the organism senses and responds to a pH stimulus (**Weinrick et al., 2004**). Regarding to molecular biological studies of antibiotic resistance and *Nigella sativa* oil against *Staph. aureus* strain, plasmid profiles of chosen treatments (controle, 550 µg/ml. CE, 350 µg/ml. AM, 20% oil, 550 µg/ml. CE+20% oil and 350 µg/ml. AM+ 20% oil) were detected. The results revealed that the absence of plasmid in all specimens by Gel electrophoresis using λ Hind III DNA marker. Then, the response of the organism to antibiotic, oil or mixture may be due to chromosomal DNA and not plasmid DNA similarly **Marian (2004)** found that, no clear correlation existed between the antibiotic susceptibility pattern of the most resistant strains and their plasmid profiles which may need further researches. Also, **Aysel and Özgür (2002)** denoted that fifty percent of studied strains harbored plasmids and association between occurrence of plasmids and resistance to antibiotics and heavy metals was observed.

A rapid and simple plasmid isolation procedure was developed for the epidemiological analysis of plasmid-mediated antimicrobial resistance. Various bacteria, such as strains of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Staphylococcus aureus*, could be analyzed. The plasmid DNA obtained could be directly used restriction endonuclease analysis without further purification. In addition , this method made it possible to analyze several cultures at the same time(**Takahashi and Nagano, 1984**)

Neu (1989) reported that, microorganisms that became or were intrinsically resistant to antimicrobial agents act through three major mechanisms; prevention of the drug from reaching its receptor site, production of altered target and destruction or modification of the agent. Also bacteria resistant as occurred due to chromosomal changes or the presence of plasmid and transposons.(**Tenover and McGowan, 1996**).

During evolution microorganisms had mechanisms of adaptation to the damaging factors of external environment. The display of biochemical mechanisms of stability was defined by genetic determinants chromosomal and plasmids nature.(**Kozitskaya and Vinnikov, 1999**).

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حساسية الستافيلوكوكاس اورييس 13 لمفعول المفرد و المدمج للسيفرادين , الامبسيللين
و زيت حبة البركة
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الملخص

ثلاث عينات طبية مختلفة تم اختيارهم فى هذا البحث . خمس سلالات سالمونيلا تايفى , 18 اشيريشيا كولاي و 15 استافيلوكوكاس اورييس تم عزلهم من عينات براز , بول و مخاط من معامل مستشفيات جامعة الزقازيق . كل السلالات الممرضة تم اختبار حساسيتهم ل 9 مضاد حيوى مختلفة لكل نوع و خمس زيوت طبيعية باستخدام طرق انتشار اقراص اجار و فتحات اجار على الترتيب . كلا من سلالات سالمونيلا تايفى 5 و الستافيلوكوكاس اورييس 13 مقاومة للسيفرادين (Ce) و حساسة للامبسيللين (Am). اقل تركيز مثبط من السيفرادين لكل من سالمونيلا تايفى 5 و الستافيلوكوكاس اورييس 13 كان 900 ميكروجرام / مل و 1000 ميكروجرام / مل على الترتيب . فى حين ان اقل تركيز مثبط للامبسيللين لكل من سالمونيلا تايفى 5 و الستافيلوكوكاس اورييس 13 كان 500 ميكروجرام / مل و 600 ميكروجرام / مل على الترتيب . تم اختبار الحساسية للعزلات لخمس انواع من الزيوت الطبيعية (زيت الزعتر , زيت البابونج , زيت حبة البركة , زيت الثوم و زيت الليمون) . كل العزلات السالبة لجرام الممرضة كانت مقاومة للزيوت الطبيعية فى حين العزلات الموجبة لجرام كانت حساسة . اقل تركيز مميت من زيت حبة البركة للستافيلوكوكاس اورييس 13 كانت 40% . التركيزات المميتة للعلاج بالدمج للستافيلوكوكاس اورييس 13 كانت 825 ميكروجرام / مل من السيفرادين مع 20% زيت حبة البركة و 525 ميكروجرام / مل من الامبسيللين مع 4% زيت حبة البركة . علاوة على ذلك تم تعيين الظروف المثلى المؤثرة على حساسية العزلات للمضادات الحيوية و الزيوت الطبيعية و كانت عند درجة حرارة تحضين 37 م لمدة 48 ساعة و رقم هيدروجينى 7 . تتأثر حساسية الستافيلوكوكاس اورييس 13 للمعالجات الفردية و المدمجة من السيفرادين (Ce) , الامبسيللين (Am) و زيت حبة البركة . من تحليل تخطيط البلازميد للستافيلوكوكاس اورييس 13 عدم وجود بلازميد لفعل الفرد او المدمج بالسيفرادين (Ce) , الامبسيللين (Am) و زيت حبة البركة .

ENVIRONMENTAL IMPLICATIONS OF SUCCESSIVE MINERAL FERTILIZATION ON SOIL AND PLANT GROWN THEREON

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ABSTRACT

This work aimed at following up the implications of the successive use of different mineral nitrogen, phosphate, and potassium fertilizers on accumulation of some heavy metals (Pb, Cd, Co and Ni) in El-Noubaria (loamy calcareous soil) and El-Bostan (non-calcareous sandy soil) as well as the plants grown thereon.

Sites covering lands of different cultivation periods (<5, 10 and >20 years) within both of the investigated soils were sampled. Likewise, plants grown at these sites were sampled. Both the soil and the plant samples were analyzed for the aforementioned metal ions. Furthermore, the waters drained through the investigated soils were sampled.

The obtained results showed that prolonging period of cultivation was associated with increasing concentrations of the investigated elements in both soils and plants grown thereon. However, DTPA-extractable contents of the metal ions were grater in the sandy soils than in the calcareous one. Also, concentrations of the different metal ions in the drainage water of the calcareous soil were less than the corresponding ones of the sandy soil.

Results indicated also that there is exposure being in children higher than adults in accordance with metal concentrations trend in soils and biota (grains of maize and wheat plants).

INTRODUCTION

Increasing agricultural production faces many challenges in most developing countries to increase production growth and consequently to satisfy the food demands for growing populations. There are several factors which cause high variability in the yield, among them the proper use of fertilizers in terms of type, quantity and nutrients used (El-Dewiny, 2001). Morvedit (1987) stated that the long-term use of phosphate fertilizers increased plant uptake of heavy metals such as Cd and Pb. Zee et al, (1990) stated that application of heavy metals as impurities in commercial fertilizers resulted in heavy metals accumulation in soil and plants.

Kandil (1997) reported that the wide use of different mineral fertilizers i.e. phosphate and nitrogen and potassium fertilizers to the soil caused an increase in heavy metal levels in the soil such as Cd, Pb, Co and Ni. He found that the application of nitrogen alone or in combination with phosphate or potassium generally increased Fe, Cd, Co and Cr content in both shoot and root of red radish plant in comparison with unfertilized treatment.

El-Dewiny (2001) found that ammonium nitrate contains 0.48- 1.9, 2.1- 2.8, 2.04- 2.72, and 6.4 mg kg⁻¹ of Cd, Co, Ni and Pb, respectively and urea fertilizer

contains 0.48- 2.16, 0.7- 3.5, 2.04- 2.7 and 12.8- 2.4 mg kg⁻¹ Cd, Co, Ni, and Pb respectively. She added that potassium chloride contains 3.36- 3.84, 15.4- 23.8, 2.4- 38.4 and 8.84- 19 mg kg⁻¹ of Cd, Co, Pb and Ni, respectively. Potassium sulphate contains 3.12- 15.8, 2.04- 41.6, 84 and 17.7- 35.4 mg kg⁻¹ of Cd, Co, Pb, and Ni, respectively.

Hala et al. (2003) pointed out that normal super phosphate fertilizer contains 2.61, 5.86 and 13.89 mg kg⁻¹, granulated super phosphate fertilizer contains 2.03, 5.67 and 19.57, while, rock phosphate contains 6.55, 1.95 and 15.66 mg kg⁻¹ of Cd, Pb and Ni, respectively. They also pointed out that potassium sulphate fertilizer contains 0.02, 3.13 and 7.11 mg kg⁻¹ of Cd, Pb and Ni, respectively.

Triple super phosphate contains 23 and 44 mg kg⁻¹ of Pb and Ni, respectively (**FAO, 2007**).

MATERIALS AND METHODS

This work was carried out during the growing seasons (2005/2006), to follow up the effects of long-term successive use of different mineral fertilizers in Egypt on accumulation of heavy metals in soils as well as the plants grown thereon.

Soils of the current study were sandy in El-Bostan village and loamy to clay loamy in El-Nubaria, El-Behira governorate. These soils are different in their properties and cultivation period <5, 10 and >20 years. These soils were sampled at depths of (0-30, 30-60, and 60-120 cm). Plant samples (root- shoot- grains) were taken from maize and wheat crops grown on the sampled soils within the seasons 2005 and 2006.

Methods of analyses:

Particle size distribution was determined by the international pipette method as described by **Piper (1950)**.

-Soil reaction (pH values) was determined in 1:2.5 soils: water suspensions by the method described by **Page (1982)**.

-Electrical conductivity (EC) of the soil paste extract was conducted according to **Page et al. (1982)**.

-The soluble cations and anions in soil paste extract were determined according to standard methods described by **Page et al., (1982)** as follows:

- Calcium and magnesium by titration with versinate method.

- Sodium and potassium by the flame photometer.

- Carbonate and bicarbonate by titration against HCl.

- Chloride by titration with silver nitrate.

- Sulphate by the difference between cations and anions.

- Calcium carbonate content was determined using the Calcimeter (**Jackson, 1967**).

- Contents of available Cd, Co, Ni and Pb were extracted using ammonium bicarbonate diethylene triamine penta acetic acid (AB-DTPA) according to **Soltanpaur (1991)** and determined by Induced Coupled Plasma (ICP).

- The total contents of Pb, Cd, Co and Ni of soil were extracted by digesting the soil samples with aqua regia (concentrated hydrochloric and nitric acids 3:1 v:v) according to **Cottenie et al. (1982)** and determined by the Inductively Coupled Plasma (ICP).

- Drainage water samples were collected from the studied soils in the two growing seasons. The contents of Pb, Cd, Co and Ni of drainage water were determined as done with the soils.
- Dried plant materials (shoot, root, grains of wheat and maize plants) were digested using a mixture of concentrated sulphuric- perchloric acids according to the procedure of *Chapman and Pratt (1961)*.

Human health aspect:

The non carcinogenic pollutants (chronic exposure rates) is calculated by the following equation according to United States Environmental Protection Agency (USEPA), (1997) and Agency of Toxic Substances and Disease Registry (ATSDR), (2003)

$$ADD = (C \cdot IR \cdot ED \cdot EF) / (BW \cdot AT)$$

Where:

ADD: is the average daily dose of the chemical ($\text{mg kg}^{-1} \text{ day}^{-1}$).

C: Is the concentration of the chemical in the media (soil or biota) (mg kg^{-1}).

IR: is the ingestion rates (mg day^{-1})

ED: is the exposure duration (years).

EF: is the exposure frequency (days/year).

BW: is the average of body weight (kg).

AT: is the averaging time, the period which exposure is averaged (days).

The obtained ADD is used in determining the Hazard Quotient or Hazard Index (HI) by using the following equation according to *United States Environmental Protection Agency (US EPA), (1996); Brown, (1998); Thornton, (2004)*.

$$HQ \text{ or } HI = ADD / R_fD$$

This is the ratio of the average daily dose (ADD, $\text{mg kg}^{-1} \text{ day}^{-1}$) of a chemical to a reference dose (R_fD , $\text{mg kg}^{-1} \text{ day}^{-1}$) defined as the maximum tolerable daily intake of a specific metal that does not result in any deleterious health effect (*WHO, 2001*).

If HQ or HI > 1.00 then the ADD of a media (soil or biota or both) exceed the R_fD , indicating that there is a potential risk associated with the metal.

Table (1) Some physical properties of the investigated soils

Cultivation period	CaCO ₃ g kg ⁻¹	SP %	Particle size distribution (%)				Textural class
			Coarse sand	Fine sand	Clay	Silt	
A-El-Bostan villages (sandy soils)							
< 5 years	20.90	19.66	90.62	4.00	3.88	1.47	Sand
10 years	14.90	20.00	89.15	3.48	4.06	3.32	Sand
>20 years	43.90	22.66	86.11	3.63	6.00	4.19	Sand
B-El-Nubaria Research Center villages (loamy calcareous soils)							
< 5 years	345.10	51.21	27.51	17.81	18.73	35.942	Loam
10 years	426.00	55.98	23.18	15.87	24.59	36.36	Loam
>20 years	370.60	61.25	20.11	15.34	27.03	37.52	Clay loam

Table (2) Some chemical properties of the investigated soils

Cultivation period	Crop	Concentration mmol _c L ⁻¹							EC dS m ⁻¹	pH
		Na ⁺	K ⁺	Ca ⁺⁺	Mg ⁺⁺	Cl ⁻	HCO ₃ ⁻	SO ₄ ⁻⁻		
A-El-Bostan sandy soils										
<5	Maize	2.70	0.12	1.55	1.63	0.12	1.70	4.18	0.55	8.74
	Wheat	3.00	0.13	1.92	0.95	0.18	2.74	3.09	0.71	8.67
10	Maize	2.96	0.18	2.57	1.95	0.24	2.61	4.81	0.78	9.00
	Wheat	3.11	0.33	3.34	2.09	0.25	3.06	5.65	0.9	7.59
>20	Maize	3.10	0.32	3.09	5.22	0.32	2.72	8.69	1.21	8.65
	Wheat	3.40	0.40	6.21	3.24	0.34	4.76	8.15	1.40	7.64
B-El-Nubaria Research Center soils (calcareous soils)										
<5	Maize	6.58	0.66	15.0	5.00	2.97	3.84	20.4	3.11	7.85
	Wheat	5.80	1.31	9.34	3.61	3.25	3.32	13.5	2.20	8.89
10	Maize	8.62	1.07	18.0	8.69	6.93	3.81	25.6	3.70	7.73
	Wheat	9.90	1.41	9.11	3.96	7.58	1.24	15.7	2.61	8.77
>20	Maize	11.7	0.64	14.0	11.0	9.90	3.92	23.5	3.74	7.86
	Wheat	11.8	1.16	15.7	11.1	11.0	5.08	23.6	4.12	8.17

RESULTS AND DISCUSSION

Total contents of the investigated heavy metals in soil:

Data presented in Table 3 and 4 reveal that prolonging the period of cultivation of the studied non-calcareous sandy and loamy calcareous soils was associated, generally with pronounced increase in total contents of the investigated elements. The mineral fertilization practices are thought to be the most important factor in this concern; however, the contents attained of these elements varied, with variation in physical, chemical and biological properties of soils, beside the variations in the chemical behaviors of the investigated elements themselves.

Initial concentrations of the investigated elements in the calcareous soils seemed, generally, higher than the corresponding ones of the sandy soil. Moreover, putting these soils under cultivation was associated with more obvious increases in soil contents of these elements and the increases were more obvious with prolonging periods of cultivation. This finding is in agreement with the results of *Kabata-Pendias, and Pendias (2001)*.

Retention of elements in the loamy calcareous soils would be greater than in the sandy soil since the drain pores and rate of infiltration of the sandy soil would be much higher than in the loamy calcareous soil.

All the investigated elements tended to be highest in the surface layers and tended to decrease with depth. This is probably due to one or more of the followings: (i) the presence of these metal ions as impurities in the surface applied fertilizers (*Mortvedt and Giordano, 1977 and Zee et al., 1990*) (ii) formation of insoluble compounds among these metal ions and the different soil components (*Jones, 1998 and Gadd, 1999*) (iii) the relatively high pH of soil which enhances precipitation reaction (*Lee and Saunders, 2003*).

Table (3) Total content of the investigated elements in the studied sandy soils.

cultivation period	Season	Depth (cm)	Concentration mg kg ⁻¹			
			Pb	Cd	Co	Ni
Control- Zero time		0-30	0.00	0.00	0.17	0.91
		30-60	0.00	0.00	0.137	0.90
		60-120	0.00	0.00	0.08	0.76
<5 years	1 st season	0-30	1.35	0.11	1.58	2.21
		30-60	1.05	0.09	1.27	2.12
		60-120	0.98	0.06	0.83	1.78
	2 nd season	0-30	2.11	0.12	1.71	4.70
		30-60	1.22	0.11	1.29	3.48
		60-120	1.12	0.09	1.01	2.09
10 years	1 st season	0-30	4.46	0.22	2.11	4.59
		30-60	3.41	0.2	1.83	4.01
		60-120	3.52	0.2	1.91	4.24
	2 nd season	0-30	6.21	0.31	2.63	4.92
		30-60	4.05	0.21	2.05	4.11
		60-120	4.24	0.20	2.12	4.25
>20 years	1 st season	0-30	6.32	0.29	2.58	6.38
		30-60	5.07	0.2	2.27	3.58
		60-120	4.90	0.16	2.32	3.61
	2 nd season	0-30	7.23	0.30	2.78	6.64
		30-60	6.84	0.22	3.01	5.01
		60-120	6.57	0.23	2.65	5.64

Table (4) Total content of the investigated elements in the loamy calcareous soils.

cultivation Period	Season	Depth (cm)	Concentration (mg kg ⁻¹)			
			Pb	Cd	Co	Ni
Control- Zero time		0-30	0.02	0.01	0.185	2.05
		30-60	0.01	0.01	0.180	1.24
		60-120	0.00	0.01	0.100	1.1
<5 years	1 st season	0-30	4.89	0.32	2.259	9.81
		30-60	2.51	0.10	2.091	8.77
		60-120	2.06	0.09	0.890	8.09
	2 nd season	0-30	5.65	0.38	3.410	11.51
		30-60	3.01	0.11	2.110	8.96
		60-120	2.11	0.10	0.970	8.14
10 years	1 st season	0-30	6.21	0.51	4.110	12.93
		30-60	4.52	0.31	3.220	10.51
		60-120	2.67	0.15	1.050	10.05
	2 nd season	0-30	9.14	0.59	5.010	14.09
		30-60	5.010	0.371	3.310	11.060
		60-120	3.110	0.172	1.080	10.210
>20 years	1 st season	0-30	10.140	0.713	6.880	17.660
		30-60	7.640	0.521	6.400	14.740
		60-120	7.240	0.232	3.210	14.620
	2 nd season	0-30	12.430	0.914	7.090	19.510
		30-60	8.110	0.541	6.890	15.810
		60-120	7.420	0.235	3.230	14.580

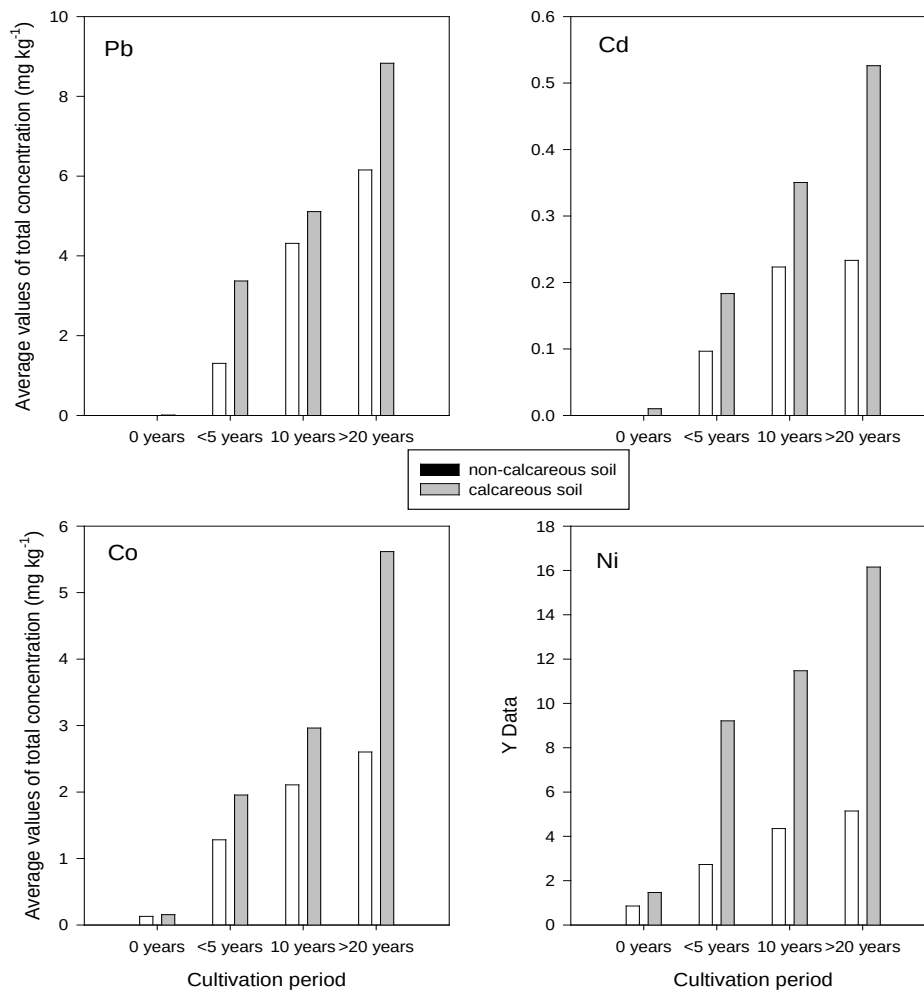


Fig (1) Average values of total heavy metals in soil as affected by the cultivation period

AB-DTPA extractable elements:

Data presented in Tables 5 and 6 show values of AB-DTPA extractable elements in the studied soils. Cultivation of the studied soils caused their contents of AB-DTPA extractable elements to increase and the increases were more pounced in soils by prolonging period of cultivation. Pb, Cd, Co and Ni increased from being 0.00, 0.00, 0.005 and 0.035 mg kg⁻¹, respectively in the surface layer of the control sandy soil (the non-cultivated soil) to reach 0.51, 0.06, 0.08 and 0.46 mg kg⁻¹, respectively in the surface layer of the sandy soil cultivated for more than twenty years (the first season- summer season). The corresponding values attained in the second season (winter season) were 0.56, 0.08, 0.1 and 1.2 mg kg⁻¹, respectively. Regarding effect of cultivation and consequently fertilization of the loamy calcareous soil, data in Table 6 reveal that Pb, Cd, Co and Ni increased from 0.10, 0.02, 0.05 and

0.32 mg kg⁻¹, respectively in the surface layers of the non-cultivated soil to of 0.87, 0.08, 0.12 and 0.68 mg kg⁻¹, respectively in the calcareous soil cultivated for more than twenty years.

Table (5) AB-DTPA extract of the investigated elements in the sandy soils

cultivation	Season	Depth (cm)	Concentration (mg kg ⁻¹)			
Period			Pb	Cd	Co	Ni
Control- Zero time		0-30	0.00	0.00	0.01	0.04
		30-60	0.00	0.00	0.00	0.02
		60-120	0.00	0.00	0.00	0.02
<5 years	1 st season	0-30	0.24	0.02	0.03	0.09
		30-60	0.15	0.02	0.04	0.08
		60-120	0.42	0.01	0.03	0.07
	2 nd season	0-30	0.46	0.02	0.09	0.21
		30-60	0.28	0.02	0.05	0.12
		60-120	0.31	0.01	0.03	0.12
10 years	1 st season	0-30	0.23	0.02	0.09	0.47
		30-60	0.26	0.02	0.05	0.65
		60-120	0.11	0.02	0.04	0.54
	2 nd season	0-30	0.47	0.04	0.10	1.10
		30-60	0.28	0.04	0.07	0.76
		60-120	0.12	0.03	0.04	0.58
>20 years	1 st season	0-30	0.51	0.03	0.08	0.46
		30-60	0.45	0.06	0.07	0.85
		60-120	0.44	0.05	0.07	0.84
	2 nd season	0-30	0.56	0.08	0.10	1.20
		30-60	0.51	0.07	0.09	0.97
		60-120	0.48	0.06	0.08	0.85

Table (6) AB-DTPA extract of the investigated elements in the loamy calcareous soils.

cultivation	Season	Depth (cm)	Concentration (mg kg ⁻¹)			
Period			Pb	Cd	Co	Ni
Control- Zero time		0-30	0.10	0.02	0.05	0.32
		30-60	0.11	0.01	0.06	0.31
		60-120	0.09	0.01	0.04	0.29
<5 years	1 st season	0-30	0.57	0.05	0.04	0.44
		30-60	0.56	0.05	0.02	0.40
		60-120	0.36	0.00	0.02	0.40
	2 nd season	0-30	0.63	0.05	0.07	0.46
		30-60	0.64	0.06	0.03	0.43
		60-120	0.55	0.00	0.03	0.40
10 years	1 st season	0-30	0.46	0.05	0.09	0.53
		30-60	0.27	0.03	0.04	0.27
		60-120	0.29	0.03	0.06	0.21
	2 nd season	0-30	0.76	0.10	0.11	0.69
		30-60	0.41	0.04	0.09	0.41
		60-120	0.40	0.04	0.08	0.29
>20 years	1 st season	0-30	0.87	0.08	0.12	0.68
		30-60	0.43	0.02	0.10	0.46
		60-120	0.30	0.02	0.04	0.29
	2 nd season	0-30	1.12	0.10	0.16	0.80
		30-60	0.59	0.04	0.12	0.66
		60-120	0.35	0.02	0.06	0.34

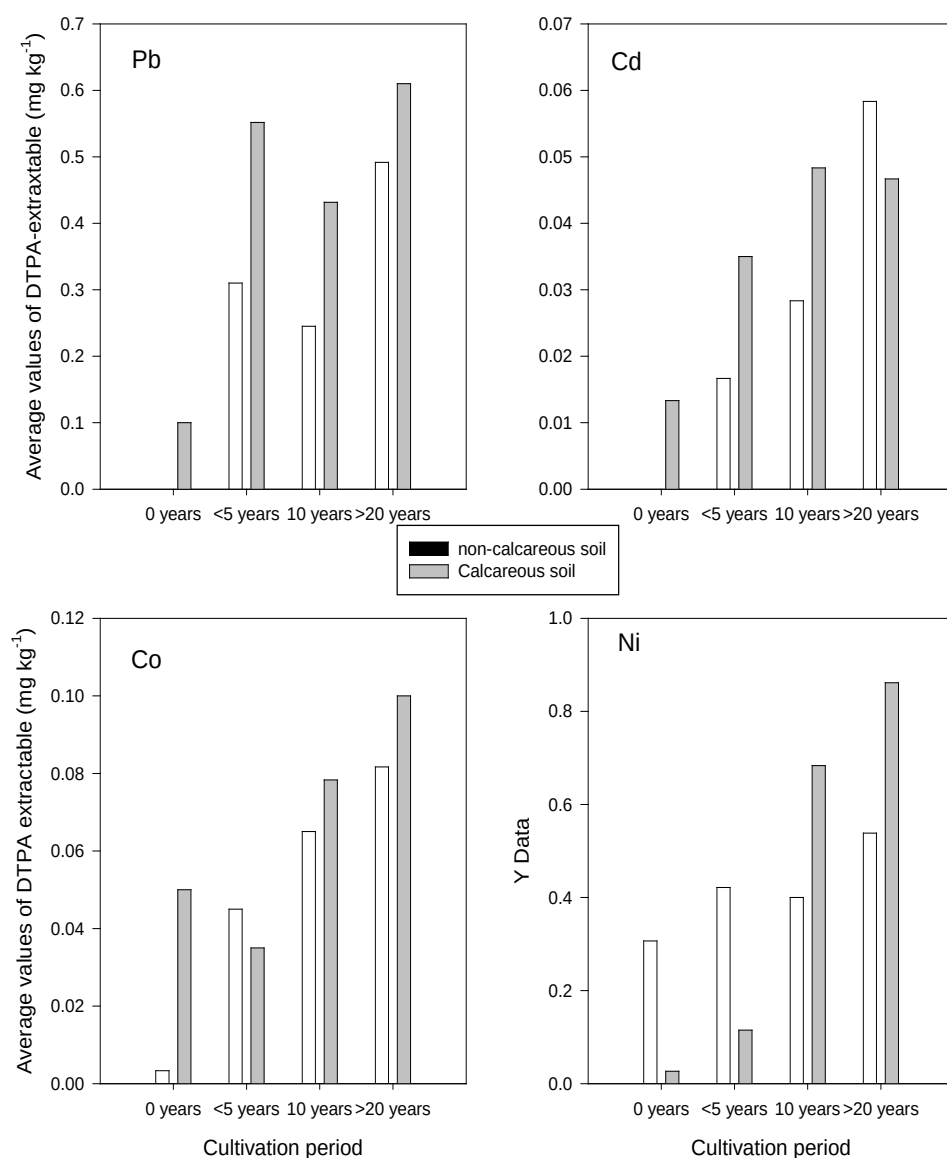


Fig. (2) Average values of DTPA extractable heavy metals in soil as affected by the cultivation period

Generally, the highest concentrations of the AB-DTPA extractable elements were detected in the surface or the sub surface soil layers whereas the lowest concentrations were detected in the deepest layers. This is in agreement with *Badawy (1993)*.

Effect of long term use of mineral fertilizers in different soils on heavy metal contents of plants grown thereon:

Data presented in Tables 7 and 8 show concentrations of the investigated elements in the different organs of the maize and wheat plants grown on the studied soils. It can be seen that prolonging the period of cultivation (and consequently fertilization with mineral fertilizers) could increase gradually concentrations of those elements in plant.

This finding was true in plants grown in the sandy soils as well as those grown in the loamy calcareous soils. It is worthy to mention that concentrations of the investigated heavy metals in the different organs of the plants grown on the sandy soil cultivated for the different studied periods seemed almost double the corresponding concentrations of these metal ions in the calcareous soil.

Thus, it can be deduced that the available content and not the total content of the metal ions is the effective factor in determining the plant content of these elements in plant. As a matter of fact, this finding proves the importance of CaCO_3 in reducing availability of different elements in soil. The hydrolysis of CaCO_3 cause soil pH to be raised hence the availability to be decreased. These results stand in well agreement with those of *Balba, (1995)*.

Effect of long term use of mineral fertilizers in different soils on heavy metal contents of their drained water:

Data presented in Table 9 show that the concentrations of the different investigated elements in the drainage water tended to decrease with increasing period of cultivation regardless of type of the studied soil. However, concentrations of the different elements in the drainage water of the loamy calcareous soil were less than the corresponding ones of the sandy soil regardless of the period of cultivation. Precipitation of heavy metals in the calcareous soil through reaction with the excess of the hydroxide ions and/or CaCO_3 may account for the lower concentrations of these elements in the drainage water of the calcareous soils than the sandy ones. The higher permeability of the sandy soil is an additional factor for increasing concentrations of the different elements in the drainage water. *Maurice (1990)* went almost to similar findings.

Table (7) Total contents of the investigated elements in the plants grown on the sandy soils.

Cultivation period	Season	Organ	Concentration mg kg ⁻¹			
			Pb	Cd	Co	Ni
<5 years	1 st season	Shoot	0.116	0.091	0.177	2.425
		Root	0.212	0.110	0.960	3.900
	Maize plant	Grains	0.116	0.050	0.114	2.550
	2 nd season	Shoot	0.187	0.100	0.075	3.146
		Root	0.230	0.110	0.500	3.930
	Wheat plant	Grains	0.096	0.091	0.095	1.630
10 years	1 st season	Shoot	0.220	0.120	0.190	3.887
		Root	0.350	0.120	1.016	4.270
	Maize plant	Grains	0.240	0.061	0.287	3.525
	2 nd season	Shoot	0.213	0.100	0.091	4.525
		Root	0.260	0.150	0.875	4.150
	Wheat plant	Grains	0.250	0.101	0.210	2.120
>20 years	1 st season	Shoot	0.272	0.141	0.400	5.670
		Root	0.400	0.190	1.000	7.900
	Maize plant	Grains	0.500	0.083	0.300	5.530
	2 nd season	Shoot	0.225	0.175	0.200	6.650
		Root	0.321	0.250	1.000	6.200
	Wheat plant	Grains	0.360	0.126	0.250	2.550

Table (8) Total contents of the investigated elements in the plants grown on the studied loamy calcareous soils.

Cultivation Period	Season	Organ	Concentration mg kg ⁻¹			
			Pb	Cd	Co	Ni
<5 years	1 st season	Shoot	0.633	0.011	0.160	2.210
		Root	1.020	0.060	0.220	2.210
	Maize plant	Grains	1.033	0.000	0.090	1.500
	2 nd season	Shoot	0.220	0.090	0.080	1.570
		Root	0.234	0.100	0.240	1.600
	Wheat plant	Grains	0.025	0.051	0.030	0.790
10 years	1 st season	Shoot	0.870	0.095	0.170	2.366
		Root	0.880	0.090	0.210	2.510
	Maize plant	Grains	2.050	0.036	0.110	2.140
	2 nd season	Shoot	0.400	0.110	0.110	2.050
		Root	0.375	0.120	0.330	1.980
	Wheat plant	Grains	0.110	0.072	0.100	1.350
>20 years	1 st season	Shoot	1.200	0.110	0.210	3.980
		Root	0.860	0.120	0.850	3.900
	Maize plant	Grains	2.456	0.041	0.150	3.510
	2 nd season	Shoot	0.475	0.150	0.370	2.150
		Root	0.525	0.175	0.420	2.410
	Wheat plant	Grains	0.119	0.075	0.150	1.900

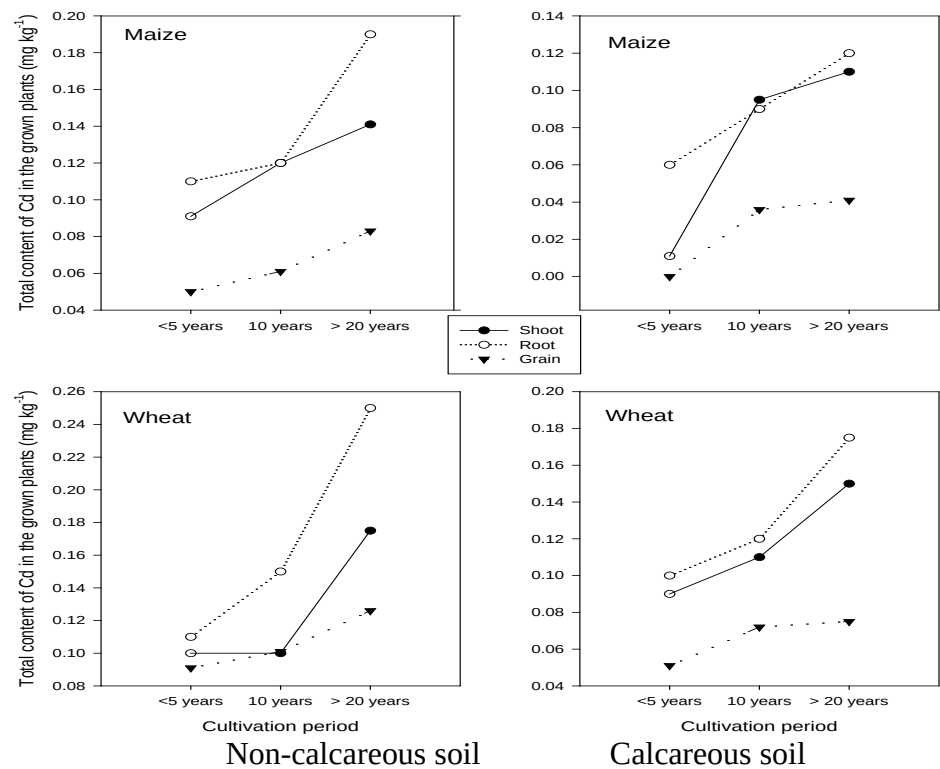


Fig. (3) Total contents of the Cd in the grown plants

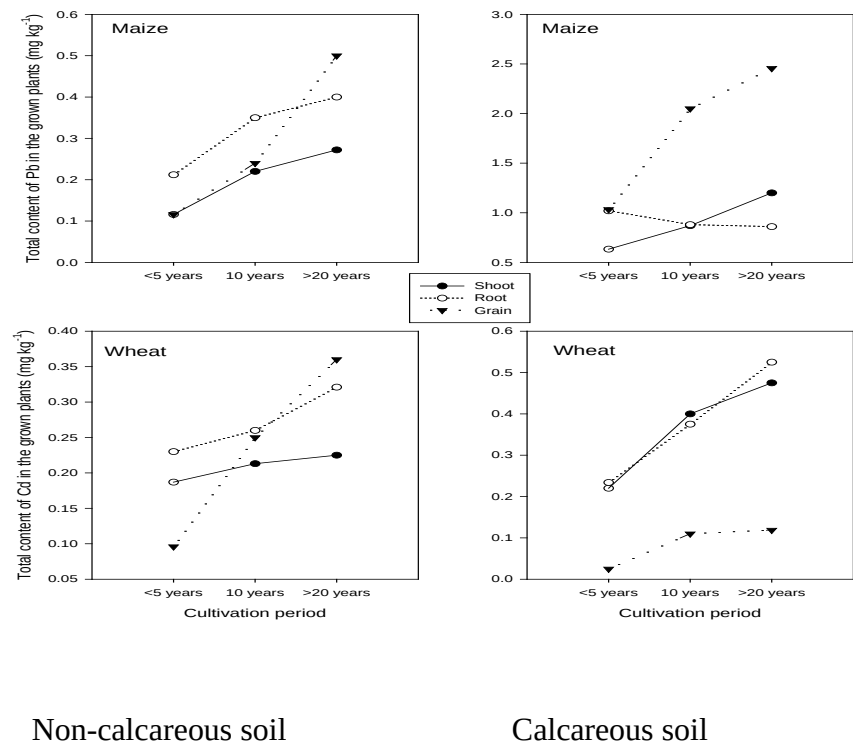


Fig (4) Total contents of Pb in the grown plants

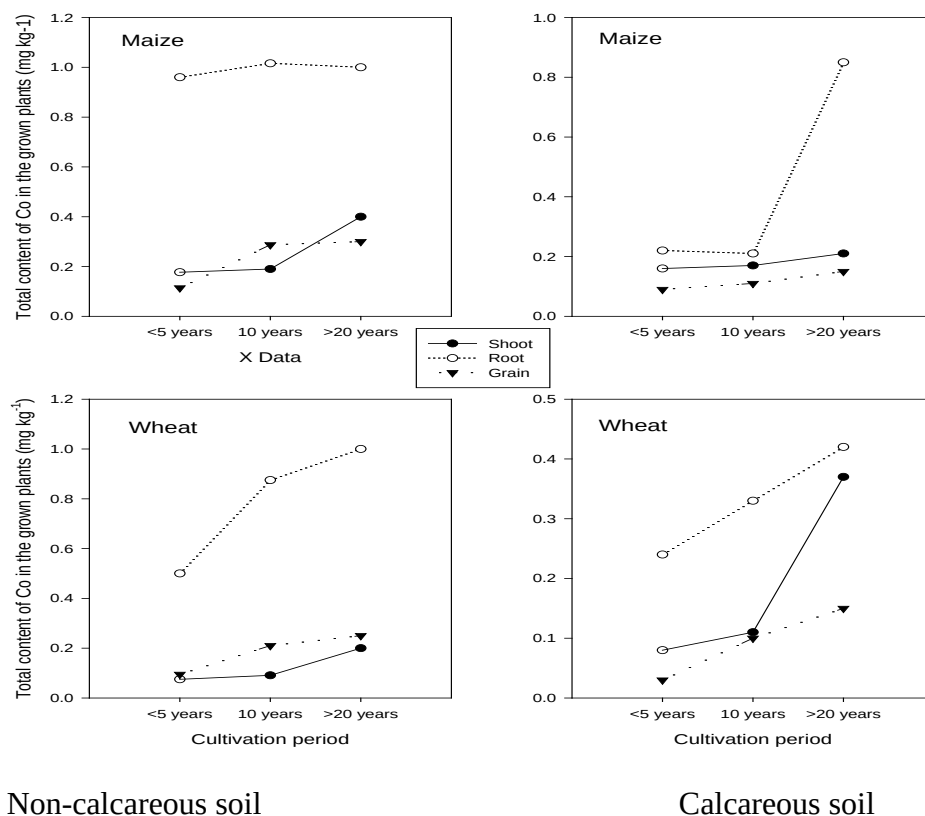


Fig. (5) Total contents of Ni in the grown plants

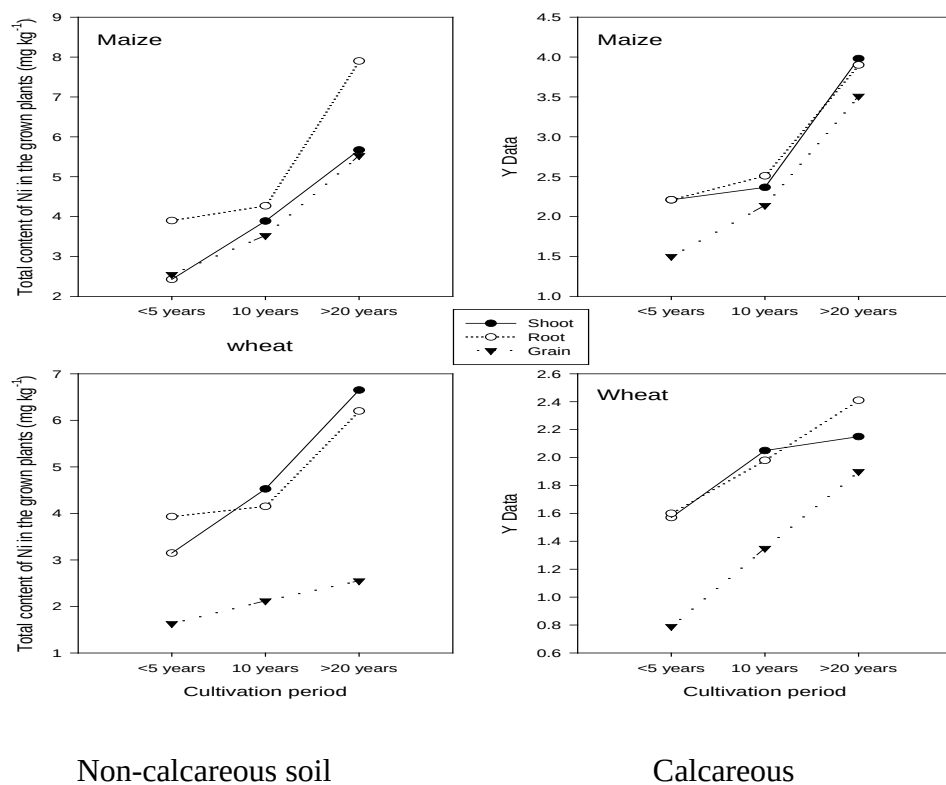


Fig (6) Total contents of Co in the grown plants

Table (9) Drainage water contents of investigated elements in the studied soils (sandy and loamy calcareous soils).

Soil /Cultivation period	Season	$mg\ L^{-1}$			
		Pb	Cd	Co	Ni
Sandy/<5 years	1 st season	nd.			
	2 nd season				
Sandy/10years	1 st season	0.71	0.12	0.91	3.24
	2 nd season	0.41	0.09	0.83	2.02
Sandy/>20 years	1 st season	0.58	0.09	0.74	2.08
	2 nd season	0.26	0.05	0.52	1.00
Calcareous/<5 years	1 st season	0.5	0.03	0.42	1.98
	2 nd season	0.23	0.00	0.35	0.85
Calcareous /10years	1 st season	0.5	0.01	0.35	1.45
	2 nd season	0.24	0.009	0.21	0.92
Calcareous />20 years	1 st season	0.5	0.03	0.42	1.98
	2 nd season	0.23	0.00	0.35	0.85

nd: not detected

Human health aspects:

Data presented in Tables 10 and 11 show the calculated amounts of ingested daily metals from soils and biota (grains of maize and wheat plants) by population groups of Egyptian citizen (adult and children).

It is obvious from the above-mentioned tables that there is high exposure in children health than in adults. There were Pb and Co hazards for both adults and children health, whereas, there were Cd and Ni hazards for children health in case of half consumption of biota than adults in the loamy calcareous soils with cultivation period >20 years.

In Brief, it could be concluded that, long-term successive use and application of mineral fertilizers increased, Pb, Cd, Co and Ni in soils as well as plants grown thereon and would affect human health. Therefore, minimizing the use of mineral fertilizers and looking for more suitable kinds of fertilizers may be helpful in avoiding contamination of soils and ground water with heavy metals. An important aspect in

this concern is the extent to which soils subjected to pollution with these metals vary widely depending not only on the kind of the mineral fertilizer but also on the nature of the soil and plant grown thereon.

Table (10) Calculated Average Daily Dose of studied heavy metals in the in the sandy soil (cultivation period >20 years)

Exposure way in Egypt	Element -A.D.D (mg kg ⁻¹ day ⁻¹) and H.I.											
	Cd			Co			Pb			Ni		
	A.D.D	H.I		A.D.D	H.I		A.D.D	H.I		A.D.D	H.I	
Soil Adult	3.26E-08	0.000033	Safe	3.83E-06	0.003829	Safe	9.68E-06	0.002765	Safe	9.30E-06	0.000186	Safe
Soil Child	5.09E-07	0.000509	Safe	5.36E-05	0.053600	Safe	1.35E-04	0.038706	Safe	1.30E-04	0.002604	Safe
Wheat Adult	3.94E-04	0.394369	Safe	1.43E-03	1.425429	Hazard	2.38E-03	0.678776	Safe	2.63E-02	0.525508	Safe
Wheat Child - half adult consumption	1.38E-03	1.380290	Hazard	4.99E-03	4.989000	Hazard	8.32E-03	2.375714	Hazard	9.20E-02	1.839278	Hazard
Wheat Child - quarter adult consumption	6.90E-04	0.690145	Safe	2.49E-03	2.494500	Hazard	4.16E-03	1.187857	Hazard	4.60E-02	0.919639	Safe
Maize Adult	3.84E-04	0.383760	Safe	7.61E-04	0.761429	Safe	1.07E-03	0.304571	Safe	7.77E-03	0.155331	Safe
Maize Child -half adult consumption	1.34E-03	1.343160	Hazard	2.67E-03	2.665000	Hazard	3.73E-03	1.066000	Hazard	2.72E-02	0.543660	Safe
Maize Child - quarter adult consumption	6.72E-04	0.671580	Safe	1.33E-03	1.332500	Hazard	1.87E-03	0.533000	Safe	1.36E-02	0.271830	Safe
Soil+Biota Adult	7.78E-04	0.778161	Safe	2.19E-03	2.190686	Hazard	3.45E-03	0.986112	Safe	3.41E-02	0.681025	Safe
Soil+Biota Child -half adult consumption	2.72E-03	2.723959	Hazard	7.71E-03	7.707600	Hazard	1.22E-02	3.480420	Hazard	1.19E-01	2.385542	Hazard
Soil+Biota Child- quarter adult consumption	1.36E-03	1.362234	Hazard	3.88E-03	3.880600	Hazard	6.16E-03	1.759563	Hazard	5.97E-02	1.194073	Hazard

Note: A.D.D.: Average daily dose

H.I: Hazard index

Table (11) Calculated Average Daily Dose of studied heavy metals in the in the loamy calcareous soil (cultivation period >20 years)

Exposure way in Egypt	Element -A.D.D (mg kg ⁻¹ day ⁻¹) and H.I.											
	Cd			Co			Pb			Ni		
	A.D.D	H.I		A.D.D	H.I		A.D.D	H.I		A.D.D	H.I	
Soil Adult	8.93E-08	0.000089	Safe	9.98E-06	0.009979	Safe	1.61E-05	0.004606	Safe	2.66E-05	0.000531	Safe
Soil Child	1.39E-06	0.001395	Safe	1.40E-04	0.139700	Safe	2.26E-04	0.064486	Safe	3.72E-04	0.007434	Safe
Wheat Adult	1.95E-04	0.194809	Safe	7.13E-04	0.712714	Safe	1.17E-02	3.334145	Hazard	1.66E-02	0.332600	Safe
Wheat Child - half adult consumption	6.82E-04	0.681830	Safe	2.49E-03	2.494500	Hazard	4.08E-02	11.669509	Hazard	5.82E-02	1.164100	Hazard
Wheat Child - quarter adult consumption	3.41E-04	0.340915	Safe	1.25E-03	1.247250	Hazard	2.04E-02	5.834754	Hazard	2.91E-02	0.582050	Safe
Maize Adult	2.28E-04	0.228429	Safe	4.57E-04	0.456857	Safe	3.62E-04	0.103554	Safe	5.78E-03	0.115554	Safe
Maize Child -half adult consumption	8.00E-04	0.799500	Safe	1.60E-03	1.599000	Hazard	1.27E-03	0.362440	Safe	2.02E-02	0.404440	Safe
Maize Child - quarter adult consumption	4.00E-04	0.399750	Safe	8.00E-04	0.799500	Safe	6.34E-04	0.181220	Safe	1.01E-02	0.202220	Safe
Soil+Biota Adult	4.23E-04	0.423326	Safe	1.18E-03	1.179550	Hazard	1.20E-02	3.442306	Hazard	2.24E-02	0.448685	Safe
Soil+Biota Child -half adult consumption	1.48E-03	1.482725	Hazard	4.23E-03	4.233200	Hazard	4.23E-02	12.096434	Hazard	7.88E-02	1.575974	Hazard
Soil+Biota Child- quarter adult consumption	7.42E-04	0.742060	Safe	2.19E-03	2.186450	Hazard	2.13E-02	6.080460	Hazard	3.96E-02	0.791704	Safe

H.I: Hazard index

Note: A.D.D.: Average daily dose

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EFFECTIVENESS OF BIO-CONTROL AGENTS AGAINST TOMATO SOIL BORNE PATHOGENS

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ABSTRACT

This research was carried out to study the efficiency of bio-control agents for controlling root-rot and wilting diseases of tomato. Obtained results showed that *Trichoderma harzianum*-I, *Pseudomonas fluorescens*-II and *Bacillus subtilis*-I were the best strains for controlling *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium oxysporum* .sp *lycopersici*. Also, the seed dressing of tomato and soil drenching with bio-control agents gave the lowest records of disease severity of tomato, while, seed soaking only gave the highest records. Generally, it could be recommended that the application of bio-control agents for tomato at sowing was more efficient for controlling of fungal soil borne diseases fungi. Application of such inocula minimizes the hazard effects of fungicides, protect the environment from pollution and maintenance of the human health.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill) is one of the most important vegetable crops in Arab Republic of Egypt. The A. R. E. government is pressing hard to increase the production of tomato to face the increasing demand of the populations and to increase the exportation. It is well known that, several fungal diseases attack tomato plants during all stages of growth causing a considerable reduction in both yield quality and quantity. Damping-off, root rots and wilting are among the important diseases. Root rot pathogens such as *Rhizoctonia solani* and *Sclerotium rolfsii* attack the roots and stem base of tomato (AbdEl-Wahab, 2004 and Morsy, 2005).

Both *Rhizoctonia solani* and *Sclerotium rolfsii* were causing high damage in tomato cultivations especially in Nile Delta and valley soils. *Fusarium* tomato wilting was among the most deleterious diseases of tomato seedlings either in the nurseries or in fields after transplanting. It was widely spread in many parts of the world, and Egypt as well especially in the newly reclaimed lands of El-Behera, Ismailia and Kafer El-Sheikh governorates.

Biological control had attracted the interest because of increasing regulation and restriction of pesticides or unsuccessful control attempts by other means. Biological control for soil-borne pathogens by antagonistic microorganisms is potential especially for soil-borne diseases because these pathogens are difficult to be controlled with specific fungicides (Moussa *et al.*, 2006 & 2007).

The excessive use of broad spectrum or persistent chemicals might results in soil contamination, fungicidal resistance or other harmful effects (Maloy, 1993). Biological control was usually more enduring with no toxic residue in nature's food chains, safe for

application and cheaper in cost. The present investigation was conducted to study the survey of the tomato wilting and root-rot diseases of tomato; as well as isolation, purification and identification of the causal microorganisms. Finally, the investigation of the effect of certain bio-control agents (fungi and bacteria) on controlling root- rot and wilting diseases of tomato has been discussed.

MATERIALS AND METHODS

Field survey for the causal microorganisms of root-rot and wilting diseases

Survey of root-rot and wilting diseases were carried out on tomato plantations at different regions in five governorates namely El-Beheira, Kafr-El-Sheikh, El-Menoufia, El-Qalubia and El-Gharbia. Samples of infected tomato seedlings were collected and used for isolating the causal microorganisms.

Isolation of the causal microorganisms

Tomato plants which showed damping-off and root-rot symptoms were thoroughly washed, cut into small pieces and surface sterilized with 0.5% sodium hypochlorite. Pieces were plated into Petri dishes containing potato dextrose agar medium. Plates were incubated at 28°C for 3-5 days and examined for fungal growth.

Identification of the causal fungi

Purified of endophytic fungi were microscopically identified according to their cultural and morphological features. The key of the genus *Fusarium* written by **Nelson et al (1983)** was followed in this respect. The other fungal genera were identified according to **Barnett & Hunter (1987)**.

Pathogenicity tests

Identified fungal strains (*F. oxysporum* f.sp *lycopersici*, *R. solani* and *S. rolfii*) were tested under greenhouse conditions for their pathogenicity using susceptible tomato cultivar Strain B.

Experimental soil infestation

R. solani and *S. rolfii* inocula 3% (fungal growth: soil, w/w) were thoroughly mixed with sterilized soil. Whereas, experimental soil infested with *F. oxysporum* at rate of 6×10^7 spores/ kg sterilized soil (**AbdEl-Wahab, 2004**).

Disease assessment

Pre and post emergence damping-off were recorded after 2 and 4 weeks of sowing, respectively with *R. solani* and *S. rolfii* as well after 30 and 60 days with *F. oxysporum*.

Diseases severity was assessed after 8 weeks from transplantation. Diseases severity was scored for *R. solani* and *S. rolfii* according to **O'sullivan & Kavanagh (1991)**.

For the assessment of *Fusarium* wilting, the disease severity was according to **Hassan (1992)**.

Effect of inoculum potential

Soil was infested with different rates i.e. 1, 3, 5, 7 and 9 % of soil weight for *R. solani* and *S. rolfsii* meanwhile, soil was infested with different levels of *F. oxysporum* i.e 1×10^7 , 3×10^7 , 6×10^7 , 9×10^7 and 1×10^7 spore/ml. The percentages of pre- and post-emergence damping-off were calculated.

Varietals reaction

Eight tomato cultivars were tested for their susceptibility to root- rot and wilting by causal pathogens; *F. oxysporum f.sp lycopersici*, *R. solani* and *S. rolfsii* under greenhouse conditions. The tested cultivars were Castle rock, UC-97, Peto-86, Strain-B, Super strain B, Marmande, Money maker and Ace. *R. solani* and *S. rolfsii* were used for soil infestation at a rate of 3.0 % of soil weight (w/w) while, *F. oxysporum f.sp lycopersici* was used at a rate of 6×10^7 spores/pot, 3 kg soil/pot. Disease indices were recorded as the percentages of pre- and post-emergence damping-off.

Interaction between isolates under greenhouse conditions

R. solani, *S. rolfsii* and *F. oxysporum f.sp lycopersici* were used for soil infestation with the same inoculum potential mentioned above. Disease indices were recorded as the percentages of pre- and post-emergence damping-off.

Isolation and identification of biological control agents

Rhizosphere soil of healthy tomato plants were collected from natural heavily diseased fields to isolate different bacteria, actinomycetes and fungi genera emphasized antagonistic properties against isolated pathogenic fungi.

Isolation process was carried out according to the methods described by **Labeda (1990)**, **Waksman & Lechevalier (1961)** and **Kiraly (1974)** for bacteria, actinomycetes and fungi, respectively, While identification process was carried out using **Bergey's Manual of Systematic Bacteriology (1994)** for bacteria and actinomycetes and **Barnett & Hunter (1987)** for fungi.

Antagonistic effect of biological control agents

Several antagonistic microorganisms were isolated from tomato rhizosphere and identified as *T. hamatum*, *T. harzianum*, *T. viride*, *Gliocladium virens*. Two isolates of actinomycetes were identified as *Streptomyces* sp and *S. aureofaciens*. Nine antagonistic bacterial isolates were identified as *Ps. fluorescense*, *B. subtilis*, *Ps. aeruginosa* and *Ps. chlororophis*.

The above mentioned strains were tested for their antagonistic effects on growth of the most virulent strains of *R. solani*, *S. rolfsii* and *F. oxysporum f.sp. lycopersici* *in vitro* and *in vivo*.

In vitro

Petri dishes contain gliotoxin fermented medium (**Brain & Hemming, 1945**) was used to monitor the antagonistic effect of bio-control (fungi) against pathogenic fungi. On the other hand, plates contained nutrient glucose agar medium **Dowson (1957)** and starch nitrate agar medium **Waksman & Lechevalier (1961)** were used to determine the antagonistic effect of bacterial and actinomycetal strains, respectively against the pathogenic fungi.

The percentage of mycelial growth reduction was calculated according to the formula proposed by **Fokkema (1973)** as follows:

$$\text{Reduction \%} = \frac{C - T}{C} \times 100$$

Where: C = growth of the pathogenic fungus in control plates, T = growth of the pathogenic fungus in dual organisms plates

In vivo

In greenhouse experiment, the efficient antagonistic strains were evaluated for their ability to protect tomato plants using the commercial cultivar Strain B against artificial inoculation with soil-borne pathogens of damping-off and wilting diseases.

Efficiency of the bio-control agents, which proved to be more antagonistic *in vitro* was examined under greenhouse conditions for disease control.

Pots were filled with sterilized soil and infested with *R. solani* or *S. rolfsii* at a rate of 3.0% (w/w). While, *F. oxysporum* f.sp *lycopersici* was used at a rate of (6×10^7 spores/pot, 3 kg soil), After 4 days of soil infestation, *T.harzianum*, *T. viride*, *T. hamatum* and *Gliocladium virens* were applied at a rate of 5g/kg soil. Antagonistic bacteria were used at a rate of 50ml/pot (1 ml contained 2.2×10^8 cells). Actinomycetes were used at a rate of 50ml/pot (1 ml contained 7×10^6 spores) (**Hadar et al. 1979**). *Vitavax-thiram* was used at a rate of 0.15% for comparison. Five seeds of cv. Strain B were sown in each pot; 7 days after infestation. Percentages of pre- and post-emergence damping-off were recorded.

Efficiency of various seed treatments

The antagonistic effect of more efficient biological control microorganisms i.e. *B. subtilis* -I (2.5×10^8 cfu/ml), *T. harzianum*-I (2×10^7 spore/ml), as well the influence of *Vitavax- thiram* 0.15% (1.5g/kg seeds) against *R. solani*, *S. rolfsii* and *F. oxysporum* f.sp *lycopersici* was investigated using the following treatments:

Seed soaking

Seeds of tomato cv. Strain B were immersed in suspension of *T. harzianum*-I (5 ml/L), cell suspension of *B. subtilis*-I (5 ml/L) and solution of *Vitavax- thiram* (1.5gm/kg seeds) for 30 minutes before seeding.

Five tomato seeds were planted in pots containing soil infested with the pathogenic fungi. Pre-and post-emergence damping-off were recorded.

Seed dressing

Ten grams of Strain B tomato cultivar seeds were thoroughly mixed with *T. harzianum*, *B. subtilis* and Vitavax- thiram in presence of a dilute gum solution 5% in plastic bags using shaking for 10 minutes .Treated seeds were permitted to dry for 6 hrs before planting. Then five seeds from Strain B cultivar were planted in each pot containing infested soil with pathogenic fungi. Percentages of pre- and post-emergence damping-off were recorded.

RESULTS AND DISCUSSION

Survey and isolation of wilting and root-rot causal microorganisms

Data in **Table (1)** revealed that the highest infection percentage was recorded in El-Beheira governorate .While, the lowest infection percentage was observed in El-Qalubia governorate. The highest disease infection percentage was recorded in El-Nubaria followed by Sakha and Etay El-Baroud.

Data in **Table (1)** also indicated that 244 fungal isolates were secured. These isolates included 72 isolates of *R. solani* followed by *F. oxysporum f.sp. lycopersici* including 59 isolates and *S. rolfii* included 35 isolates .

Moreover, the highest isolate numbers of *R. solani* and *F. oxysporum f.sp lycopersici* were recorded in El-Beheira followed by Kafr El-Sheikh. While the lowest isolate numbers of *R. solani* and *F. oxysporum f.sp lycopersici* were recorded in El-Qalubia .

Table 1. Identity, occurrence and frequency of root-rot and wilting fungi isolated from diseased tomato plants collected from different governorates .

Governorates	Locations	Infection (%)	Strains							Total
			A	B	C	D	E	F	G	
El-Beheira	El-Nubaria	34	10	6	3	2	2	0	0	23
	Damanhour	24	7	5	3	2	3	2	0	22
	Etay-El Baroud	29	7	9	1	4	0	0	0	21
Mean		29.0	24	20	7	8	5	2	0	66
% Frequency			36.4	30.3	10.6	12.1	7.6	3.0	0.0	100
Kafr El-Sheikh	Sakha	30	9	5	2	4	2	0	0	22
	Sidy-salem	22	6	7	6	1	2	1	0	23
	Mean	26.0	15	12	8	5	4	1	0	45
% Frequency			33.3	26.7	17.8	11.1	8.9	2.2	0.0	100
El-Menoufia	El-Bagour	18	7	7	4	2	1	3	0	24
	Shebeen-El-kcoum	16	6	3	3	4	4	0	0	20
	Mean	17.0	13	10	7	6	5	3	0	44
% Frequency			29.6	22.7	15.9	13.6	11.4	6.8	0.0	100
El-Qalubia	Toukh	15	5	3	3	2	5	3	1	22
	Kaha	10	4	5	3	5	1	2	2	22
	Mean	12.5	9	8	6	7	6	5	3	44
% Frequency			20.5	18.2	13.6	15.9	13.6	11.4	6.8	100
El-Gharbia	Tanta	12	7	2	3	5	3	2	0	22
	Kafr-El Zayat	16	4	7	4	2	3	3	0	23
	Mean	14.0	11	9	7	7	6	5	0	45
% Frequency			24.4	20.0	15.6	15.6	13.3	11.1	0.0	100
Over all mean		19.7	72	59	35	33	26	16	3	244
% Frequency			29.5	24.2	14.3	13.5	10.7	6.6	1.2	100

A) *Rhizoctonia solani*

B) *Fusarium oxysporum*

C) *Sclerotium rolfii*

D) *Fusarium* sppE) *Macrophomina* sppF) *Verticillium* sppG) *Pythium* spp

On the other hand, the highest isolate number of *S. rolfsii* was recorded in Kafr El-Sheikh followed by El-Beheira and El-Gharbia . The lowest isolates number of *S. rolfsii* was recorded in El-Qalubia .These results were in harmony with those reported by **Moustafa and Khafagi (1992)** and **Abd EI-Wanis (2001)** who isolated *R. solani* and *F. oxysporum f.sp lycopersici* from tomato seedlings showing damping-off symptoms.

Also, **Abd-El-Wahab (1997 & 2004)** found that *Fusarium oxysporum f.sp lycopersici* was the main soil-borne pathogenic fungus of tomato. While **Yuan et al. (1990)** found that all isolates of *S. rolfsii* from tomato diseased plants were highly pathogenic. **Ristaino et al. (1994)** found that *S. rolfsii* was one of the most destructive soil-borne pathogens of cultivated and wild plants grown in South Eastern of USA.

Identification

The isolated fungi from different locations were identified as *F. oxysporum f.sp. lycopersici*, *R. solani* and *S. rolfsii* .

Pathogenicity test

Data in **Table (2)** indicated that tested fungi differed in their virulence against tomato plants. In this respect, *F. oxysporum f.sp lycopersici* Sa-F₂ strain seemed to be more aggressive which caused the highest percentage of pre- and post-emergence damping-off . EB-R₁ and Sa-R strains showed the highest percentage of pre- and post-emergence damping-off.

Results in **Table (2)** also indicated that *S. rolfsii* NB-S and Kz-S strains were more suppressive, they recorded the highest percentages of pre- and post-emergence damping-off.

These results were in agreement with those obtained by **Shehata (2001)** who found that *R. solani* caused the highest percentage of pre-emergence damping-off and gave the lowest of healthy survival plants compared with the other fungi .

Table 2. Pathogenicity of pathogenic fungi under greenhouse conditions

<i>Fusarium oxysporum f.sp. lycopersici</i>				
Isolate code No.	Locations	Damping-off		Survived plants
		Pre	Post	
NB-F1	El-Nubaria	0.0 ^d	46.7 ^{ab}	53.3 ^d
NB-F2	El-Nubaria	0.0 ^d	53.3 ^a	46.7 ^d
D-F	Damanhour	20.0 ^{ab}	20.0 ^{abc}	60.0 ^{cd}
EB-F1	Etay-El Baroud	13.3 ^{bc}	33.3 ^{abc}	53.3 ^d
EB-F2	Etay-El Baroud	13.3 ^{bc}	26.7 ^{abc}	60.0 ^{cd}
Sa-F1	Sakha	13.3 ^{bc}	33.3 ^{abc}	53.3 ^d
Sa-F2	Sakha	20.0 ^{ab}	46.7 ^{ab}	33.3 ^e
SS-F	Sidy-salem	20.0 ^{ab}	13.3 ^{bc}	66.7 ^{bcd}
Ba-F	El-Bagour	6.7 ^{cd}	33.3 ^{abc}	60.0 ^{cd}
SH-F	Shebeen El-koum	6.7 ^{cd}	20.0 ^{abc}	73.3 ^{a-d}
To-F	Toukh	0.0 ^d	6.7 ^c	93.3 ^a
Ka-F	Kaha	0.0 ^d	20.0 ^{abc}	80.0 ^{abc}
Ta-F	Tanta	6.7 ^{cd}	6.7 ^c	86.7 ^{ab}
KZ-F	Kafr-El-Zayat	6.7 ^{cd}	26.7 ^{abc}	66.7 ^{bcd}
<i>Rhizoctonia solani</i>				
NB-R	El-Nubaria	66.7 ^a	6.7 ^e	26.7 ^{bc}
D-R	Damanhour	40.0 ^{bc}	20.0 ^{cde}	40.0 ^{abc}
EB-R1	Etay-El Baroud	53.3 ^{ab}	26.7 ^{bcd}	20.0 ^c
EB-R2	Etay-El Baroud	26.7 ^{cd}	20.0 ^{cde}	53.3 ^a
Sa-R	Sakha	40.0 ^{bc}	40.0 ^b	20.0 ^c
SS-R	Sidy-salem	53.3 ^{ab}	6.7 ^e	40.0 ^{abc}
Ba-R	El-Bagour	26.7 ^{cd}	40.0 ^b	33.3 ^{abc}
SH-R	Shebeen EL-koum	26.7 ^{cd}	20.0 ^{cde}	53.3 ^a
To-R1	Toukh	53.3 ^{ab}	20.0 ^{cde}	26.7 ^{bc}
To-R2	Toukh	20.0 ^{cd}	26.7 ^{bcd}	53.3 ^a
Ka-R	Kaha	26.7 ^{cd}	13.3 ^{de}	60.0 ^a
Ta-R	Tanta	13.3 ^d	33.3 ^{bc}	53.0 ^a
KZ-R	Kafr El-Zayat	13.3 ^d	53.3 ^a	46.7 ^{ab}
<i>Sclerotium rolfsii</i>				
NB-S	El-Nubaria	40.0 ^b	53.3 ^a	6.7 ^d
EB-S	Etay-El Baroud	33.3 ^b	20.0 ^c	46.7 ^a
Sa-S	Sakha	40.0 ^b	26.7 ^{bc}	33.3 ^{bc}
Ba-S	El-Bagour	26.7 ^b	46.7 ^{ab}	26.7 ^c
To-S	Toukh	26.7 ^b	33.3 ^{abc}	40.0 ^{ab}
KZ-S	Kafr El-Zayat	60.0 ^a	33.3 ^{abc}	6.7 ^d

Means followed by letter(s) within each column, are not significantly different from each other at 1% level.

Abd-El-Wahab (2004) found that *F. oxysporum f.sp. lycopersici* isolates, *R. solani* and *S. rolfsii* were the most aggressive fungal isolates.

Inoculum density

Data in **Table (3)** revealed that the percentage of disease severity was significantly increased by increasing inoculum density of the three pathogenic fungi.

Regarding the *F. oxysporum* f.sp *lycopersici*, the obtained data showed that two strains of NB-F₂ and Sa-F₂ significantly varied in their aggressiveness. Sakha (Sa-F₂) strain caused the highest percentage of disease severity.

Spore density of 6x10⁷/ml recorded moderate percentage of pre- and post-emergence damping-off as well moderate percentage of survived plants . Respecting the *R. solani*, EB-R₁ and Sa-R significantly varied in their aggressiveness.

Inoculum densities (3 and 5% /kg soil) for EB-R₁ and Sa-R strains recorded moderate percentage of pre- and post-emergence damping-off. Similar trend of results was observed with *S. rolfsii* investigated strains.

These findings were in agreement with **Abd-El-Wahab (2004)** who indicated that an increase in the inoculum potential of *F. oxysporum* was associated with a corresponding increase in disease severity. Meanwhile, **Shehata (2001)** showed that the highest percentage of infection with *R. solani* and *S. rolfsii* gradually increased with the increasing of inoculum density.

Table 3: Effect of different inoculum densities of virulent pathogenic fungal strains on tomato plants (Strain B cultivar) under greenhouse conditions

Conc. Spores/ml	<i>F. oxysporum</i>		Conc. gm/kg soil	<i>R. solani</i>		Conc. gm/kg soil	<i>S. rolfsii</i>	
	%damping-off			%damping-off			%damping-off	
	Pre	Post		Pre	Post		Pre	Post
El-Nubaria (NB-F ₂)			Etay- El-Baroud (EB-R ₁)			El-Nubaria (NB-S)		
1×10 ⁷	0.0 ^c	13.3 ^c	1	6.7 ^d	20.0 ^a	1	0.0 ^d	33.3 ^{ab}
3×10 ⁷	13.3 ^{ab}	26.7 ^b	3	33.3 ^c	26.7 ^a	3	26.7 ^c	40.0 ^a
6×10 ⁷	26.7 ^{ab}	20.0 ^{bc}	5	53.3 ^b	20.0 ^a	5	53.3 ^{bc}	26.7 ^b
9×10 ⁷	26.7 ^{ab}	40.0 ^a	7	60.0 ^b	26.7 ^a	7	60.0 ^b	33.3 ^{ab}
12×10 ⁷	40.0 ^a	40.0 ^a	9	80.0 ^a	13.3 ^a	9	80.0 ^a	20.0 ^b
Sakha (Sa-F ₂)			Sakha (Sa-R)			Kafr-El-Zayat (KZ-S)		
1×10 ⁷	0.0 ^b	26.7 ^b	1	0.0 ^c	20.0 ^b	1	0.0 ^c	20.0 ^a
3×10 ⁷	6.7 ^b	33.3 ^{ab}	3	6.7 ^c	33.3 ^a	3	20.0 ^{bc}	26.7 ^a
6×10 ⁷	13.3 ^b	40.0 ^{ab}	5	26.7 ^b	26.7 ^{ab}	5	40.0 ^{ab}	20.0 ^a
9×10 ⁷	33.3 ^a	40.0 ^{ab}	7	53.3 ^a	26.7 ^{ab}	7	46.7 ^a	26.7 ^a
12×10 ⁷	40.0 ^a	46.7 ^a	9	66.7 ^a	26.7 ^{ab}	9	60.0 ^a	26.7 ^a

Means followed by letter(s) within each column, are not significantly different from each other at 1% level.

Varietal reaction

Data in **Table (4)** showed that the tested cultivars greatly differed in their reaction against the wilting and root-rot diseases. The cultivars Castle Rock, Super strain-B and UC-97 showed relative resistance to *F. oxysporum* f.sp *lycopersici* Sa- F₂. In contrast, Ace cultivar followed by Marmand proved to be the most susceptible cultivars. Meanwhile, Strain-B, Peto-86 and Money Maker were considered moderate cultivars.

Data in **Table (4)** also revealed that Castle-rock and Supper Strain-B were considered the most resistant cultivars to *R. solani* EB-R₁. Money Maker, Ace and Marmand cultivars were the most susceptible cultivars. Meanwhile, Strain-B, UC-97 and Peto-86 were considered moderate cultivars.

Moreover, the obtained results emphasized that the cultivars Castle-rock and Supper Strain-B were found resistant to *S. rolfsii*, NB-S strain. Meanwhile, Money Maker followed by Ace cultivars was the most susceptible. On the other hand, UC-97 and Peto-86 were moderate cultivars.

These results were in line with the findings of **Moustafa and Khafagi (1992)** who found that the tested tomato cultivars differed in their infection reaction by changing the kind of isolate. **Samy (1995)** found that Castle Rock and Ace cultivars were resistant and susceptible, respectively to both *F. oxysporum* f.sp. *lycopersici* and *S. rolfsii*.

Meanwhile **Shehata (2001)** studied varietal resistance of ten tomato cultivars to infection with *R. solani* and found that cvs. Strain-B followed, by Ace and cv. Marmand proved to be the most susceptible ones showing the highest percentages of infected plants. In contrast, Castle Rock was the least susceptible cultivars.

Table 4. Effect of varietals reaction of eight tomato varieties infected with the most virulent pathogenic fungi under greenhouse Conditions

Tomato varieties	<i>F. oxysporum</i> (Sa-F ₂)		<i>R. solani</i> (EB-R ₁)		<i>S. rolfsii</i> (NB-S)	
	%damping-off		%damping-off		%damping-off	
	Pre	Post	Pre	Post	Pre	Post
Castle-rock	0.0 ^d	6.7 ^c	6.7 ^f	13.3 ^a	0.0 ^f	13.3 ^a
Super train B	0.0 ^d	13.3 ^c	13.3 ^f	13.3 ^a	20.0 ^e	13.3 ^a
Strain-B	13.3 ^{bcd}	40.0 ^{ab}	53.3 ^b	13.3 ^a	53.3 ^{cd}	20.0 ^a
UC-97	6.7 ^{cd}	20.0 ^{bc}	33.3 ^{de}	13.3 ^a	26.7 ^e	13.3 ^a
Peto-86	13.3 ^{bcd}	26.7 ^{abc}	20.0 ^{ef}	13.3 ^a	33.3 ^{de}	20.0 ^a
Marmand	33.3 ^a	40.0 ^{ab}	46.7 ^{cd}	13.3 ^a	66.7 ^b	6.7 ^a
Money Maker	20.0 ^{abc}	40.0 ^{ab}	80.0 ^a	13.3 ^a	86.7 ^a	13.3 ^a
Ace	26.7 ^{ab}	46.7 ^a	53.3 ^b	33.3 ^a	60.0 ^{bc}	26.7 ^a

Means followed by letter(s) within each column, are not significantly different from each other at 1% level.

Interaction between fungal strains

Data presented in **Fig (1)** revealed that combination between the three pathogenic fungi gave the highest percentages of pre-emergence. *R. solani* and *S. rolfsii* mixture recorded 73.3 and 20.0% pre- and post-emergence damping-off, respectively. While, the percentage of healthy survived plants was 6.7%.

On the other hand, *F. oxysporum* f.sp *lycopersici* and *S. rolfsii* mixture recorded the lowest percentage of pre- and post-emergence damping-off being 33.3 and 46.7%, respectively. While, the percentage of healthy survived plants was 20.0%.

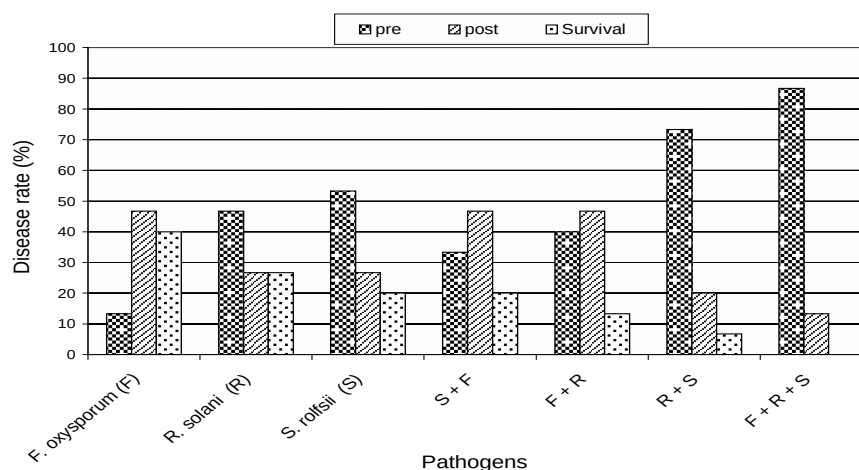


Fig 1. Interaction effect between pathogenic fungi on tomato plants under greenhouse conditions

These results were in agreement with those obtained by **Moustafa and Khafagi (1992)** who found that infested soil with *F. oxysporum* f.sp *lycopersici* and *R. solani* in combination increased infection reaction to the Fusarium wilting and root-rot.

Antagonistic effect of biological control agents

Antagonistic effect for biocontrol agents was performed to select the more potent isolates for using in subsequent experiments in this research. Screening showed that nine bacterial isolates were identified as *Ps. fluorescens*-I, *Ps. fluorescens*-II, *Ps. fluorescens*-III, *Ps. aeruginosa*-I, *Ps. aeruginosa*-II, *Ps. chlorophis*, *B. subtilis*-I, *B. subtilis*-II and *B. subtilis*-III.

Fungal isolates were identified as *T. harzianum*-I, *T. harzianum*-II, *T. harzianum*-III, *T. hamatum*-I, *T. hamatum*-II, *T. viride* and *G. virens* and showed antifungal activity against the three pathogenic fungi *F. oxysporum* f.sp *lycopersici*, *R. solani* and *S. rolfsii*. Actinomycetes exhibited antagonistic effects against pathogenic fungi were identified as *Streptomyces aureofaciens*, *Streptomyces* sp. These results were in line with the findings of **Frommell et al. (1991)** who isolated 29 *Pseudomonas* spp from rhizosphere of tomato and tested for their ability to reduce growth of *F. oxysporum* f.sp *lycopersici* and *R. solani* *in vitro*. Also, **Phae et al. (1990)** and **Abd-El-Wahab (2004)** isolated different microorganisms from rhizosphere of healthy tomato plants which were identified as *T. harzianum*, *T. viride*, *B. subtilis* and *B. cereus*.

Data in **Table (5)** illustrated in **Figs (2, 3 and 4)** demonstrated that *T. harzianum*-II was the best strain since recorded 76.8% in growth reduction of *F. oxysporum* f.sp *lycopersici* followed by *T. harzianum*-I (74.1%) in growth reduction. At the meantime, *B. subtilis*-I and *B. subtilis*-II were the best bacterial strains as biological control since they recorded 65.6 and 61.8% of growth reduction of *F. oxysporum* f.sp *lycopersici*, respectively. The lowest growth reduction of the same pathogen was observed in case of *Ps. aeruginosa*-II being 25.6%

compared with control. On the other hand, *T. harzianum*-I and *T. harzianum*-II were the best strains and showed the highest antagonistic effect against *R. solani*. These strains resulted the highest percentages of growth reduction of *R. solani* being 56.8 and 49.3%, respectively. *B. subtilis*-I was the best bacterial strain and recorded 38.9% of growth reduction of *R. solani*. While, *Streptomyces* sp and *Gliocladium virens* were the lowest strains since they recorded 2.22 and 1.85% of growth reduction of *R. solani*, respectively. *Ps. aeruginosa*-I recorded 14.1% in growth reduction of *R. solani*.

T. harzianum-I and *T. harzianum*-II recorded the highest percentage of growth reduction of *S. rolfii* being 64.1 and 59.3%, respectively. *B. subtilis*-I recorded 48.2% growth reduction.

Streptomyces sp and *Gliocladium virens* showed the lowest percentage in growth reduction of *S. rolfii* being 30.7 and 7.8%, respectively. These results were in agreement with those obtained by **Cherif and Benhamou (1990)** and **Monaco et al. (1991)** who mentioned that the antagonistic effect of *T. harzianum*, *T. koningii* and *T. viride* inhibited the growth of *F. oxysporum* f.sp *lycopersici*, *S. rolfii* and suppressed the germination of *R. solani* *in vitro*. The antagonistic effect of *Trichoderma* spp might be attributed to producing cell wall degrading enzymes glucanase and chitinase which lyse the cell wall of the pathogenic fungi, inhibition of the host mycelium and hyphal penetration by *Trichoderma*

Khalifa (1991) found that *T. harzianum* suppressed the growth of *F. oxysporum* f.sp *lycopersici* and a direct contact was observed between the two fungi. Thereafter, *T. harzianum* grew over mycelium of *F. oxysporum* f.sp *lycopersici*.

El-Kafrawy (2002) found that eight isolates of *T. harzianum* and three from both *T. hamatum* and *T. koningii* could kill all the sclerotia of *S. rolfii*

Table 5. Effect of different bio-control agents on mycelial growth of different pathogenic fungi (*F. oxysporum* f.sp *lycopersici*, *R. solani* and *S. rolfii*) *in vitro*

Biocontrol agent strains	<i>F. oxysporum</i> (Sa-F ₂)		<i>R. solani</i> (EB-R ₁)		<i>S. rolfii</i> (NB-S)	
	Linear growth (mm)	Reduction (%)	Linear Growth (mm)	Reduction (%)	Linear growth (mm)	Reduction (%)
<i>Ps. fluorescens</i> -I	51.0 ^g	43.3 ⁱ	66.7 ^d	25.9 ⁱ	58.7 ^{de}	34.8 ^{jk}
<i>Ps. fluorescens</i> -II	38.3 ⁱ	57.4 ^g	63.3 ^{ef}	29.6 ^{gh}	52.7 ^h	41.5 ^g
<i>Ps. fluorescens</i> -III	51.3 ^{fg}	42.9 ⁱ	64.7 ^{de}	28.2 ^{hi}	49.3 ⁱ	45.3 ^f
<i>B. subtilis</i> -I	31.0 ^l	65.6 ^{cd}	55.0 ^h	38.9 ^e	46.7 ^g	48.2 ^e
<i>Ps. aeruginosa</i> -1	61.0 ^d	32.2 ^k	77.3 ^c	14.1 ^j	57.0 ^{efg}	36.7 ^{hij}
<i>B. subtilis</i> -II	34.3 ^{jk}	61.8 ^{ef}	62.0 ^{fg}	31.1 ^{fg}	55.0 ^g	38.9 ^h
<i>Ps. aeruginosa</i> -II	67.0 ^b	25.6 ^l	61.0 ^{fg}	32.2 ^{fg}	56.0 ^{fg}	37.8 ^{hi}
<i>B. subtilis</i> -III	63.0 ^c	30.0 ^k	62.7 ^{ef}	30.4 ^{gh}	59.7 ^d	33.7 ^k
<i>Ps. chlorophis</i>	48.0 ^h	46.7 ^h	59.7 ^g	33.7 ^f	52.3 ^h	41.8 ^g
<i>Streptomyces</i> sp	53.0 ^{ef}	41.1 ^{ij}	88.0 ^{ab}	2.2 ^{kl}	62.3 ^c	30.7 ^l
<i>St. aureofaciens</i>	62.7 ^{cd}	30.3 ^k	86.0 ^b	4.4 ^k	57.7 ^{def}	35.9 ^{ijk}
<i>T. harzianum</i> -I	20.7 ^o	74.1 ^b	40.3 ^l	56.8 ^a	32.3 ⁿ	64.1 ^a

<i>T. hamatum</i> -I	29.7 ^l	67.1 ^c	49.0 ^j	45.6 ^c	40.0 ^l	55.6 ^c
<i>T. harzianum</i> -II	25.7 ^m	71.5 ^b	54.3 ^{hi}	39.6 ^{de}	41.7 ^{kl}	53.7 ^{cd}
<i>T. viride</i>	36.0 ^j	60.0 ^{fg}	52.3 ⁱ	41.8 ^d	46.7 ^j	48.2 ^e
<i>T. harzianum</i> -II	22.7 ⁿ	76.8 ^a	45.7 ^k	49.3 ^b	36.7 ^m	59.3 ^b
<i>T. harzianum</i> -III	33.3 ^k	62.9 ^{de}	47.7 ^{jk}	47.1 ^{bc}	43.0 ^k	52.2 ^d
<i>G. virens</i>	54.0 ^e	40.0 ^j	88.3 ^{ab}	1.8 ^{kl}	83.0 ^b	7.80 ^m
Control	90.0 ^a	0.00 ^m	90.0 ^a	0.0 ^l	90.0 ^a	0.0 ⁿ

Means followed by letter(s) within each column, are not significantly different from each other at 1% level.

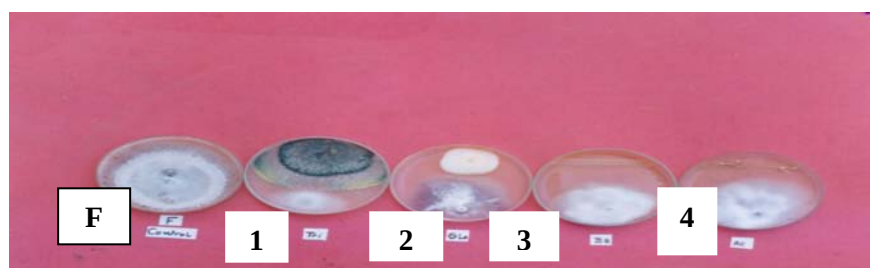


Fig 2. Effect of different bio-control agents on mycelial growth of *F. oxysporum f.sp. lycopersici* in vitro.

F = *F.oxysporum*

1= *T. harzianum*- II

2 = *Gliocladium virens*

3 = *Bacillus subtilis*-I

4 = *Streptomyces aureofaciens*

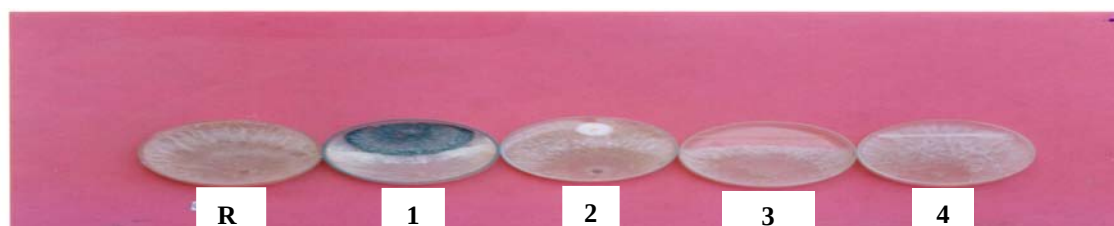


Fig 3. Effect of different bio-control agents on mycelial growth of *R. solani* in vitro.

R = *R. solani*

1= *T. harzianum*-II

2 = *Gliocladium virens*

3 = *Bacillus subtilis*-I

4 = *Streptomyces aureofaciens*

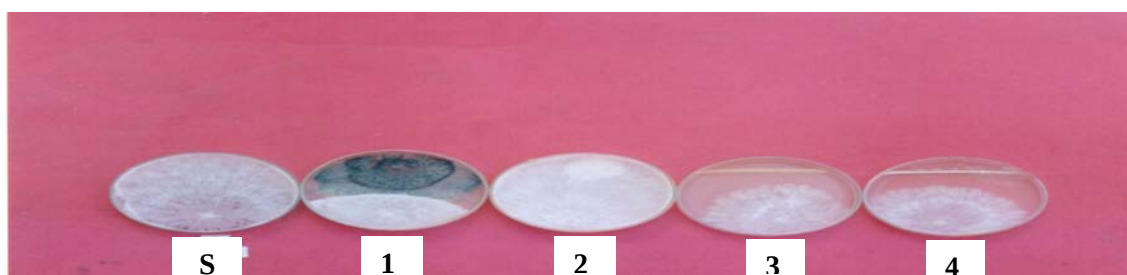


Fig 4. Effect of different bio-control agents on mycelial growth of *S. rolfsii* in vitro.

S = *S. rolfsii* 1 = *T. harzianum*- II 2 = *Gliocladium virens*
 3 = *Bacillus subtilis*-I 4 = *Streptomyces aureofaciens*

Meanwhile, **Asaka and Shoda (1996)** and **Sadler (1996)** found that *B. subtilis* strongly suppressed the growth of *F. oxysporum*, *S. rolfsii* and *F. solani* responsible for tomato wilting and damping off *in vitro* by producing antifungal and basillomycin and subtilisin antibiotics against several pathogens.

Estimation of biological control agents activity under greenhouse conditions

Data in **Table (6)** showed that all tested antagonists decreased tomato wilting disease caused by *F. oxysporum f.sp lycopersici*. In this respect, the strains of bacteria had less antagonistic effect than fungal strains. *T. harzianum*-I, *B. subtilis*-I and *T. hamatum*-II were the highly effective antagonists since they recorded the lowest percentages of pre- and post-emergence damping-off.

Also, data in **Table (6)** showed that *T. harzianum*-I and *B. subtilis*-II were the best strains for controlling of *R. solani*. While, *Ps. aeruginosa*-I recorded the lowest antagonistic effect.

Results in **Table (6)** also emphasized that *T. harzianum*-I was the best strain for controlling *S. rolfsii* followed by *B. subtilis*-I and *T. harzianum*-II. On the other hand, *Streptomyces* sp and *B. subtilis*-III recorded the lowest antagonistic effect for controlling of *S. rolfsii*. These results are in harmony with those reported by **Durman et al. (1999)** who found that *T. harzianum* and *T. viride* were more effective than other antagonistic fungi when tested in the greenhouse as seed treatments against *R. solani* and *F. oxysporum*.

Morsy (2005) proved the role of *Ps. synxantha* as antagonistic strain against *R. solani* and *F. solani* in rhizosphere of tomato. **Moussa et al. (2007)** reported that *Ps. synxantha*, *B.*

subtilis and *B. amylolique faciens* suppressed disease incidence of peanut root-rot caused by *R. solani*

Also, Niknejad *et al.* (2000) and Tsahouridou and Thanassouloupoulos (2002) found that the lower percentage of root-rot and wilting disease severity of tomato plants were obtained when the soil was firstly infested with *T. harzianum* and *T. viride*, and then re-infested with the highly pathogenic isolate of the pathogen. *T. harzianum* decreased the population of *F. oxysporum* f.sp *lycopersici* in rhizosphere, while the population of *T. harzianum* increased up to the 4th week after transplanting.

While, Sabaratnam and Traquair (2002) and Abd-El-Wahab (2004) found that tomato seed treatment with *T. harzianum*, *T. viride*, *G. virens*, *B. subtilis*, *Ps. fluorescens*, and *Streptomyces* recorded the maximum protection against pre- and post-emergence damping-off caused by *F. oxysporum* f.sp *lycopersici*, *R. solani* and *S. rolfsii* and reduced the disease incidence .

Table 6: Effect of different bio-control agents on root-rot and wilting diseases of tomato plant under greenhouse conditions

Treatments	<i>F. oxysporum</i> (Sa-F ₂)		<i>R. solani</i> (EB-R1)		<i>S. rolfsii</i> (NB-S)	
	% damping-off		% damping-off		% damping-off	
	pre	post	pre	post	pre	post
<i>Ps. fluorescens</i> -I	0.0 ^e	53.3 ^{ab}	60.0 ^b	33.3 ^a	53.3 ^{bcd}	26.7 ^{abc}
<i>Ps. fluorescens</i> -II	13.3 ^{cde}	26.7 ^{cd}	46.7 ^{bcd}	26.7 ^{ab}	40.0 ^{def}	13.3 ^{bc}
<i>Ps. fluorescens</i> -III	0.0 ^e	26.7 ^{cd}	53.3 ^{bc}	33.3 ^a	33.3 ^{d-g}	13.3 ^{bc}
<i>B. subtilis</i> -I	0.0 ^e	20.0 ^{cde}	33.3 ^{def}	13.3 ^{ab}	20.0 ^{fg}	13.3 ^{bc}
<i>Ps. aeruginosa</i> -I	26.7 ^{bc}	33.3 ^{bcd}	80.0 ^a	13.3 ^{ab}	46.7 ^{cde}	40.0 ^a
<i>B. subtilis</i> -II	20.0 ^{bcd}	33.3 ^{bcd}	26.7 ^{ef}	6.7 ^b	53.3 ^{bcd}	13.3 ^{bc}
<i>Ps. aeruginosa</i> -II	33.3 ^{ab}	20.0 ^{cde}	26.7 ^{ef}	26.7 ^{ab}	40.0 ^{def}	33.3 ^{ab}
<i>B. subtilis</i> -III	26.7 ^{bc}	40.0 ^{abc}	40.0 ^{cde}	20.0 ^{ab}	66.7 ^{abc}	26.7 ^{abc}
<i>Ps. chlorophis</i>	26.7 ^{bc}	13.3 ^{de}	26.7 ^{ef}	13.3 ^{ab}	26.7 ^{efg}	33.3 ^{ab}
<i>Streptomyces</i> sp	33.3 ^{ab}	26.7 ^{cd}	80.0 ^a	13.3 ^{ab}	80.0 ^a	6.7 ^c
<i>St. aureofaciens</i>	46.7 ^a	26.7 ^{cd}	80.0 ^a	6.7 ^b	66.7 ^{abc}	6.7 ^c
<i>T. harzianum</i> -I	0.0 ^e	13.3 ^{de}	20.0 ^f	13.3 ^{ab}	20.0 ^{fg}	6.7 ^c
<i>T.hamatum</i> -I	6.7 ^{de}	20.0 ^{cde}	33.3 ^{def}	20.0 ^{ab}	33.3 ^{d-g}	13.3 ^{bc}
<i>T.hamatum</i> -II	13.3 ^{cde}	6.7 ^e	53.3 ^{bc}	13.3 ^{ab}	33.3 ^{d-g}	6.7 ^c
<i>T.viride</i>	20.0 ^{bcd}	26.7 ^{cd}	53.3 ^{bc}	6.7 ^b	40.0 ^{def}	26.6 ^{abc}
<i>T.harzianum</i> -II	0.0 ^e	26.7 ^{cd}	20.0 ^f	20.0 ^{ab}	13.3 ^g	20.0 ^{abc}
<i>T.harzianum</i> -III	13.3 ^{cde}	40.0 ^{abc}	33.3 ^{def}	20.0 ^{ab}	33.3 ^{d-g}	13.3 ^{bc}
<i>Gliocladium virens</i>	26.7 ^{bc}	26.7 ^{cd}	46.7 ^{bcd}	20.0 ^{ab}	53.3 ^{bcd}	13.3 ^{bc}
Vitavax-thiram	0.0 ^e	13.3 ^{de}	20.0 ^f	6.7 ^b	13.3 ^g	6.7 ^c
Control	20.0 ^{bcd}	60.0 ^a	73.3 ^a	20.0 ^{ab}	80.0 ^a	20.0 ^{abc}

Means followed by letter(s) within each column, are not significantly different from each other at 1% level.

Meanwhile, **Barbosa *et al.* (1995)** and **Arndt *et al.* (1998)** reported that inoculation of seeds and roots by *Ps. fluorescense* significantly increased seedling emergence rate, reduced disease incidence and severity of damping-off. Also, **Kandoh *et al.* (2001)** and **Shehata (2001)** reported that *B. subtilis* (RB14) had the ability to protect tomato plants against infection by *F. oxysporum* and *R. solani*.

Effect of seed treatment with the potent strains of biocontrol agents and fungicide on root-rot and wilting diseases

This trial was carried out under greenhouse conditions using the more potent strains to study the effect of seed treatments namely seed soaking, seed soaking +soil drenching, seed dressing and seed dressing + soil drenching combined with three treatments namely fungicide (vitavax thiram), *T. harzianum*-I and *B. subtilis*-I.

Regarding the effect of abovementioned treatments on disease severity caused by *F. oxysporum* f.sp *lycopersici*, data in **Table (7)** revealed that seed dressing + soil drenching gave the lowest recorded of pre- and post-emergence damping-off. Meanwhile, seed soaking gave the lowest effect of seed treatment being 21.65 and 25.03 for pre- and post-emergence damping-off, respectively.

On the other hand, fungicide gave the best treatment for controlling Fusarium wilt followed by *T. harzianum*-I compared with control which recorded (10.0 and 13.3%); (10.0 and 18.35%) and (79.98 and 68.33%) for pre- and post-emergence damping-off , respectively.

Table 7. Effect of various treatments with biocontrol agents, fungicide, *Fusarium oxysporum* f.sp *lycopersici*, *Rhizoctonia solani* and *Sclerotium rolfsii* on tomato plants under greenhouse conditions

Treatments	<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i> (Sa-F ₂)							
	Pre				Post			
	Seed soaking	Seed soaking +soil drenching	Seed dressing	Seed dressing + soil drenching	Seed soaking	Seed soaking +soil drenching	Seed dressing	Seed dressing + soil drenching
<i>B. subtilis</i> -I	13.3 ^{bc}	20.0 ^b	13.3 ^{bc}	6.7 ^{cd}	40.0 ^a	26.7 ^b	20.0 ^b	26.7 ^b
<i>T. harzianum</i> -I	20.0 ^b	13.3 ^{bc}	6.7 ^{cd}	13.3 ^{bc}	26.7 ^b	20.0 ^b	20.0 ^b	6.7 ^c
Vitavax-thiram	20.0 ^b	13.3 ^{bc}	6.7 ^{cd}	0.0 ^d	6.7 ^c	13.3 ^{bc}	13.3 ^{bc}	6.7 ^c
Control	33.3 ^a	33.3 ^a	33.3 ^a	33.3 ^a	26.7 ^b	26.7 ^b	26.7 ^b	26.7 ^b
Treatments	<i>Rhizoctonia solani</i> (EB-R ₁)							
	Pre				Post			
	Seed soaking	Seed soaking +soil drenching	Seed dressing	Seed dressing + soil drenching	Seed soaking	Seed soaking +soil drenching	Seed dressing	Seed dressing + soil drenching
<i>B. subtilis</i> -I	46.7 ^{cd}	53.3 ^b	40.0 ^c	26.7 ^{de}	40.0 ^a	20.0 ^{cd}	13.3 ^{de}	13.3 ^{de}
<i>T. harzianum</i> -I	40.0 ^c	40.0 ^c	33.3 ^d	20.0 ^e	40.0 ^a	26.7 ^{bc}	6.7 ^e	13.3 ^{de}
Vitavax-thiram	33.3 ^d	20.0 ^e	13.3 ^{ef}	13.3 ^{ef}	33.3 ^{ab}	26.7 ^{bc}	20.0 ^{cd}	6.7 ^e
Control	80.0 ^a	80.0 ^a	80.0 ^a	80.0 ^a	13.3 ^{de}	13.3 ^{de}	13.3 ^{de}	13.3 ^{de}
Treatments	<i>Sclerotium rolfsii</i> (NB-S)							
	Pre				Post			
	Seed soaking	Seed soaking +soil drenching	Seed dressing	Seed dressing + soil drenching	Seed soaking	Seed soaking +soil drenching	Seed dressing	Seed dressing + soil drenching
<i>B. subtilis</i> -I	60.0 ^b	60.0 ^b	46.7 ^{bc}	40.0 ^{bc}	13.3 ^a	6.7 ^{ab}	0.0 ^b	6.7 ^{ab}
<i>T. harzianum</i> -I	53.3 ^b	46.7 ^{bc}	20.0 ^{cd}	13.3 ^d	6.7 ^{ab}	0.0 ^b	13.3 ^a	6.7 ^{ab}
Vitavax-thiram	40.0 ^{bc}	33.3 ^c	20.0 ^{cd}	13.3 ^d	6.7 ^{ab}	6.7 ^{ab}	6.7 ^{ab}	0.0 ^b

Control	80.0 ^a	80.0 ^a	80.0 ^a	80.0 ^a	6.7 ^{ab}	6.7 ^{ab}	6.7 ^{ab}	6.7 ^{ab}
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Means followed by letter(s) within each column, are not significantly different from each other at 1% level.

Table (7) indicated that seed dressing + soil drenching exhibited the best treatment which recorded the lowest percentage of pre- and post-emergence damping-off . While, seed soaking recorded the highest percentage of pre- and post- emergence damping-off .

On the other hand, fungicide was the best treatment for controlling of *R. solani* which recorded 19.98 and 21.68 % of pre- and post-emergence damping-off, respectively, followed by *T. harzianum*-I which recorded 33.33 and 21.68 % compared with control which recorded 80.0 and 13.3 % for pre- and post-emergence damping-off, respectively.

Respecting the effect of four seed treatments combined with three treatments by fungicide (vitavax thiram), *T. harzianum*-I and *B. subtilis*-I on disease severity caused by *S. rolfsii*, results in **Table (7)** revealed that seed dressing + soil drenching was the best treatment. These results were in agreement with those obtained by **Askew and Taing (1994)** and **Ghonim (1999)** who found that the application of *T. harzianum* as seed dressing and soil drenching gave the best control for root-rot of tomato caused by *F. oxysporum* f.sp *lycopersici* and tomato damping-off of caused by *R. solani*, whereas soaking of tomato seedling roots in *T. harzianum* spores suspension caused the lowest effect. Also, **Shehata (2001)** and **Guo et al. (2004)** found that seed dressing + soil drenching by rizolex-T, vitavax thiram and vitavax captan markedly reduced the percentage of infected tomato seedlings caused by *R. solani*. While, **Arndt et al. (1998)** and **Ghonim (1999)** reported that treatment of tomato seeds with *B. subtilis* and *Pseudomonas* strains reduced tomato wilting disease severity caused by *F. oxysporum* f.sp *lycopersici* and damping-off caused by *R. solan*.

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الملخص العربي

فعالية المقاومة البيولوجية ضد مسببات أمراض الطماطم الموجودة بالتربة

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تم إجراء هذا البحث لدراسة كفاءة الميكروبات التي تستخدم في مجال المقاومة البيولوجية لمقاومة أمراض أعفان الجذور والذبول في الطماطم . من أهم النتائج المتحصل عليها أن فطر *T. harzianum*-I وبكتيريا *B. subtilis*-I و *Ps. fluorescens*-II كانت من أكفأ السلالات التي عزلت وتم استخدامها في مقاومة فطريات أعفان الجذور والذبول في الطماطم وهي :

R. solani, *S. rolfsii* and *F. oxysporum* f.sp *lycopersici*

كذلك أوضحت النتائج أن تغليف بذور الطماطم مع إضافة لقاحات المقاومة البيولوجية سألقة الذكر إلى التربة أدى إلى الحصول على نتائج جيدة من حيث خفض نسبة الإصابة بالفطريات الممرضة سألقة الذكر بينما عملية نقع بذور الطماطم مع إضافة اللقاحات إلى التربة كانت أقل كفاءة من حيث خفض نسبة الإصابة. وعموماً في ضوء نتائج هذه الدراسة فإنه يجب أن يوصي باستخدام بعض الميكروبات ذات المقدرة على تثبيط أو إحباط الفطريات الممرضة وذلك لتقليل الاعتماد على استخدام المبيدات الفطرية في مقاومة مثل هذه الفطريات وهذا يؤدي إلى حماية البيئة من التلوث والحفاظ على صحة الإنسان .

STUDIES ON THE RESISTANT AND SUSCEPTIBILITY OF SOME PATHOGENIC BACTERIAL ISOLATES TO β -LACTAM ANTIBIOTIC

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ABSTRACT

Seventy seven bacterial isolates were isolated from different sources from Zagazig University Hospitals 28, 21 & 28 isolates from wound, respiratory & urine infections respectively. These isolates were tested against 10 β -lactam antibiotics. Most Gram-positive isolates were resistant to at least 6 β -lactam antibiotics while Gram-negative isolates were resistant to at least 8 antibiotics.

The MIC against β -lactam antibiotics (ampicillin, amoxycillin and cephalexin) was determined, only 9 isolates have higher level of β -lactam resistant (MIC >5000 μ g/ml) against of ampicillin and only 7 isolates have higher level of β -lactam resistant (MIC >5000 μ g/ml) against amoxycillin and cephalexine. The most resistant isolates were identified as *Pseudomonas aeruginosa* (5 isolates) , *Staphylococcus aureus* (2 isolates) and as *Bacillus cereus* (2 isolates).

The combination of sub-MICs of tested antibiotics with Amoxycillin led to a decrease in its MIC for the three *Ps.aeruginosa* tested isolates ranged from 100 to 3500 μ g/ml as compared with 5000 μ g/ml. The maximal decrease was obtained with erythromycin, azithromycin ,gentamycin , chloramphenicol and tetracyclin, respectively. While minimal decrease was obtained with clindamycin.

Plasmid isolates from the highly β -lactam resistant *Ps.aeruginosa* were detected and their DNA profiles were screened. The resistant of isolate were due to the presence of plasmid. Furthermore the protein patterns of the tested isolates were similar .Finger printing techniques and (RAPD-PCR) were used for enzymatically synthesizing defined sequences of DNA of tested isolates.

INTRODUCTION

Bacterial pathogens resistant to various β -lactam antibiotics represent a dangerous problem in the clinical field. Bacterial resistant to antibiotics may be attributed to decrease permeability of the antibiotic (Legakis et al ., 1978 and 1989) or disruption of bacterial envelop or the outer layer in gram- negative cells (Yaneyama and Nakae ., 1993) or to increase lipid biosynthesis in the bacterial cells(Conard and Gilleland., 1981 ; Brissette et al., 1982 and Abu- Shady et al., 1994).

The most important type of resistant to β -lactam agents in clinical medicine was that due to β -lactamases enzymes produced by bacteria, these enzymes capable of hydrolyzing the cyclic

amide bond in the β -lactam ring of β -lactam antibiotic (penicillin, cephalosporins and related compounds) to produce inactive degradation products. The production of β -lactamase by bacteria, therefore, play a prime role in their resistance to β -lactam antibiotics (**Matthew & Harris, 1976; Arisawa et al., 1983; Wood et al ., 1996**). Generally, bacteria with the highest level of resistance were isolated from environments contaminated with antimicrobial agents e.g. hospitals, fish frames, sewage effluents and waste water (**Datta., 1969; Smith., 1969; Grabow & Prozesky., 1973; Fontaine & Hoadley., 1976 ;Toranzo et al., 1984;McPherson & Gealt., 1986; Gauthier & Breittmayer., 1990 &1992 and Senda et al., 1996**). However, resistant bacteria had also been isolated from apparently nonselective environments (**Mach & Grimes, 1982; Levy, 1992 and Kruse & Sorum, 1994**).

Pseudomonas aeruginosa was a clinically trouble some Gram negative pathogen that caused wide range of opportunistic infections and nosocomail outbreaks (**Lowbury et al, 1970 and Koch&Hoiby,1993**). *Ps.aeruginosa* isolates generally demonstrated resistance to various antimicrobial agents .There resistance to multiple antimicrobial agents was often associated with the production of specific enzymes, such as β -lactamases (**Lodge et al, 1990; Senda et al .,1996 and Marchandin et al.,2000**). *Ps.aeruginosa* was usually resistant to penicillin, ampicillin, cephalothin, tetracycline, chloramphenicols and amino glycosides. There also the catabolic plasmids carrying genes coding for the enzymes required in the breakdown of substances like naphthalene, salicylate and toluene. Some of these plasmids have a wide host range including most Gram- negative species and therefore *Ps.aeruginosa* is also a potential vector for the spread of drug resistance to other species (**Williams et al ., 1984; O'Morchoe et al ., 1988 and Kruse & Sorum ., 1994**).

Zaid (2001) studied the resistance patterns of *Ps.aeruginosa* isolates to β -lactam antibiotics and found that all isolates under investigation were resistant to at least 7 of tested antibiotics, and two isolates were resistant to 11 antibiotics. **El-Awady (2005)** found that the prevalence of resistant among (64) *Ps. aeruginosa* isolated in the study was high for most antibiotics tested. The most effective antibiotic against *Ps.aeruginosa* strains was Imipenem. While the highest percentage of resistant isolates were observed for ampicillin and piperacillin (100%).

EL-Aidy (2008) found that the prevalence of resistant among four *Ps.aeruginosa* isolated was high for most effective antibiotics tested. Imipenem was the most effective antibiotic while the highest percentage of resistant were observed for claforan, gentamycin, velosef,

chloramphenicol, rifampicine, tetracyclin, flumox, penicillinG , vancomycin, amoxacillin, cfr , erthromycin and ampicillin (100%) followed by oxacillin while streptomycin , nigram, STX (75%).

Resistant to antimicrobial agents may be mediated by genes that were encoded on the host cell chromosome, plasmids or transposons, which were capable of integrating into the plasmid and /or into chromosomes (Joly et al., 1986 ; Williams et al., 1988 ; Agodi et al., 1990 , 1995 and Tenover & McGowan., 1996).

PCR was used for direct detection and amplification of several types of bacteria. For example on *Ps.aeruginosa* and on *Serratia marcescens*(Arakawa et al., 1995 ; Kureishi et al ., 1994; Poole et al., 1996 ; Vos et al., 1997; Liao & Hancock, 1997 and Ziha- zarifi et al ., 1999)

The objective of the present study is therefore to evaluate the activity of some β -lactam antibiotics which communally used in clinical field against some clinical bacterial isolates from different sources (wound ,respiratory and urine infection).Moreover isolation of plasmids &apply (RAPD-PCR) for determination of DNA finger print of selected highly resistant isolates were carried out.

MATERIAL AND METHODS

1-Sample collection: -

Medical specimens of pus, sputum and urine were collected from different Zagazig University Hospitals, Egypt. Samples were collected in period from October 2001 to March 2002.

2-Isolation, Purification &Identification of bacterial isolate.

Sample were collected from wound, respiratory and urine infection as pus ,sputum and urine by sterile swab , clean dry cups or aspiration of end tracheal tubes and sterile container (respectively). All collected sample were transferred to laboratory within few hours, a pure culture were maintained on slant of nutrient agar at 4-5 °C for further studies. The cultural, cell morphological and physiological characteristics of the selected isolates, were studied. Media and reagent were prepared according to stander procedures as described by(Lennette et al 1979). Identification was carried out by traditional biochemical tests according to (Sneath et al., 1986; Holt et al., 1994 and Murray et al.,1999) .

3-Ant biotic susceptibility test:

The susceptibility patterns (antibiograms) of the collected isolates to different β -lactam antibiotics had been carried out according to Bauer- Kirby single disc diffusion method (Bauer et al., 1966).

The isolates were tested against 10 β -lactam antibiotics namely aztreonam, ceftriaxone, cephalexin, penicillinG, ampicillin, methicillin, amoxycillin, amoxycillin /clavulanic acid, cloxacillin and sulperazone.

4-Determination of the Minimum Inhibitory Concentrations (MIC_S) and Minimum Bactericidal Concentrations (MBC_S)

The minimal inhibitory concentration (MIC) of ampicillin, amoxycillin and cephalexine for each isolate was determined by standard dilution technique (**Washington and Sutter, 1980**).

Approximately 10^6 colony-forming units one ml of bacterial suspension was inoculated into nutrient broth containing varying dilutions of ampicillin, amoxycillin, and cephalexine each alone, the final concentration ranged from 0.375 to 50 mg/ml medium. Tubes were incubated at 37 °C for 18-20 hrs and the MICs recorded as the lowest concentrations that inhibit the growth completely.

To determine minimal bactericidal concentration (MBC) 100 μ l was taken from each MIC concentration as well as the lower concentration and introduced into nutrient agar medium. The plates were incubated at 37°C four 24 hours .The minimal bactericidal concentration(MBC) is the lowest concentration at which no growth occurs on solid media.

5-Determination of (MIC_S) of highly resistant *Ps.aeruginosa* to some antibiotics:-

The minimal inhibitory concentration (MIC) of azithromycin, gentamycin, clindamycin, tetracyclin, chloroamphenicol and erythromycin for three selected isolated were determined by using the previous standard broth dilution technique. Approximately 10^6 colony forming units(c.f.u) .(0.1 ml of bacterial suspension) was inoculated into nutrient broth containing varying dilution of each antibiotic .

6- β -lactamase activity:

β -lactamase activity was determined in both cell free broth (extra cellular) and in bacterial cells (intra cellular) by micro-iodometric method as described by **Matthew et al.(1979)**.

7-Plasmid isolation: Detection of plasmid DNA in the three tested *Ps.aeruginosa* isolates were done according to (**Maniatis et al., 1982**).

8-Polyacrylamide Gel Electrophoresis (PAGE): Proteins of highly β -lactam resistant *Ps.aeruginosa* isolates were separated by PAGE according to the method of **Davis,(1964)**.

9-Random amplified polymorphic DNA Polymerase Chain Reaction (RAPD-PCR):

Finger printing techniques and PCR-based genomic profiling assay for *Ps.aeruginosa* had been described by (Hassan et al., 1994).

RESULTS

Table 1 shows that the 77 collected bacterial isolates are distributed into 51 Gram positive bacterial isolates (66.23%) and 26 Gram negative isolates (33.76%). Table 1 also shows that 36.5% from all isolates are from wound infection, 27% from all collected isolates were from respiratory infection and isolates from urine infection represent 36.5% also.

Antibiotic susceptibility test

The data in table 2 revealed that, 33.3% of Gram positive isolates were susceptible to amoxycillin/clavinc acid, 13.7% were susceptible to both cloxacillin and ceftriaxone, 3.9% were susceptible to both ampicillin and methicillin and one isolates 1.9% were susceptible to amoxycillin. On the other hand, all or most isolates were resistant to penicillinG 100%, sulperazone 100%, amoxycillin 98.1%, ampicillin 96.1%, aztreonam 96.1% and cephalixin 90.2%. Also the antibiotic activity of β -lactam antibiotics against 26 Gram negative isolates collected from different clinical specimens. 7.7% of total Gram-negative isolates were susceptible to both methicillin and amoxycillin/clavinc acid and two isolates (3.8%) were susceptible two aztreonam. All or most isolates were resistant to the following antibiotic: penicillinG (100%), cephalixin (100%), amoxycillin (100%), coloxacillin (100%) and sulperazone (100%). Also (96.2%) of total Gram negative isolates were resistance to both ampicillin and aztrtreonam, (92.3%) of total Gram negative isolates were resistance to amoxycillin/clavanic acid and (88.5%) were resistant to ceftriaxone.

Table 3 show that all gram positive isolates collected from wound infection (16 isolates) were resistant to the following antibiotic; penicillinG , sulprazone, ampicillin , amoxycillin , amoxycillin/alavinic acid and cloxacillin. Also,(15 isolates) represent 93.8% from collected strains of the same source were resistant to azetronem , ceftraxione ,cephalexin and methacillin . All sample collected from respiratory infection were resistant to two antibiotic penicillinG and sulprazone. Also, 18 samples represent 94.7% were resistant to azetroneom, ampicillin and amoxicillin. But 78.9%, 52.6%, 31.6%, and 26.3% from the same samples were resistant to cephalixin, ceftraxone, methaicillin ,amoxycillin/clavinc acid and cloxacillin respectively. All samples which collected from urine were resistant to azetroneom, cephalixin,

pencillin G, amoxycillin and sulprazone but about 81.3% were resistant to both ceftraxione and cloxacillin.

Table 4 shows that the total numbers of Gram negative isolates collected from different sources (26 isolates) were resistant to the following antibiotic penicillin, cephalixin, amoxycillin, cloxacillin and sulperazone.

All collected samples from the wound infection were resistant to ampicillin , methicillin and sulperazone, 91.7% of total sample collected from wound infection were resistant to both amoxycillin /clavinc acid and azetronem, and 83.3% of the same sample source were resistant to ceftriaxone. Two sample collected from respiratory infection were resistant to seven examined β -lactam antibiotic. But only one of them were resistant to other three antibiotic ceftrizxone, methicillin and amoxycillin. All gram negative isolates collected from urine infection (12 isolate) were resistant to the following antibiotic: azetreonam, ceftraxone, cephalixin, penicillinG, amoxycilin, amoxycillin/Clavinc acid, collaxcillin and sulperazone , and 91.7% of the samples were resistant to both ampicillin and methicillin.

Screening of β -lactam resistant isolate

Table 5 shows that number of resistant isolates growing on the surface of nutrient agar medium supplemented with ampicillin 375 μ g/ml were (57 isolates) is higher than that growing in the same medium supplement with amoxyicillin or cephalixin (52 isolates) on the same condition.

Susceptibility of β -lactam resistant isolates to tested β -lactam antibiotics.

The results were represented in table 6, only 7 isolates had higher levels of amoxycillin – resistant and cepalexine each alone, and 9 isolates for ampicillin (MIC>5000 μ g/ml), this resistance may be raised from its ability to produce the enzyme that catalyzes and hydrolysis β -lactam ring present in beta-lactam antibiotic.

Identification of highly resistant isolates

The cultural morphological and physiological characteristic of the nine isolates achieved the higher level of resistant to beta-lactam antibiotics (MIC>5000 μ g/ml) , which known as isolates number 8, 9, 17, 22, 46 and 76 were identified as *Ps.aeruginosa*, isolates No. 22 and 64 were identified as *S.aerues* and isolates No.13 and 65 were identified as *B.cereus* according to (Sneath et al.,1986;Holt et al., 1994 and Murray et al.,1999) .

MICs and MBCs of *Pseudomonas aeruginosa* isolates to some antibiotics.

Table 7 showed the MICs and MBCs of Gentamycin, azithromycin, clindamycin, tetracycline, chloroamphenicol and erathromycin for highly resistant *Ps. aeruginosa* ranged from 100 to 3500 µg/ml, with respect that the lowest MIC_s & MBC_s were achieved with erathromycin while the highest with clindamycin.

The effect of sub-MICs of some antibiotics on the MIC of amoxycillin on *Ps. aeruginosa* isolates.

Results in table 8 revealed that the combination of sub-MICs of tested antibiotics with amoxycillin led to a decrease in its MIC for the three tested isolates ranged from 100 to 3700 µg/ml as compared with the control (5000 µg/ml). The maximal ascending decrease was obtained with erthromycin ,azithromycin ,gentamycin , cholroamphenicol and tetracyclin, respectively. While the highest resistant was obtained with clindamycin .

β- lactamase activity of *Ps.aeruginosa* isolates.

The data in table 9 clearly reveal that the bacterial cells of the three, isolates had a very low β-lactamase activity. On the otherhand the enzyme activities were higher in the cell free broth. The three isolates can be arranged in the following decreasing order of β-lactamase activity: isolate No. 76 > isolate No. 46 > isolate No. 17.

Detection of plasmid profile:

Fig 1 showed that lane (1), (2) and (3) the presence of plasmid in the tested *Ps.aeruginosa* , isolates No 17 , 46 & 76 with molecular weight 58578, 47683 and 45960 Kbp which indicated the resistance to the tested antibiotics due to plasmids.

Protein pattern:-

The protein finger print technique was carried out for the selected *Ps.aeruginosa* to confirm the identification of isolates. Results presented in fig 2 and tables 10 showed in the electrophotogram (scan of protein profiles or finger prints of the total cellular proteins of the vegetative cells of tested isolates No. 17, 46 and 76 were fractionated in denatured gel by electrophoresis (SDS-PAGE). The protein profiles isolates No. 17, 46 and 76 contains 27, 26 and 26 respectively. As in tables 10, 11&12 and figs 3&4 which showed that comparison of protein patterns from 3 isolates were similar especially in the high molecular weight parts of

the gels. Most of the bands in each isolate could be matched with bands in each of the others. As shown in tables 10, 11 & 12.

The result in fig 3 & 4 and table 10 shows in the electrophoregram (scan of protein profiles or fingerprints) of total cellular proteins *Ps.aeruginosa* isolates revealed from 26 to 27 discrete protein bands.

Random amplified polymorphic DNA polymerase Chain Reaction (RAPD-PCR).

This analysis is another version of employing the PCR technology for differentiating living organisms from each others. It was also used different DNA primers, however, these primers were not specific to certain region of any gene but they were designed to be short and they come in a package of primers each set and if they found their complementary sequences they do amplify the region of the gene between them to produce PCR pattern. Therefore, the process is totally random and the products should be different for different isolates except the identical organisms, they should produce the same PCR product profile pattern. Genomic DNA had been used in RAPD-PCR. Nine primers from random primers kits were used and only five of them produce meaningful results, these primers were (C₄ , C₇ , Z₁ , Z₅ and B₈) as in figures 5 & 6 .

Phylogenetic position relation between the three isolates:

In order to determine the phylogenetic relation between the three tested isolates, the genomic DNA of the bacteria was extracted and the DNA was amplified by RAPD-PCR. The nucleotide sequence of the amplified DNA RAPD fragment of three isolates was determined and compared fig 7.

DISCUSSION

Production of β -lactamase by the pathogenic bacteria especially those having β -lactamase activity, was well documented by several investigator (**Richmond and Sykes, 1973; Farrar and Odle., 1976 ; Olsson et al., 1979 ; Brown., 1988 ; Abdel Fatah et al, 1993&1994 ;Bush,1991 and Hamouda, 2000 ;Zaid ,2001 and El- Aidy 2008**). Studying the occurrence of bacterial pathogen resistant to β -lactam antibiotic in clinical field was observed by many author, (**Nour El-Din, 1999**) ,found the high level of resistance to cepharadin among 135 isolate obtained from different sources. (**Hamouda,2000**) found high level of resistant to amoxycillin from 102 isolates from different sources.(**Zaid, 2001**) study the resistant of 94

strains of *Ps.aeruginosa* to 12 β -lactam antibiotics and found that all tested organisms were resistant to at least 7 antibiotics (penicillin ,ampicillin ,amoxycillin, amoxycillin /clavanic acid, cepalexin, cephaoridin and cloxacillin).

The present work was concerned with studying of resistant to β -lactam antibiotic and β -lactamase production of clinical specimens isolated from different sources at Zagazig University Hospitals, Egypt. The present study was initiated to made isolation, purification and initial morphological identification by Gram-stain to 77 clinical specimens isolated from different sources. Data showed that 28 were isolated from urinary tract infection, 21 from respiratory infection and 28 from wound infections. The studying of the antibiotic susceptibility patterns of 77 isolates against 10 β -lactam antibiotics was investigated by using single diffusion method by **Bauer et al., 1966**. Antibiotics include the following aztreonam, ceftriaxone, cephalixin, penicillin G, ampicillin, methicillin, amoxycillin, amoxycillin / clavinc acid, cloxacillin and cefoperazone / sulbactam (sulprazone).

The results in this study showed that Gram-positive isolates were susceptible to (amoxycillin / clavanic acid) 33.3%, 13.7 % were susceptible to ceftriaxone and cloxacillin and 3.9 were susceptible to both ampicillin and metaicillin and 1.9 were susceptible to amoxycillin. 7.7% susceptible to methacillin and amoxycillin/clavanic acid , 3.8% susceptible to aztreonam. **Bonfiglio et al., 1998** concluded that the different levels of resistance were found are in accordance with the high use of drug in different centers.

This result matched with the study of **Giacommetti et al ., 2000** who reported that a very low susceptibility rate (17% sensitivity) was found for ampicillin . Also matched with study of **EL-Awady ,2005** who reported that penicillin G and ampicillin showed the lowest activity against all tested Gram-positive and Gram-negative bacterial isolates. Also matched with the study of **El- Aidy 2008** who found that penicillin G and ampicillin showed low activity against all the tested Gram-positive isolated and Gram-negative isolates. The susceptibly patterns of penicillin G were 38.8%, 15.3%, 0%and 0% against *Staph.aureues*, *K.penumoniae* , *E.coli* and *Ps.aeruginosa* respectively. The susceptibility patterns of Ampicillin were 33.3%. 16.6%, 15.3% and 0% against *E.coli*, *Staph.aureues*, *K.penumoniae* and *Ps.aeruginosa*. On the other hand these results not match with the study of **Matsukawa et al. (2001)** who found that Penicillin and first to second generation Cephalosporin(old β -lactam antibiotics) can prevent bacterial infection except those caused by resistant microorganism.

Furthermore, result showed that Gram-positive and Gram-negative isolate were resistant to at least 6 and 8 antibiotics from 10 tested β -lactam antibiotics, respectively. the result were nearly in accordance with the results of **Zaid ,(2001)** who found that all *Ps.aeruginosa* isolates were resistant to at least 7 antibiotics from 12 β -lactam of tested antibiotics.

In this study screening of β -lactam resistant isolate were performed by inoculating the collected isolates on Nutrient agar plate containing conc.375ug/ml medium from 3 β -lactam antibiotics each alone. The isolates which grow in these conditions are the highest resistant one to these antibiotics. The result showed that 57, 52and 52 each out of 77 isolates collected were grow on the media containing conc 375 μ g/ml of ampicillin, amoxycillin and cephalixin, respectively.

Minimum inhibitory concentrations (MICs) of the isolates grow in media containing 375 μ g/ml were performed by using dilution method the result revealed that only 7 isolate achieved higher level of β -lactamase (MIC >5000 μ g/ml) with amoxicillin, and 9 isolate achieved higher β -lactamase activity (MIC > 5000 μ g/ml) with ampicillin and cephalixin each alone. This resistant may be arised from their ability to produce the enzyme that catalyzes and hydrolysis of β -lactam ring present in β -lactam antibiotic (**Mathew & Harris, 1976; Roy et al., 1992; Kunugita and Hyodo., 1995 and Arpin et al 1996**).

Despite improvement in antibiotic therapy, *Ps.aeruginosa* remained the most prominent Gram negative rods causing hospital associated infections and a major source of morbidity and mortality in an intensive care unit (**Morrison &Wenzel, 1984; Wade & Schimpff, 1988 and Yee et al., 1996**).

In the present investigation identification of highly resistant β -lactam antibiotics (9 isolates) were performed. Isolates No. 8, 9, 17, 46, 76 were identified as *Ps.aeruginosa* , isolates No. 22 and 64 were identified as *Staph.aureus* and isolates No. 13, 65 were identified as *B.cereus* according to their cultural morphological and physiological characteristics (**Holt, 1994**). These results matched with the study of (**Nour El-Din, 1999**) who found that 102 isolates from 135 isolates had cephalosporinase activities. Among them 52 isolates exerted high cephalosporinase activity. Only 8 isolates from them had the higher levels of cephalosporin resistance (MIC>5000 μ g/ml). and 2 isolates belonging to *Salmonella typhi*, 2 isolates

belonging to *Ps.aeruginosa* , 2 isolates belonging to *E.coli* and 2 isolates belonging to *Staph.aureus*. Also by matching the results with **Hamouda, 2000** who recorded that 102 isolates had β -lactamase activities. Among them 42 isolates exerted high β -lactamase activities. Only 4 isolates from them had levels of amoxycillin-resistant ($MIC > 5000 \mu g/ml$), and four isolates identified as *Proteus penneri*, *Ps.aeruginosa* , *Proteus vulgaris* and *Klebsiella pneumoniae*.

In this study 5 isolates from 9 were identified as *Ps.aeruginosa* so we chose *Ps.aeruginosa* for further studies. 3 isolates from *Ps.aeruginosa* gave high enzyme activities 5.2, 5.9, and 6.6 unit /ml culture for *Ps.aeruginosa* No. 17, 46 and 76 respectively. The results in this study revealed that a combination of sub MIC_s of some antibiotics (gentamycin, azithromycin, clindamycin, tetracyclins, chloroamphenicol and erthromycin) with amoxycillin lead to a decrease in MIC of amoxycillin for the three tested *Ps.aeruginosa* isolates from 100 to 3700 $\mu g/ml$ compared with 5000 $\mu g/ml$. Also the results recorded that the maximal decrease on the MIC obtained by the combination of amoxycillin with erthromycin ,azithromycin and gentamycin with fraction of 1/2 ,1/4 & 1/8 of their MIC_s . And the minimal decrease on the MIC obtained by combination of Sub MIC of clindamycin, tetracyclin and chloroamphenicol for all tested isolates. This results were in agreement with the study of **Hamouda , 2000** was reported that combination of sub MIC_s of erthromycin with amoxycillin lead to a decrease in MIC of amoxycillin of *K.pneumonia*, *Ps.aeruginosa*, *Pr.penneri* and *Pr.vulgaris*.

The results not matched with the study of **Nour El-Din, 1999** and **Hamouda , 2000** which recorded that combination between sub MIC of clindamycin lead to maximal decrease of MIC of β -lactam antibiotics (amoxycilin & cephadrin) for all tested isolates.

Bacterial resistance to β -lactam antibiotics was often associated with the production of β -lactamase (penicillin amidohydrolase), which hydrolyses the cyclic amide bond in the β -lactam ring of penicillins, cephalosporins, and related compounds and change these compounds to inert and ineffective agents (**Dathoff & Cullmonn., 1984; Aronoff & Shalaes., 1987; Letendre et al., 1988 ; Lodge et al., 1990 ; Bush et al., 1991; Mascaretti et al., 1999 and Yan et al ., 2000**).

The data in this study clearly revealed that the bacterial cells of the three *Ps.aeruginosa* isolates had a very low β -lactamase activity, while the enzyme activities were higher in the cell free broth.

The most distributing feature of *Ps.aeruginosa* is its broad resistance to antibiotics. This resistance may be plasmid mediated. There are at least thirteen group of plasmid based on compatibility, they carry markers conferring resistance to various antibiotics as well as chemicals (Kruse and Sorum, 1994). Plasmid DNA profile can be used as an epidemiological marker, but in combination with other typing methods and with antibiotic resistance patterns to characterize the circulation of *Ps.aeruginosa* strains and the spread of resistance in these bacteria (Millesimo et al ., 1996).

The results in this study reported that in three chosen β -lactam resistant strains of *Ps.aeruginosa* No. 17, 46 and 76 revealed presence of plasmids of sizes 48.578, 47.683 and 45.960 Kbp, respectively. These results were in accordance with the results of Gerherdt (1994) stated that plasmid sizes ranged from 1-200 Kpb.

Tables & Figures

Table 1:- Distribution of collected isolates according to their Gram stain reaction.

Source of bacterial sample	Gram positive isolates		Gram negative isolates		Total	
	No.	%	No.	%	No .	%
Wound infection	16	20.78	12	15.58	28	36.36
Respiratory infection	19	24.68	2	2.6	21	27.3
Urine infection	16	20.78	12	15.58	28	36.36
Total	51	66.234	26	33.766	77	100

Table 2:-The antibiotics susceptibility of Gram –positive and negative bacterial isolates to β -lactam antibiotics.

Antibiotic	Code	Conc. μ g/disc	Gram –positive isolates			Gram- negative isolates		
			Resistant %	Moderately susceptible %	Susceptible %	Resistant %	Moderatory susceptible %	Susceptible %
Aztreonam	AT M	30	96.1%	3.9 %	0.0 %	96.2 %	0.0 %	3.8 %
Ceftriaxone	CR O	30	74.50%	11.8 %	13.7 %	88.5 %	11.5 %	0.0 %
Cephalexin	CL	30	90.2%	9.8 %	0.0%	100 %	0.0 %	0.0 %
Penicillin G	P	10	100%	0.0 %	0.0 %	100 %	0.0 %	0.0 %
Ampicillin	AM	10	96.1%	0.0 %	3.9 %	96.2 %	3.8 %	0.0 %
Methicillin	ME T	5	86.3%	9.8 %	3.9 %	92.3 %	0.0 %	7.7 %
Amoxycillin	AM X	30	98.1%	0.0 %	1.9%	100 %	0.0 %	0.0 %
Amoxycillin /Clavinc Acid	AM C	30	66.7%	0.0 %	33.3%	92.3 %	0.0 %	7.7 %
Cloxacillin	OB	5	66.7 %	19.6 %	13.7 %	100 %	0.0 %	0.0%
Cefoperazone (75μg)/Sulbactam (30μg) (sulperazone)	SCF	10 5	100.0%	0.0 %	0.0%	100 %	0.0 %	0.0 %

Table 3:- Percentage of resistant of Gram positive isolate to β -lactam antibiotic according to source of isolation.

antibiotics Source of isolation	No. of examined isolate	Number of resistant isolate																			
		ATM		CRO		CL		P		AM		MET		AMX		AMC		OB		SCF	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Wound infection	16	15	93.8	15	93.8	15	93.8	16	100	16	100	15	93.8	16	100	16	100	16	100	16	100
Respiratory infection	19	18	94.7	10	52.6	15	78.9	19	100	18	94.7	17	89.5	18	94.7	6	31.6	5	26.3	19	100
Urine infection	16	16	100	13	81.3	16	100	16	100	15	93.8	12	75	16	100	12	75	13	81.3	16	100

The total examined isolates were 51

ATM Aztreonam, CRO Ceftriaxone, CL Cephalexin, P Penicillin G, AM Ampicillin, MET Methicillin, AMX Amoxycillin, AMC Amoxycillin /Clavinc Acid, OB Cloxacillin, SCF Cefoperazone (75 μ g)/Sulbactam (30 μ g) (sulperazone).

Table 4:-Percentage of resistant of Gram negative isolate to β -lactam antibiotic according to source of sample.

antibiotics	No. of examined isolate	Number of resistant isolate																			
		ATM		CRO		CL		P		AM		MET		AMX		AMC		OB		SCF	
		No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Source of isolation																					
Wound infection	12	11	91.7	10	83.4	12	100	12	100	12	100	12	100	12	100	11	91.7	12	100	12	100
Respiratory infection	2	2	100	1	50	2	100	2	100	2	100	1	50	2	100	1	50	2	100	2	100
Urine infection	12	12	100	12	100	12	100	12	100	11	91.7	11	91.7	12	100	12	100	12	100	12	100

Table 5:- Screening of resistant isolates to different β -lactam antibiotic (375 μ g/L) The total examined isolates were 26

β -lactam antibiotic	Resistant isolates	% of resistance
Ampicillin	57	74.02
Amoxycillin	52	67.53
Cephalexin	52	67.53

ATM Aztreonam, CRO Ceftriaxone, CL Cephalexin, P Penicillin G, AM Ampicillin, MET Methicillin, AMX Amoxycillin, AMC Amoxycillin /Clavinc Acid, OB Cloxacillin, SCF Cefoperazone (75 μ g)/Sulbactam (30 μ g) (sulperazone).

Table 6: Susceptibility of β -lactam -Resistant Isolates to β -lactam antibiotics

Number of viable bacteria			MIC (μ g/ml)
ampicillin	amoxycillin	Cephalexine	
57	52	52	>375
50	47	45	>500
55	40	40	>750
52	38	37	>1000
50	37	33	>1250
46	34	30	>1500
43	33	27	>1750
39	30	26	>2000
35	28	25	>2250
33	26	25	>2500
30	25	23	>2750
30	24	22	>3000
27	24	22	>3250
25	24	21	>3500
25	23	20	>3750
24	22	19	>4000
20	18	17	>4250
17	12	14	>4500
14	11	12	>4750
9	7	7	>5000

Table 7:- MIC and MBC for *Pseudomans areuginosa* species No.(17) , No.(46) and No.(76) for different antibiotics.

Bacterial species	Antibiotics	MIC (µg/ml)	MBC (µg/ml)
<i>Ps.areuginosa</i> No.(17)	Azithromycin	300	≥ 500
	Gentamycin	400	≥600
	Clindamycin	3500	≥4000
	Tetracycline	3000	≥3500
	Chloroamphenicol	600	≥1000
	Erthromycin	100	≥100
<i>Ps.areuginosa</i> No.(46)	Azithromycin	400	≥500
	Gentamycin	600	≥900
	Clindamycin	3500	≥4000
	Tetracycline	3000	≥3500
	Chloroamphenicol	1000	≥2000
	Erthromycin	100	≥100
<i>Ps.areuginosa</i> No.(76)	Azithromycin	600	≥1000
	Gentamycin	800	≥1000
	Clindamycin	3500	≥4500
	Tetracycline	3000	≥3500
	Chloroamphenicol	1300	≥2000
	Erthromycin	250	≥300

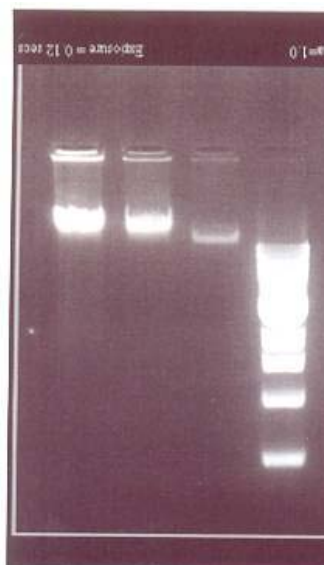
Table8:- Effect of sub MICS of Azithromycin, Gentamycin, Clindamycin, Tetracyclin, Chloroamphenicol, Erthromycin and Clavulanic acid on MIC of Amoxycillin for selected isolates *Ps.aeruginosa* No.(17) ,(46) and (46)

Antibiotics	MIC of Amoxycillin (µg/ml)			
	Sub MIC µg/ml	<i>Ps.aeruginosa</i> No.(17)	<i>Ps.aeruginosa</i> No.(46)	<i>Ps.aeruginosa</i> No. (76)
Amoxycillin	control	≥5000	≥5000	≥5000
Azithromycin	1/2	200	700	850
	1/4	250	750	1000
	1/8	350	900	1250
	1/16	500	1000	1500
Gentamycin	1/2	300	800	1000
	1/4	400	950	1100
	1/8	600	1000	1300
	1/16	700	1200	1700
Clindamycin	1/2	3000	2950	3300
	1/4	3150	3000	3450
	1/8	3200	3200	3600
	1/16	3350	3350	3700
Tetracycline	1/2	1000	1100	1150
	1/4	1150	1300	1300
	1/8	1300	1550	1600
	1/16	1450	1950	2000
Chloroamphenicol	1/2	600	1000	1300
	1/4	750	1250	1400
	1/8	800	1400	1600
	1/16	900	1500	1700
Erythromycin	1/2	200	210	300
	1/4	250	300	310
	1/8	300	350	420
	1/16	450	400	450

Table 9: β -lactamase activity of *Ps.aeruginosa* isolates (unit/ml) and their dry weight (mg/ml).

Bacterial isolates	β -lactamase activity (Unit/ml)			
	cell-free supernatant	cells culture	Total units/ml	Dry weight mg/ml culture
<i>Pseudomonas aeruginosa</i> (17)	3.9	0.5	4.4	1.39
<i>Pseudomonas aeruginosa</i> (46)	4.2	0.7	4.9	1.4
<i>Pseudomonas aeruginosa</i> (76)	5.1	0.5	5.6	1.5

Lane 1 lane 2 lane 3 lane 4

**Fig(1)**Plasmid profileLane 1 = *Ps.aeruginosa* No. 17Lane 2 = *Ps.aeruginosa* No 46Lane 3 = *Ps.aeruginosa* No 76

Lane 4= Marker

Table 10: The number of the bands of cellular protein and its molecular weight of highest concentration of proteins bands in relation to (SDS-PAGE)

Bacterial strains	Total No. of the bands	M.wt of highest conc of protein
<i>Ps.aeruginosa</i> No.17	27	466.407
<i>Ps.aeruginosa</i> No.46	26	387.194
<i>Ps.aeruginosa</i> No. 76	26	352.538

Lane 1 lane 2 lane 3 lane4

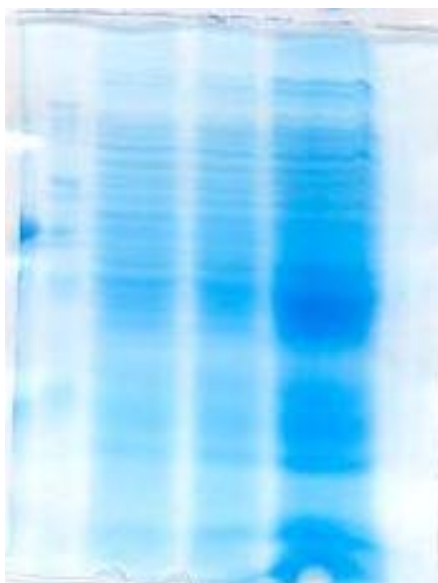


Fig (2) protein pattern

Lane 1 = marker (208.964, 119.849, 99.109, 52.799, 37.513, 28.855, 19.374, 6.894).

Lane 2 = *Ps.aeruginosa* No. 17

Lane 3 = *Ps.aeruginosa* No.46

Lane 4 = *Ps.aeruginosa* No.76

Table 11: Molecular weight's of protein products of *Ps.aeruginosa* isolates No. 17, 46 and 76.

M.wt values No. of bands	Lane 1	Lane 2	Lane 3	Lane 4
Band 1	208.964	466.407	387.194	352.538
Band 2	119.849	402.047	367.391	312.932
Band 3	99.109	382.243	322.833	228.767
Band 4	52.799	352.538	176.112	160.424
Band 5	37.513	170.803	119.417	119.272
Band 6	28.855	119.272	118.523	118.208
Band 7	-	117.711	116.993	116.402
Band 8	-	115.295	114.006	113.13
Band 9	-	109.549	108.693	109.127
Band 10	-	104.732	101.132	103.004
Band 11	-	97.152	92.273	95.197
Band 12	-	79.895	83.633	81.754
Band 13	-	65.982	63.618	60.616
Band 14	-	59.894	51.692	40.409
Band 15	-	51.692	45.807	36.169
Band 16	-	46.576	40	33.851

Band 16	-	46.576	40	33.851
Band 17	-	40.409	36.686	32.168
Band 18	-	37.657	33.851	30.331
Band 19	-	36.955	32.317	29.073
Band 20	-	34.045	27.246	26.863
Band 21	-	28.012	25.407	25.714
Band 22	-	26.173	24.105	23.338
Band 23	-	24.258	22.419	21.423
Band 24	-	22.572	20.197	18.818
Band 25	-	21.27	17.515	17.515
Band 26	-	20.197	13.608	15.753
Band 27	-	17.209		

(-)= there is no band

Lane 1 = marker

Lane 2 = *Ps.aeruginosa* No. 17

Lane 3 = *Ps.aeruginosa* No.46

Lane 4 = *Ps.aeruginosa* No.76

Table 12: RF- values of proteins products of filed *Ps.aeruginosa* isolates No. 17, 46 and 76.

R.F. No. of bands	Lane 1	Lane 2	Lane 3	Lane 4
Band 1	0.095	0.002	0.03	0.043
Band 2	0.127	0.025	0.038	0.057
Band 3	0.24	0.032	0.054	0.088
Band 4	0.338	0.043	0.106	0.111
Band 5	0.429	0.107	0.132	0.134
Band 6	0.631	0.134	0.143	0.147
Band 7	-	0.152	0.159	0.165
Band 8	-	0.174	0.182	0.188
Band 9	-	0.206	0.209	0.208
Band 10	-	0.224	0.234	0.229
Band 11	-	0.243	0.252	0.247
Band 12	-	0.275	0.268	0.272
Band 13	-	0.304	0.309	0.317
Band 14	-	0.318	0.342	0.399
Band 15	-	0.342	0.365	0.447
Band 16	-	0.361	0.403	0.485
Band 17	-	0.399	0.44	0.521

Band 18	-	0.428	0.485	0.574
Band 19	-	0.436	0.517	0.623
Band 20	-	0.481	0.669	0.678
Band 21	-	0.651	0.712	0.705
Band 22	-	0.694	0.742	0.76
Band 23	-	0.739	0.782	0.805
Band 24	-	0.778	0.834	0.866
Band 25	-	0.809	0.896	0.896
Band 26	-	0.834	0.987	0.937
Band 27	-	0.903		

(-)= there is no band

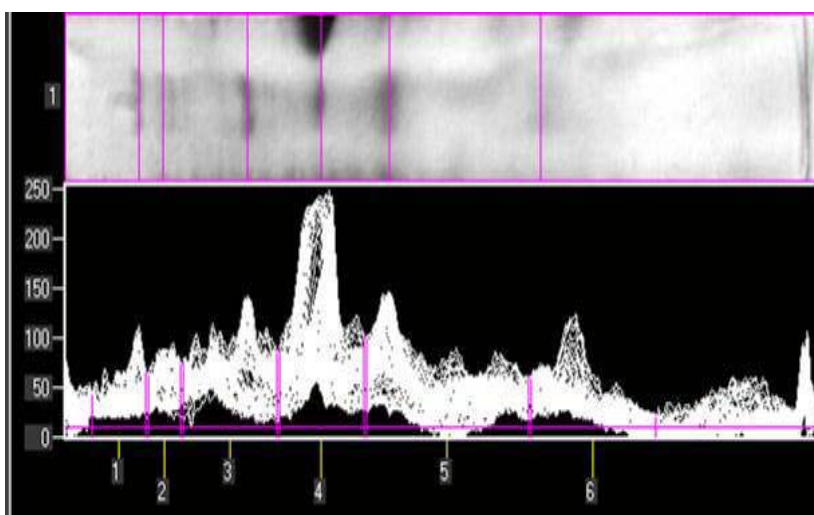
Lane 1 = marker

Lane 2 = *Ps.aeruginosa* No. 17

Lane 3 = *Ps.aeruginosa* No.46

Lane 4 = *Ps.aeruginosa* No.76

Marker lane profile:



lane 2 profile:

Ps.aeruginosa No. 17

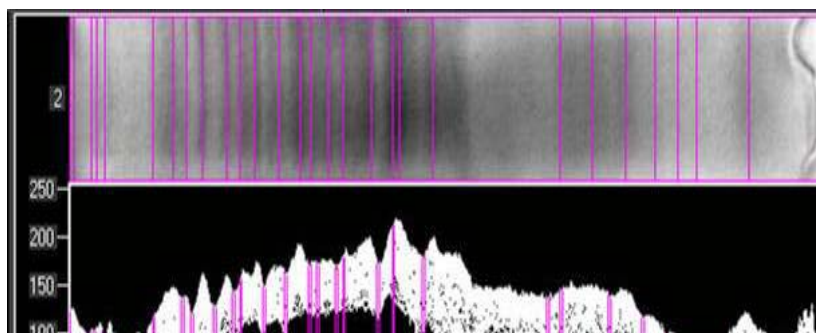
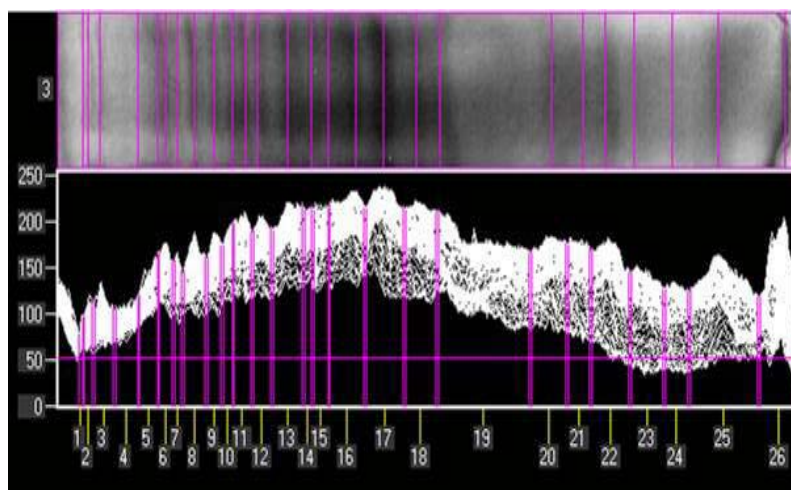


Fig 3:- showing densitometric analysis of *Ps.aeruginosa* No. 17 using software data analysis for gel proanalyzier and IBM compatible personal computer.

Lane 3 profile

Ps.aeruginosa No.46



Lane 4 profile

Ps.aeruginosa No.76

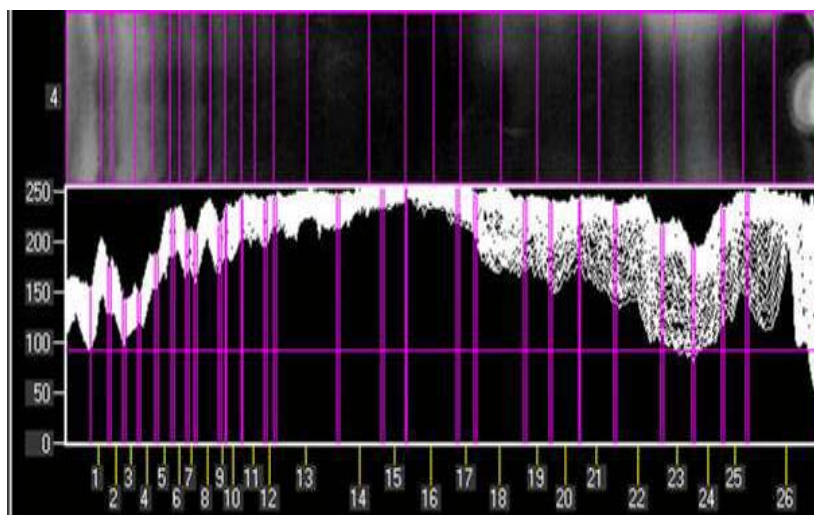


Fig 4: showing densitometric analysis of *Ps.aeruginosa* No. 46 & 76 using software data analysis for gel proanalyzies and IBM compatible personal computer.

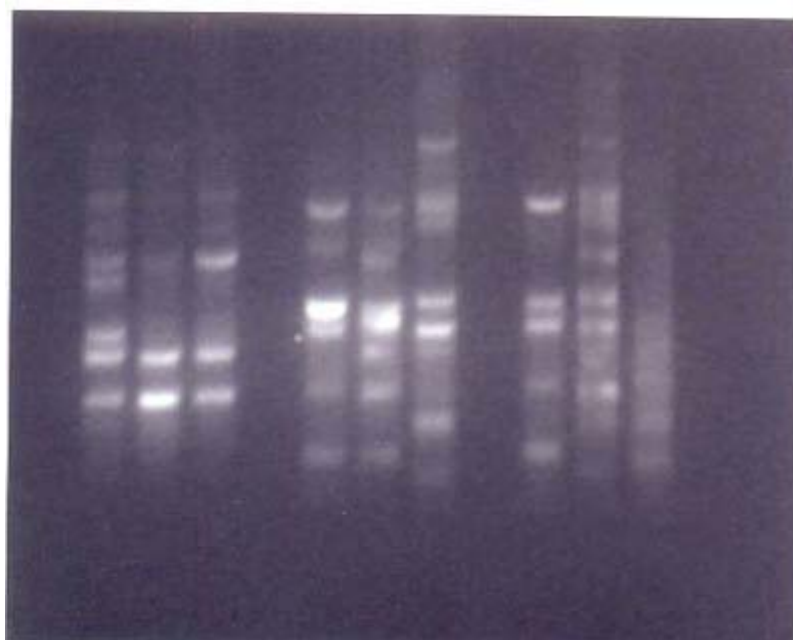


Fig 5:- RAPD-PCR of *Ps.aeruginosa* No. 17, 46 and 76 with C₄, B₈ and C₇ primers, respectively. Ethidium bromide gel electrophoresis showing RAPD-PCR using genomic DNA as a template amplified with C₄, B₈ and C₇ primers. Lane 1 to 9 represents the three *Ps.aeruginosa* isolates.

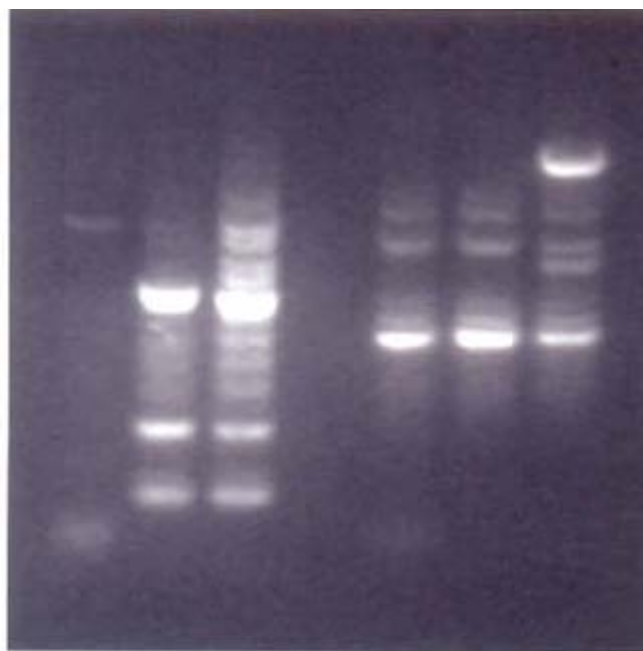


Fig 6:- RAPD-PCR of *Ps.aeruginosa* No. 17, 46 and 76 with primers Z₁ and Z₅ respectively. Ethidium bromide gel electrophoresis showing RAPD-PCR using genomic DNA as a template amplified with primers. Z₁ and Z₅ Lane 1 to 6 represent the three *Ps.aeruginosa* isolates.

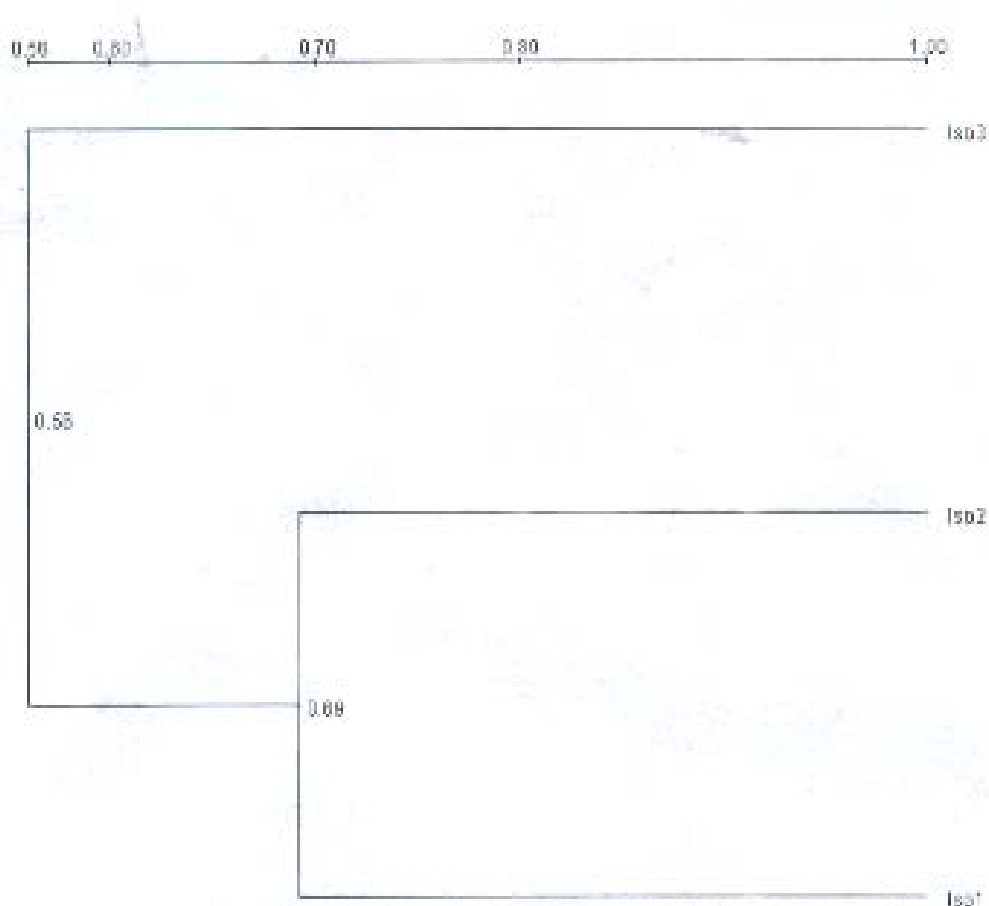


Fig 7: Dendrogram constructed with UPGMA Cluster analysis of PAPD-PCR data showing relationships among the three isolates.

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UTILIZATION OF AN INDUSTRIAL WASTE PRODUCT IN THE PREPARATION OF LOW COST CEMENT

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ABSTRACT

Blast furnace slag is a by-product of the manufacture of iron. There are huge amounts of this material annually produced, Therefore, the utilization of this waste product in the production of any industry save money and to keep the area of this factory in normal case and prevent the pollution of the atmosphere. Slag improves the mechanical and physicochemical properties of the cement paste. There are strong environmental and energy reasons for developing a wide range of pozzolanic cements. The aim of this work is to study the effect of substitution of cement with granulated slag by the determination of water of consistency, initial and final setting times, combined water, free lime contents, bulk density, total porosity and compressive strength. The results show that the addition of granulated slag increases the water of consistency, initial and final setting times, compressive strength as well as total porosity, whereas the free lime and combined water decrease with the granulated slag content.

INTRODUCTION

The problem of producing pozzolanic cement has been of considerable scientific and technological interest because such addition increases the chemical resistance to sulphate attack, impermeability, lowering heat of hydration and thermal expansion (**Hewlett, 1998**). The use of pozzolanic cement is increasing world-wide because it needs less energy for production.

Power plants using coal or rice husks as fuel and metallurgical furnaces producing pig iron, steel, copper, nickel, lead, silicon and ferrosilicon alloys are major sources of by-products. These by-products must be disposed off in a suitable way. Disposal by dumping represents a resource of waste and causes environmental problems. Exploiting the pozzolanic and the cementitious properties of these materials by incorporating as components of Portland cement concrete, represents high value of applications.

Granulated blast-furnace slag has no cementitious value; but when it is quenched and then ground, it acquires cementitious value. The process of quenching is called “granulation” and the product “granulated blast-furnace slag”.

Blast furnace slag is formed as a liquid at 1350 - 1550°C in the manufacture of iron; limestone reacts with materials rich in SiO₂ and Al₂O₃ associated with the ore or present in ash from the coke. if allowed to cool slowly, it crystallizes to give a material having no

cementing properties. If cooled sufficiently rapidly to below 800°C, it forms a glass which is latent hydration cement. Cooling is most often effected by spraying droplets of the molten slag with high-pressure jets of water. This gives a wet, sandy material which when dried and ground is called ground granulated blast furnace slag and often contains over 95% of glass.

The Tunisian blast-furnace slag has been characterized by several physicochemical methods to evaluate its hydraulic reactivity (**Samet and Chaabouni, 2004**). It has been noted that nearly all the slag is glassy, so its use as a replacement of cement is possible. This result has been confirmed by different physical tests applied to pozzolanic cements as specific surface, normal consistency, setting time, stability to expansion and the minislump.

The replacement of a part of clinker by slag has led to the following results:

- an acceptable extension of the setting time, an improvement of the rheological behavior, and a very good stability to expansion, and
- an improvement of the compressive strength at 28 days, but with a slight decrease at 7 days.

Blast-furnace slag is the most useful latent hydraulic material, because the amount produced is very large and its properties are very stable composed with other industrial by-products (**Daimon, 1980**).

Bágel (1998) studied the workability and incorporating blast furnace slag and silica fume composite cement mortars. The compressive strength and mercury intrusion were tested for plain Portland cement mortar and/or slag-cement mortar. The results showed that with high portions of slag and silica fume in the binding system, the mortars reached relatively satisfactory level of compressive strength and contributed to the significantly denser pore structure.

The aim of the present work is to establish the optimum composition of slag cement made from ground granulated blast furnace slag and OPC. Different blends were prepared from these materials and the physico-mechanical properties were studied.

EXPERIMENTAL

The materials used in this investigation were ground granulated blast furnace slag (WCS), which provided from iron steel company; Helwan, Egypt, and ordinary Portland cement (OPC) from Suez cement Company. The chemical analyses of these raw materials are shown in Table (1).

Table (1): Chemical composition of starting materials, (wt%).

Oxides Materials	SiO ₂	Al ₂ O ₃	Fe ₂ O ₃	CaO	MgO	MnO	TiO ₂	Na ₂ O	K ₂ O	BaO	SO ₃	S ⁺⁺	L.O.I
WCS	37.48	12.86	0.40	36.70	2.45	6.24	0.72	1.84	0.71	5.31	0.01	0.75	—
OPC	21.51	5.07	4.39	65.21	2.00	0.15	—	0.23	0.29	—	0.25	—	2.40

Different mixes were made by substitution 0, 10, 20, 30 wt. % of cement by slag. The mixes were denoted as M₀, MS₁, MS₂, and MS₃ as shown in Table (2).

Table (2): Mix composition of the prepared cements, (wt %).

Mix No.	OPC	Slag
M ₀	100	—
MS ₁	90	10
MS ₂	80	20
MS ₃	70	30

Each dry mix was homogenized on a roller in a porcelain ball mill with four balls for 1 h to assure complete homogeneity. The water demand for standard consistency and the setting time were measured (**ASTM Designation: C87-98, 2002**)&(**ASTM Designation: C191-01a, 2002**). The pastes were mixed with the water of consistency and moulded into stainless steel (2×2×2 cm) moulds, cured in a 100 % R.H at 23 ± 1°C for the first 24 hours, then demolded and cured under water until the desired curing time such as 3, 7, 28 and 90 days. After the predetermined curing time, the hydration of the paste was stopped by the removal of free water. About 10 grams of the crushed paste, after compressive strength testing, were dried at 105°C for about 24 hours to remove the free water. The resulted dried samples were then stored in desiccators for other physico-chemical measurements. The kinetics of hydration was followed by the determination of the free lime (**Kondo et al., 1975**), as well as combined water contents. The combined water content was determined by the ignition at 850 °C for 20

minutes of the dried paste on ignited weight basis minus the loss of the anhydrous blend. The compressive strength was determined up to 90 days. The total porosity, ξ , was calculated by the determination of bulk density, d_p , the total water, W_t , and the evaporable water content, W_e , of hardened cement paste as in the following equation:

$$\xi = (0.99 \times W_e \times d_p) / (1 + W_t) \quad (\text{Copeland, and Hayes, 1956})$$

RESULTS AND DISCUSSION

Water of Consistency and Setting Time

The results of water of consistency and setting times of pozzolanic cement pastes are shown in Fig. (1). Water of consistency of slag cement pastes increases with the slag content. This phenomenon was observed by several authors (**Sato, et al., 1986**). It seems that the high porosity of the slag is responsible for the increase of water demand (**Samet and Chaabouni, 2004**).

The slag content generally elongated the setting time which remains in conformity with the European norms (**Samet and Chaabouni, 2004**) due to the slow rate of hydration of granulated slag in comparison to Portland cement. This behavior can be attributed to the increase of water of consistency which increases the total porosity and elongates the setting time. Also, there is insufficient Ca(OH)_2 to react with slag, then the slag grains and the hydration products formed coat the cement particles which delay further hydration. Accordingly, the initial and final setting times are elongated.

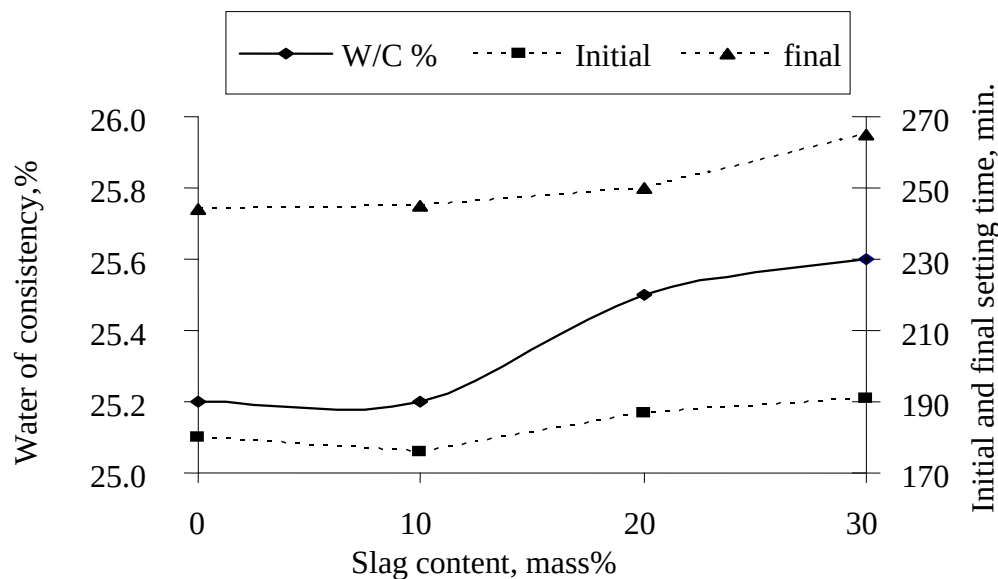


Fig. 1 Water of consistency and setting time of slag cement pastes

Combined Water Contents:

The combined water contents of hydrated slag cement pastes are graphically represented as a function of curing time in Fig. (2). It can be observed generally that the combined water contents increase gradually with curing time for all hardened cement pastes. This is attributed to the progress of hydration. With 30 % slag the combined water content is higher than OPC at 90 days This is attributed to the reaction of slag with liberated Ca(OH)_2 at later ages forming additional hydration products as calcium silicate hydrate which increase the combined water contents. The increase of combined water of 30% granulated slag is occurred at later ages (28-90 days) due to the higher rate of hydration of slag at later ages of hydration.

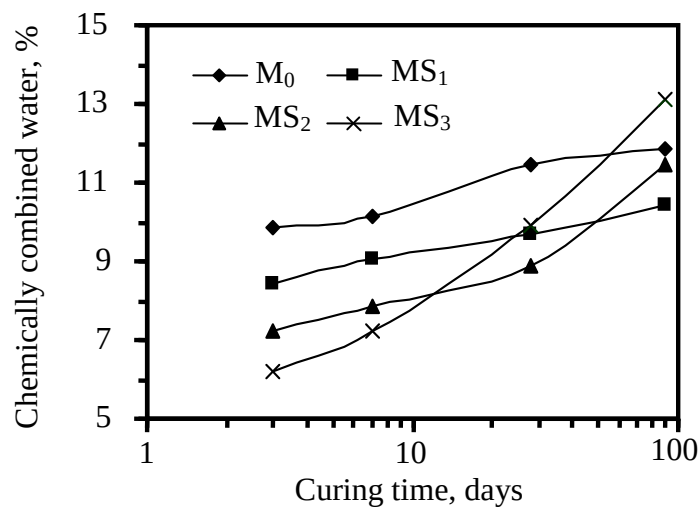


Fig. 2: Combined water contents of OPC and pozzolanic cement pastes up to 90 days

Free Lime Contents:

The free lime contents of hydrated slag cement pastes are graphically represented as a function of curing time in Fig. (3). It can be found generally that the free lime contents increase gradually with curing time for all hardened cement pastes. This is attributed to the continuous hydration of C_3S and $\beta\text{-C}_2\text{S}$ liberating hydrated lime. The increase of slag content tends to decrease the free lime contents (Salem, et al.,1995). The increase of free lime of slag cement pastes linearly with curing time up to 90 days due to the higher content of liberated lime during the hydration of Portland cement. This is sufficient to activate the hydration of granulated slag without any decrease after 28 days.

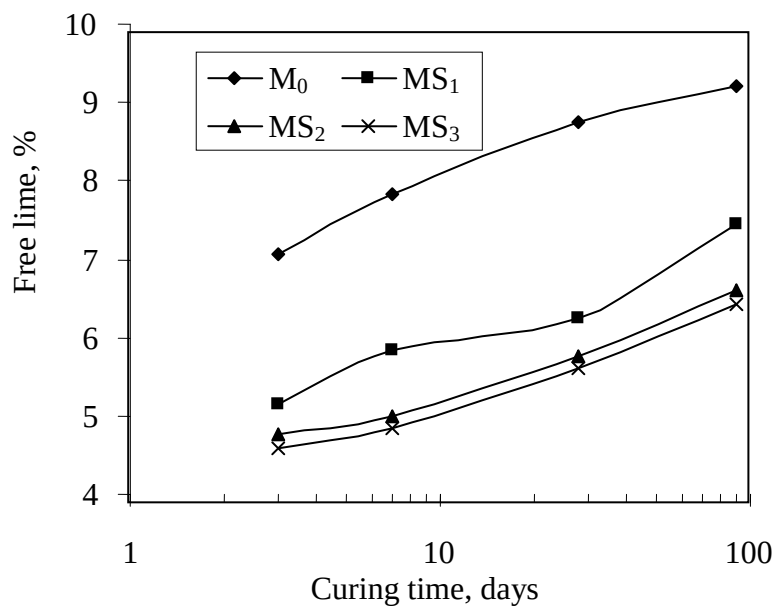


Fig. 3: Free lime contents of OPC and pozzolanic cement pastes up to 90 days

Figure (4) Shows the XRD hardened pozzolanic cement pastes containing mixture of (70% OPC + 30% WCS) cured at 3, 28 and 90 days. It is found that the intensity of characteristics lines for Ca(OH)_2 (4.92 Å, 2.62 Å, 1.92 Å and 1.79 Å), increase with curing time. This is due to the continuous hydration of $\beta\text{-C}_2\text{S}$ and C_3S . Also, the peaks of unhydrated phases of $\beta\text{-C}_2\text{S}$ and C_3S decrease with curing time due to the continuous hydration of $\beta\text{-C}_2\text{S}$ and C_3S . The CaCO_3 is also detected and overlapped by CSH at 3.03 Å.

Figure (5) Shows the FTIR patterns of hydration products of different Portland slag cement pastes (M_0 , MS_1 , MS_2 and MS_3) after 90 days curing. It is clear that, the band at 3640 cm^{-1} which is due to the stretch vibration of OH^- group of portlandite (CH) (Saika et al., 2002) decreases with the amount of slag. This is attributed to increase the amount of OPC replacement, which decreases the amount of OPC reactive phases. This leads to hinder the degree of hydration of pozzolanic cement pastes. The peak at 3443 cm^{-1} is due to water (Alons and Palomo, 2001). The band at 1436 cm^{-1} is attributed to presence of calcite, possibly formed due to carbonation of the pastes (Bented, 1974). The intensity of the band at 986 cm^{-1} which is due to Si – O stretching vibration of C S H (Saika et al., 2002) increases in MS_2 and MS_3 (indicating increasing of formation of CSH), i.e. has higher hydraulic properties.

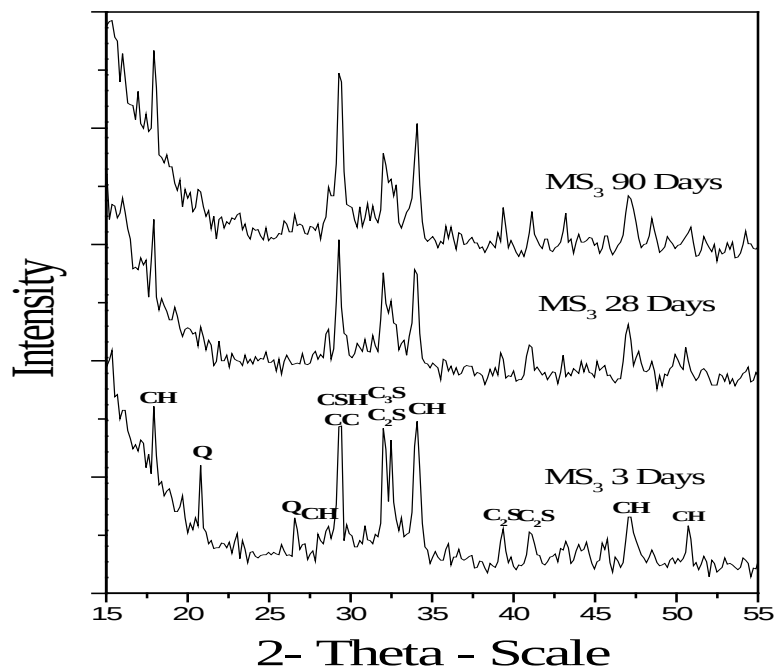


Fig. 4: X-ray diffraction patterns of hardened cement pastes containing mixture of (70% OPC + 30% WCS) immersed in tap water up to 90 days

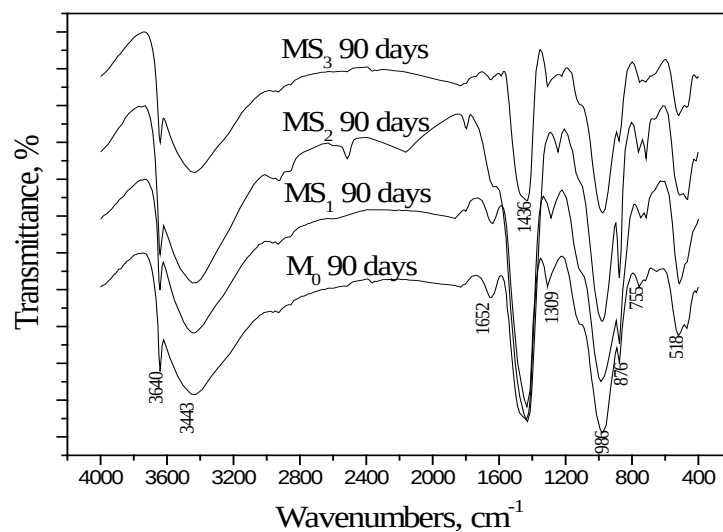


Fig. 5: Infrared spectra of OPC and pozzolanic cement pastes after 90 days

Bulk Density and Total Porosity:

The bulk density and total porosity of cement pastes cured up to 90 days are given in Figs. (6) and (7). The bulk density of the hardened cement pastes increases and the total porosity decreases with curing time due to the filling of some of the pores. The bulk density decreases and the total porosity increases with slag content. This is attributed to the increase of water of

consistency, which plays an important role in the values of bulk density and total porosity. The water of consistency of the slag pozzolanic cement paste was higher than that of OPC paste. Generally, the bulk density of the slag cement pastes is lower than the Portland cement. The calcium silicate hydrate formed during the hydration of granulated slag cement paste has low C/S as well as water content. This tends to decrease the bulk density of the cement paste.

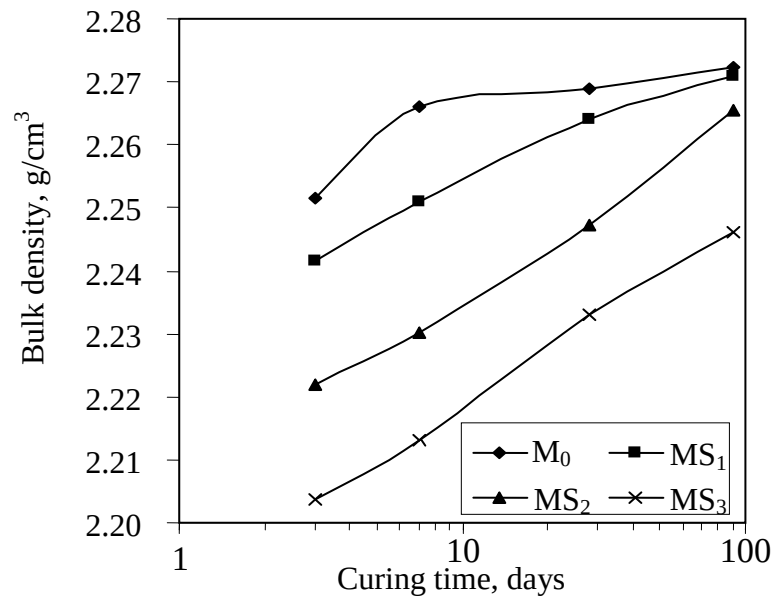


Fig. 6: Bulk density of pozzolanic cement pastes up to 90 days

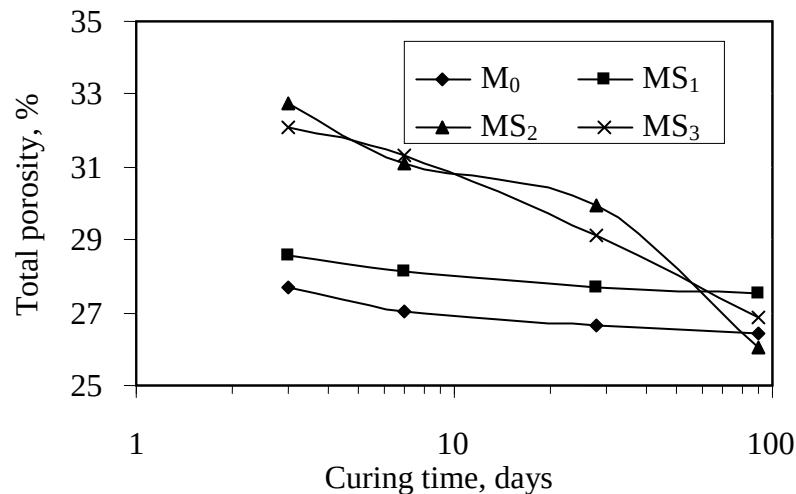


Fig. 7: Total porosity of pozzolanic cement pastes up to 90 days

Compressive Strength:

The results of compressive strength of cement pastes cured up to 90 days are graphically plotted as a function of curing time in Fig. (8). It is clear that the compressive strength increases with curing time for all cement pastes. As the hydration proceeds, more hydration products and cementing materials are formed. This leads to an increase in the compressive strength of hardened cement pastes. The hydration products possess a large specific volume than unhydrated cement compounds. Therefore, the accumulation of the hydrated products will fill a part of the originally filled spaces. This leads to a decrease the total porosity and increase the compressive strength of the cement paste. The increase of slag content decreases the compressive strength due to the increase of water of consistency as well as low hydration characteristics in comparison to OPC. Slag contributes to hydration after seven days improving the strength at medium and later ages (**Menéndez, et al., 2003**). 30 % slag gives the highest compressive strength value at 90 days. This is also due to the high amount of consumed lime that tends to increase the compressive strength of the cement paste; this result is in a good agreement with that of combined water.

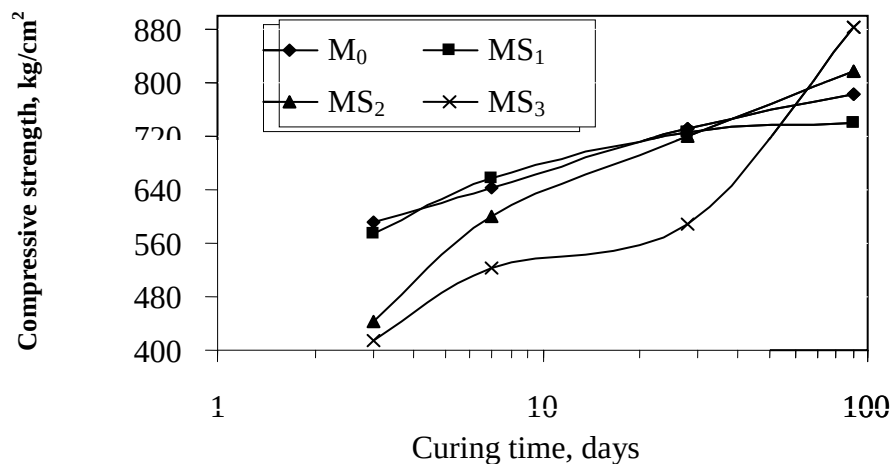


Fig. (8): Compressive strength of pozzolanic cement pastes up to 90 days

CONCLUSIONS

From the above findings it can be concluded that:

1. Slag increases the water of consistency and elongated the setting time,
2. 30% slag increases the combined water content at 90 days as well as the compressive strength,
3. The bulk density decreases and the total porosity increases with slag content, and
4. Slag decreases the free lime content.

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الملخص العربي

إستخدام أحد النفايات الصناعية فى تحضير أسمنت رخيص التكلفة

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** قسم الهندسة الكيميائية – المعهد العالى للهندسة – أكاديمية الشروق

يعتبر خبث أفران الحديد ناتج ثانوى عن صناعة الحديد. ويتم إنتاج كميات ضخمة منه كل عام. لذلك فإن إستخدام هذه النفايات فى أى صناعة يوفر المال ويحافظ على البيئة. وجد أن خبث أفران الحديد يحسن خواص الأسمنت الميكانيكية والفيزيوكيميائية. هناك العديد من الأسباب الخاصة بتوفير الطاقة والمحافظة على البيئة التى أدت إلى تطوير العديد من الأسمنتات البوزولانية. الهدف من هذا البحث دراسة تأثير إستبدال الأسمنت بخبث أفران الحديد المحبب . تم إجراء إختبارات قياس نسبة ماء الخلط وزمن الشك الابتدائى والنهائى وتعيين الماء المتحد كيميائياً وكمية الجير الحر والكثافة الكلية والمسامية وقوة تحمل الضغط. من خلال هذه الدراسة أمكن التوصل إلى بعض النتائج نوجزها فيما يلى:-

وجد أن كمية ماء الخلط وزمن الشك الابتدائى والنهائى وكذلك قوة تحمل الضغط تزداد مع زيادة خبث أفران الحديد المحبب. بينما كمية الماء المتحد كيميائياً وكمية الجير الحر تتناقص مع كمية خبث أفران الحديد المحبب.

CAMPANIAN-PALEOCENE FACIES, DEPOSITIONAL ENVIRONMENTS AND PALEOCLIMATIC CONDITIONS ALONG THE RED SEA COAST, EGYPT

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ABSTRACT

A well established Campanian to Paleocene succession (Duwi and Dakhla formations) mark the scarp faces at the Safaga-Quseir region. It is invoked by a variety of shallow and deep marine sediments with distinct lateral lithofacies and thickness variations. Microfacies analyses, X-ray mineralogy and faunal contents are improved information on seven shallowing upward cycles coeval with cool/warm cycles. During the Abu Shegela Member of Dawi Formation (Upper Campanian) a shallow sea covered the region under a long period of warm climate with two intermittent cold climatic horizons. The cold condition is documented from increases of phyllosilicates and quartz, with significant decreases in calcite and koalinte. This led to the occurrences of phosphorite and glauconitic calcareous sandstone and barren shale. These facies are laid down in inner nertic to lagoonal environments, with low oxygen conditions. Meanwhile, the Duwi and Atshan members of the Duwi Formation (Lower Maastrichtian) shows marine facies rich in macrofossils reflecting two warm/cold climatic cycles as indicated from the lithofacies and clay mineralogy. The Hamma Member of Dakhla Shale (Upper Maastrichtian) shows a short period of warm climate with one intermittent cold climatic horizon as suggested from the increases in terrestrial runoff. As well as, it is recognized by faunal decline and increasing in smectite, and the total organic matter. The Beida Member of the Dakhla Shale (Lower Paleocene) is characterized by warm climate, while its upper part is characterized by seasonal fluctuations which are reflected by alternations of fossiliferous shale and limestone succession that lay down in deep and open marine environments.

INTRODUCTION

Specific climatic conditions have been prevailed along the northern edge of the Arabian-African plate during Mid-Cretaceous-Eocene because of the paleoenographic changes. Short term fluctuations of the Campanian-Maastrichtian can be recognized in the continental shelf and upper slope areas of southwestern Tethys (Li et al., 2000). These fluctuations are expressed from global cooling and polar ice growth. Cool/arid climate is recorded from the Late Campanian, Campanian-Maastrichtian and Late Maastrichtian (~74.2, 70.2, & 68.9 Ma, Li et al., 2000). However, long term sea-level fluctuations are generally related to plate tectonic movements and particularly high ocean spreading rates (Pitmann & Golvchenko, 1991; Hallam, 1992; Miller, et al. 1991).

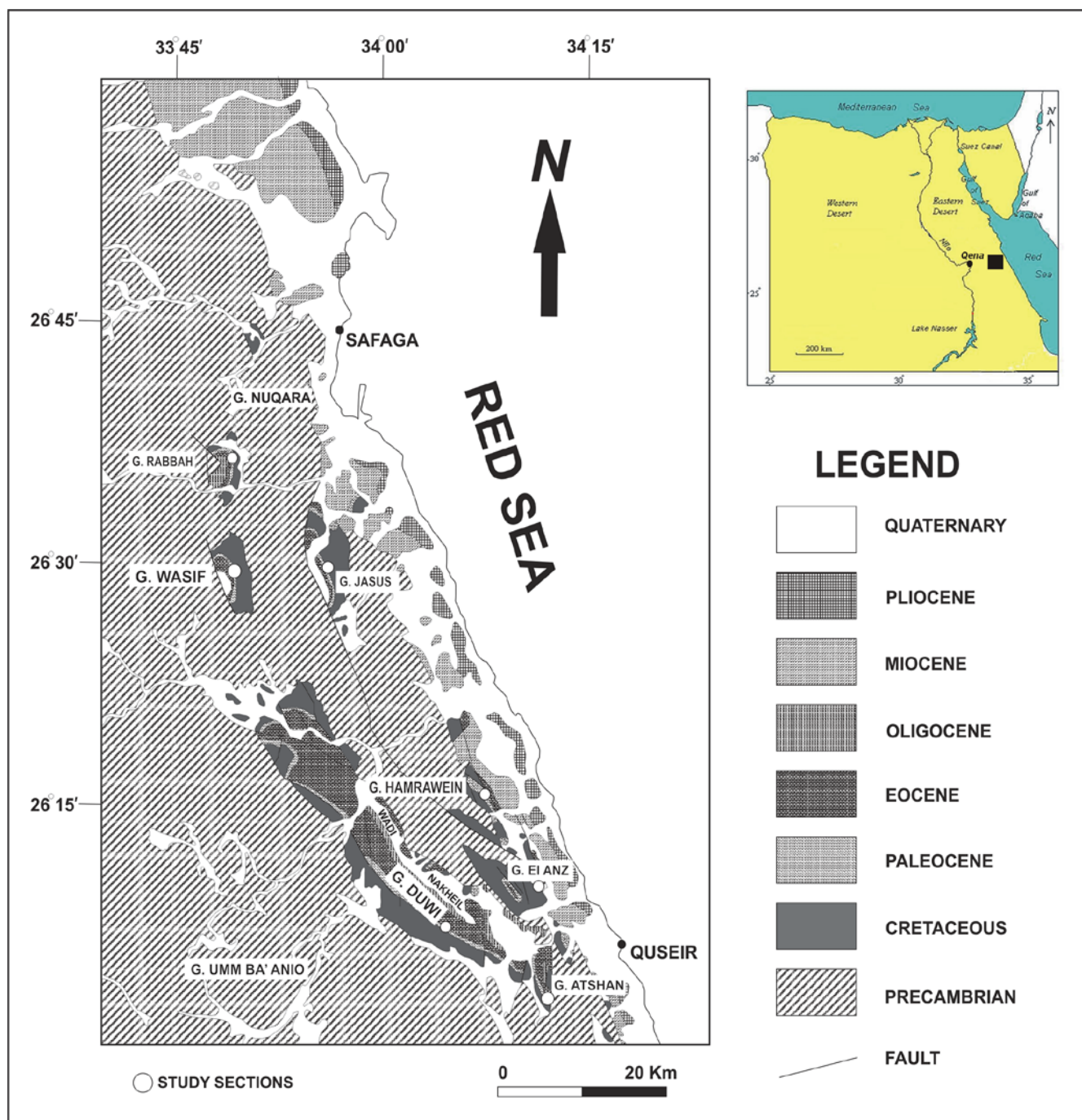


Fig.1: Geologic and location map of the study area (after geologic map of Qena Quadrangle, Egypt, 1:500,000, Geologic Survey of Egypt, 1978)

During the Early Maastrichtian sea-level transgression, open marine condition were established throughout central Egypt and to the South. During subsequent sea-level regressions and erosions were widespread and extensive in the marginal seas to the south and west coupled with local tectonic in the Western Desert (Tantawey et al., 2001). But in the Eastern Desert, Sinai and Palestine, sediment deposition is occurred in deeper middle-outer shelf environments. It was primarily interrupted by erosion due to global sea-level fluctuations (Luger et al., 1989). Also, in the Eastern Desert the sediment deposition during the Late Cretaceous and Early Paleocene was primarily controlled by large-scale rejuvenation of the pre-existing fault system with major erosion linked to active uplift during the Late Campanian to Early Maastrichtian (Hendriks et al., 1987). During the Late Maastrichtian to Early Paleocene sediment deposition was suggested also by less diverse lithologies (Hendriks & Luger 1987).

The Upper Cretaceous marine sediments of the Eastern Desert of Egypt are of economic interest due to the rich phosphatic beds of the Duwi Formation that form a part of the extensive Middle-East-North African phosphate province. Also, the Dakhla Formation is characterized by a particular interest because of its rhythmic deposition of shales and glauconite-rich lithofacies. The stratigraphic sections recorded of the Cretaceous-Tertiary transition in Egypt are variable depending on the depositional environment. In the Eastern Desert, the deposition was occurred in deeper middle to outer shelf environments where occasionally the deposition was due to global sea-level fluctuations (Sestini, 1984; Hendriks et al. 1987; Luger, 1988; Keller & Benjamini, 1991; Kassab & Keheila, 1994).

The study area extends in a northwest direction from south of Safaga to Qusier, Red Sea, between latitudes 25° 50' and 26° 67' N and longitudes 33° 45' and 34° 25' E, covering an area of about 500 km² (Fig. 1). The Cretaceous-Paleocene deposits occupy the troughs of tilted blocks, where oyster bank or reef were developed on the paleohighs and phosphorites were laid down in gentle ramps of the southern tilted blocks (Philobos, 1996). Numerous studies have been published on the geology, stratigraphy, and sedimentology of the Campanian-Paleocene marine sediments exposed in the Safaga-Qusier area (Barron & Hume, 1902; Youssef, 1957; Ansary & Fakhry, 1958; Faris & Hassan, 1959; Anwar & El-Tarabili 1960; El Akkad & Dardir, 1966; Shahin et al. (1986); Abdel Razik, 1967,1972; Soliman & Amer, 1969,1972; Issawi et al., 1971; Malak, et al., 1977; Gleen & Mansour, 1979; El-Kammar et al. (1990); Awad et al., 1992; Hassan et al. 1992; Hassanien et al. 1993;

Ismael, 1996; Khalid, 1996; Faris et al. (1999); Elewa et al., 1999,2004; Keller et al. 2002 and Ismail & Ied, 2005).

The present work deals with the lithofacies associations, depositional environments, sedimentation breaks and mineralogy of the Upper Campanian-Lower Paleocene succession which cropping out in the Safaga-Qusier region. This is achieved to assort and interpret the paleoenvironments and paleoclimatic conditions. Seven surface stratigraphic sections are measured, sampled and studied to represent the lateral and vertical facies changes marking the study area (Fig. 1). Three stratigraphic sections from the Safaga area (Gabals Wasif, Gasus and Rabbah) and four sections are measured from the Quseir area (Gabals Atshan, Duwi, El-Anz, and Hamrawein).

METHODS

The seven stratigraphic sections were measured, sampled and examined for lithological and faunal contents, bioturbations, erosion surfaces, and hardgrounds. The recorded microfacies were described, discussed, interpreted and photographed from 200 thin sections in the different rock units and localities. The terminology of Dunham (1962), Friedman (1965) and Pettijhon (1975) are used in the petrographic description. Gabals Wasif and Atshan sections were selected to represent the mineralogical composition contents of the studied rocks. X-ray diffraction analyses have been carried out principally for the whole rock of seventy seven selected bulk samples and seventy four clay samples. The oriented mounts were run under three conditions: i) In air dry state. ii) After ethylene glycol treatment. iii) After heating to 550° C for 1 hour. The total organic matter was determined by using a high temperature TOC/TNb analysis (Liquid TOC) after decarbonization. The whole rock samples were analysed for TOC concentrates. About 200 mg pulverized sample was used for this analysis. Carbonate was removed by treatment with 10% aqueous hydrochloric-acid. The residual materials were used for the determination of TOC by combustion analysis of temperatures in excess of 850C°. The evolved gas (CO₂) was measured quantitatively and simultaneously by infrared detectors and recorded as percentage of carbon. The preparation of samples and the analyses of the samples were carried out in the laboratories of the Institute of Geologie and Palaontologie, Tubingen, Germany.

Lithostratigraphy

The Cretaceous-Paleocene succession is widely distributed throughout much of the Safaga-Quseir region (Fig. 1). It is well established along its scarp faces and plateau surfaces. As such the succession provides an excellent opportunity to study the obvious lateral and vertical lithofacies changes making its enclosing sediments. The succession is dominated by many lithologic varieties, including fossiliferous shales, sandstones, claystones, limestones, dolostones, chalk, phosphorites with various micro-and macrofossils assemblages. The total exposed thickness ranges between 195 to 250m (Figs. 2 & 3). According to Said (1962), the studied sediments are assigned into two well defined formations namely from base to top: Duwi Formation and Dakhla Shale. The studied Duwi Formation and Dakhla Shale are correlated among the studied sections (Fig. 4). The following are detailed description, facies analysis, and discussion of the exposed two formations at the study area.

1. Duwi Formation (Upper Campanian-Lower Maastrichtian):

Yossef (1957) and Said (1962, 1990) extended the use of the term Duwi Formation at Safaga-Quseir region and subdivided the whole section in the Red Sea area into three members: the upper Atshan or "A" Member separated from the middle Duwi or "B" Member and lower Abu Shegela or "C" Member. The Duwi Formation in the Safaga-Quseir district comprises a heterogeneous suite of shallow marine rocks that lay stratigraphically conformable above the Quseir Variegated Shale and below the Dakhla Shale (Figs. 2 & 3). The lower contact is marked by medium-grained sandstones bed that comprises two irregular reddish shale bands while the upper contact is marked by gradational change from limestone and argillaceous limestone to calcareous shale. The three members can be described as follows.

The Abu Shegela Member is separated from the Duwi Member by variegated shales of non-marine to marginal marine sediments in the Safaga-Quseir area. This member shows variable thickness (40-80 m thick) in the study area, with a maximum thickness at Atshan section and a minimum thickness at Hamrawein section (Fig. 4). It consists of grey, green occasionally brownish shale beds and a series of phosphrite beds. It comprises three phosphorite beds (30 to 120 cm thick). The phosphorites are

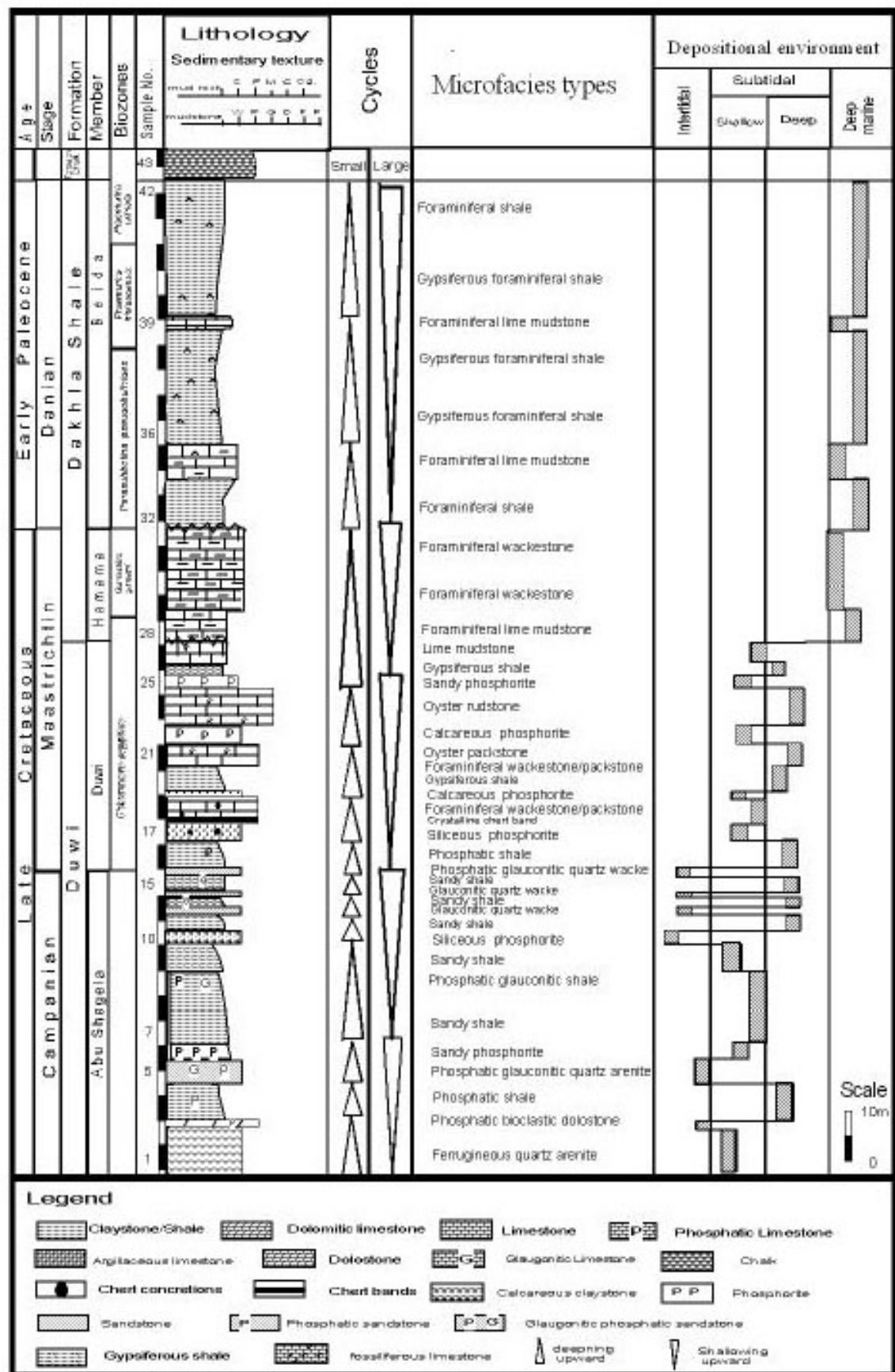


Fig. 2: Representative stratigraphic section showing sedimentological characteristics, depositional and environmental cycles of Gabal Wasif.

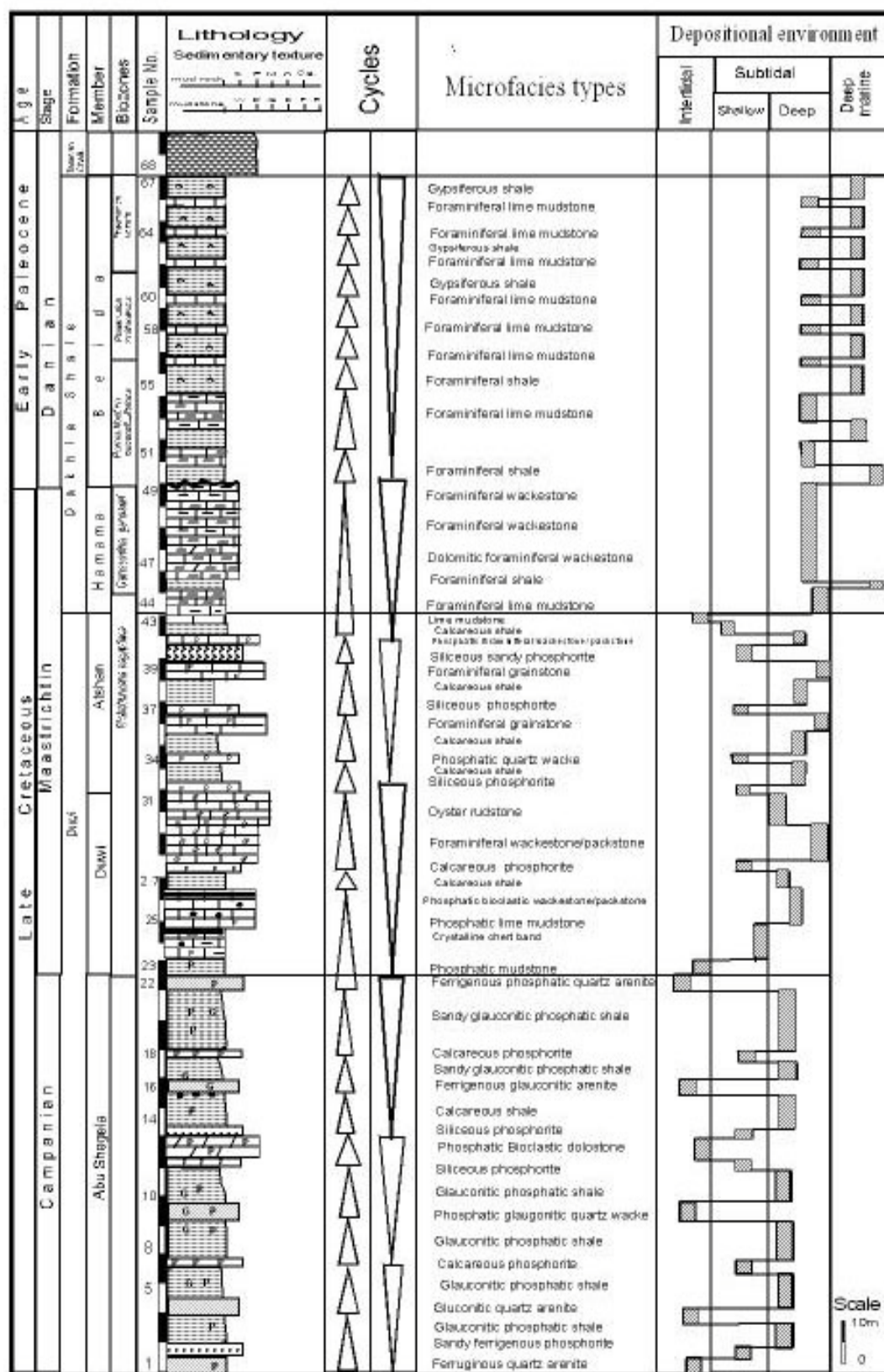


Fig. 3: Representative stratigraphic section showing sedimentological characteristics, depositional cycles and environments of Gabal Atshan (legend refers in Fig. 2).

gray to yellowish gray, black light brown, soft to hard, medium to coarse sand size; rarely pebbly, very poorly to well-sorted, calcareous and occasionally, ferruginous, gypsiferous, bituminous and siliceous. The Duwi Member (25-65m thick) is well developed at Gabal Duwi in Quseir area, it is made up of several phosphorite bands separated by thin shale, and silicified limestone beds with oyster banks. The carbonates are represented by shale in Hamrawein section. Its thickness ranges from 0.5m to 1m and its colour from gray, pale gray to yellow in colour with many vertebrate remains, coprolites and fish teeth. An important marker in this member is a thick oyster limestone bed (*Ostrea villei*), which caps and intercalates this member with assortment of bivalves (*Trigona multidentata*), gastropods and ammonites (*Lybicoceras ismaeli*). Oyster limestone beds are abundant in Quseir area relative to the Safaga area. The Atshan Member (~35m thick) is made up of three phosphorite beds separated by thin shale, chert and phosphatic limestone beds with burrows in the Atshan section at the Quseir area only. The phosphrite bed ranges in thickness from 90-170cm. and its colour varies from pale white to pale gray. The upper part of this member consists of thin phosphatic conglomerate beds intercalated with thin shale and fossiliferous limestone beds. The Duwi Formation in Quseir–Safaga region is of Campanian age in its lower part and Maastrichtian in the upper part (Youssef, 1957; El Akkad & Dardir, 1966; Issawi et al. 1971; and Obaidalla, 2000).

Lithofacies association

The lithofacies characteristics of the Duwi sediments and the faunal associations of planktonic foraminifera (according to Obaidalla, 2000) indicate deposition in an upward in shallow marine environment with oscillations from intertidal to deep subtidal.

a. Shallow subtidal massive bedded sandstone: This lithofacies represents thin interbeds throughout the Abu Shegela Member of Duwi Formation (Figs. 2 & 3). It rarely reaches up to 5m in thickness. Microscopically, sandstone lithofacies are yellowish, reddish brown, moderately hard, massive, ferruginous, highly glauconitic, phosphatic, fossiliferous and capped by iron streaks, nodules and concretions. The sandstone is classified into quartz arenite and wacke. The quartz arenite are composed of quartz grains, phosphatic, glauconitic pellets and iron-hydroxides. The quartz grains are fine to medium grained, mostly monocrystalline where the grain contacts are pointed and concave-convex, and display moderate sphericity and tight-packed

fabric, subangular to subrounded, mature, moderately sorted quartz grains. A few quartz grains are partially coated by syntiaxial silica overgrowths as a result of partial dissolution. Quartz arenite is cemented by iron-hydroxide (ferruginous quartz arenite, Pl. 1/1). Ferruginous cement is present in green sand filling the intergranular pore spaces or scattered patches and coated the quartz grains. Also, the sandstone is dominated by glauconitic pellets and phosphatic grains which form 20-35% of the rock content (phosphatic glauconitic quartz arenite, Pl. 1/2).

The quartz wackes are composed of quartz grains, colophane pellets and glauconite grains, with argillaceous matrix and badly preserved bivalve debris. The quartz grains are medium to coarse grained, subangular to angular, poorly sorted and immature. Matrix of this lithofacies is made up of argillaceous material (> 25%), glauconitic, phosphatic grains, and fine quartz grains. Quartz wacke is cemented by calcite, iron-hydroxide, and gypsum (phosphatic, calcareous and glauconitic quartz wacke). The glauconite grains are elliptical, ovoidal, rounded and irregularly-shaped due to compaction. Grain size ranges from fine to medium sands, green, yellowish green to brown partially oxidized to dark brown iron-hydroxide (Pl. 1/3 & 4). Some secondary quartz overgrowths produced by diagenetic processes are observed with rare plagioclase, some altered feldspars to sericite, rare chert, glauconite, colophane pellets and bone fragments.

The massive-sandstones are interpreted that it is virtually devoid of any morphological features denoting a lower shoreface deposits. It has been developed during low wave energy fair-weather periods. The lithofacies are resulted from very rapid deposition of suspension load in which a large amount of sands are dumped as high clastic influx from the land.

b. Deep subtidal shale/claystone: The shale/claystone is the dominant lithofacies in Duwi Formation (Figs. 2 & 3). Shale is greenish grey, reddish brown, fissile to massive calcareous, sandy and partly glauconitic (Pl. 1/5). It is barren of fossils except some plant remains and carbonaceous matter. The claystone is compact, black with iron-hydroxides and calcareous materials. The lithofacies characters of these sediments, based on association indicate deposition in an upper deep subtidal setting under calm lagoonal conditions during periods receiving appreciable influx of detrital clay size fraction. Deposition occurred below fair-weather wave base under limited water circulation, with the open sea and a relative decrease in water salinity due to fresh water influx.

PLATE 1

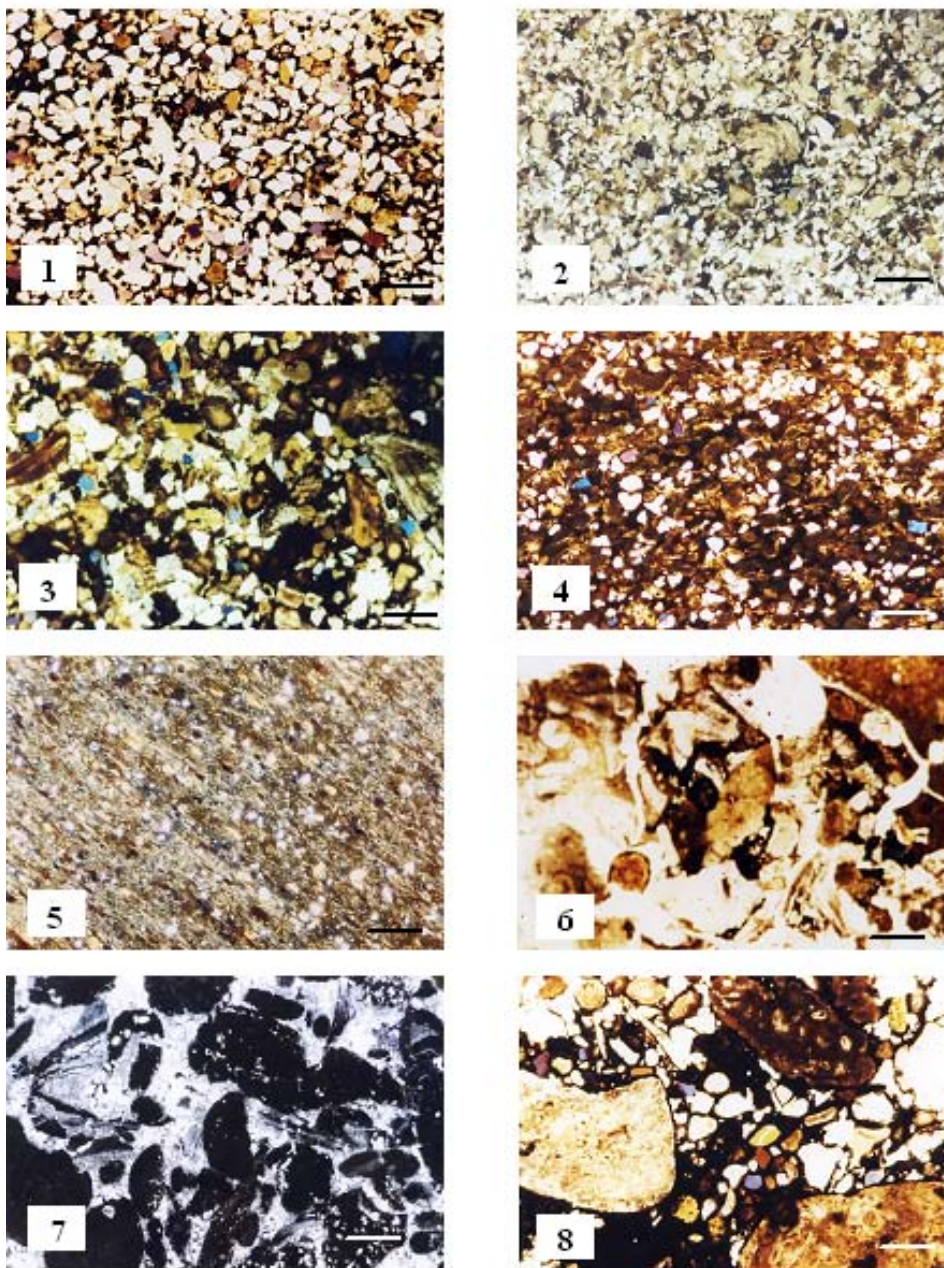


Plate
1/ 1:

Ferruginous quartz arenite, showing pointed contact between quartz grains. Some glauconite pellets are clear with iron-hydroxide, Abu Shegela Member, Duwi Formation, sample no. 1, Qusier area, p.l., bar scale= 180µm. **2:** Phosphatic glauconite quartz arenite, showing concavo-convex contact between quartz grains, poorly defined peloids are made of collophane, bone fragments and glauconite grains, Abu Shegela Member, Duwi Formation, sample no. 5, Safaga area, p.l., bar scale=180µm. **3:** Phosphatic glauconitic quartz wacke, showing different shapes (elliptical-ovoidal-rounded) of glauconite grains and bone fragments of phosphate, Abu Shegela Member, Duwi Formation, sample no. 17, Safaga area, p.l., bar scale= 75µm. **4:** Gloconitic quartz wacke, showing fine to medium quartz, glauconite grains and bone phosphatic fragments cemented by calcite, Abu Shegela Member, Duwi Formation, sample no. 16, p.l., bar scale=180µm. **5:** Laminated phosphatic sandy shale, showing foliation of clay (directive texture) with detrital quartz and collophane pellets, Abu Shegela Member, Duwi Formation, sample no. 18, Safaga area, p.l., bar scale= 180µm. **6:** Phosphorite showing coarse to medium bone fragments and collophane grains with few quartz grains, Abu Shegela Member, Duwi Formation, sample no. 16, Safaga area, p.l., bar scale= 180µm. **7:** Siliceous phosphorite showing collophane, francolite replaced by secondary silica. Cellophane grains are colloform, Abu Shegela Member, Duwi Formation, sample no. 11, Safaga area p.l., bar scale= 180µm. **8:** Sandy ferruginous phosphorite showing phosphate grains (collophan) are pebbly size, subrounded to rounded with fine to medium quartz grains cemented by iron oxides, Abu Shegela Member, Duwi Formation, sample no.2. p.l., Qusier area, bar scale= 180µm.

c. Shallow intertidal phosphorite lithofacies: The phosphorite lithofacies is encountered in the Dawi Formation (Figs. 2 & 3). They are gray to yellowish gray, black, light brown, white, soft to hard, medium to coarse grained, rarely pebbly, calcareous and occasionally, ferruginous, argillaceous, gypsiferous, bituminous and siliceous. The petrography of phosphorite is based on three categories: grains (coated and uncoated), biophases (bone fragments and fish teeth), matrix (fine material) and cement (post depositional). The phosphorites have ovulitic, equigranular and less commonly, heterogranular, organomorphic or massive textures. Their phosphatic components are represented by collophanitic grains, bone and a tooth fragments (grains and biophases). These textural relations can be recognized as fragmental texture demonstrated by coated and uncoted, the interstitial texture corresponding to the fine matrix and the crystalline texture characterized by interlocking grains, demonstrated by the cementing materials. Apatite is the main constituent in phosphorite beds in the studied area and represented by peloids (coated and non-coated grains), microfossils, and microsphere aggregates with sometimes as megafossils, bone fragments and fish remnants (Pl. 1/6-8). Uncoated grains are predominant and represent about 80% of the phosphatic grains. The grains are elongated or irregular shaped. The coated grains represent about 10 % of the phosphatic grains. They are characterized by inorganic or biogenic nuclei surrounded by a cortex of various layers of phosphatic coatings. The biophases consist mainly of bone fragments of different forms. The grains are oval, elongated or irregular, subrounded to round in shaped. The collophane grains are colloform, while the francolite and dahlite forms lath shape and prismatic grains. Some collophane grains are cracked, pitted, and replaced by silica (silicification) having poikilytopic texture. Quartz grains are fine-medium, monocrysalline, subrounded and mature. The bone fragments are replaced by microcrystalline or megacrystalline quartz. The matrix in phosphorite rocks is fine material fillings like iron hydroxides, carbonaceous materials and clay matter. Cements consist of silica, calcite, dolomite, gypsum, anhydrite and iron oxy-hydroxides. According to the common cement present, the phosphorites are divided into three microfacies namely: sandy, silicified, and calcareous phosphorites (Pl. 1/6-8). These phosphorites resulted from the upwelling of oceanic currents from the Tethys onto the broad continental shelves along its southern shore. The optimum depth for phosphate formation is 30–200m (Selley, 1988).

d. Intertidal phosphatic bioclastic dolostone: This lithofacies denotes Abu Shegela Member of the Duwi Formation (Figs. 2 &3). The dolostones are grayish brown, very hard, massive, fossiliferous, and phosphatic with common shark teeth. It consists of a mosaic of interlocking fine dolomite rhombs with many scattered fine to medium quartz grains, fish bones and teeth, foraminiferal tests and iron-hydroxide spots (Pl. 2/1). The rhombs are equigranular, idiomatic to hypidiomatic and unzoned. The size and fabric of the dolomite rhombs, lime mud relics and high phosphatic content suggest that they were formed from early diagenetic dolomitization of an original phosphatic lime-mudstone in the upper intertidal setting.

e. Shallow/deep subtidal oyster rudstone: This lithofacies is present in the Duwi Member of the Duwi Formation, which has an average thickness of about 5-10m (Figs. 2 &3). It is yellowish-gray in colour, with black patches of manganese. Oyster rudstone consists of abundant skeletal grains (oyster fragments, Pl. 2/2), with some reworked fossils highly packed in a lime mud matrix, the fabric of the fossil bank suggests deposition in shallow subtidal regime down to a depth of 40m. The presence of the characteristic calm and deep water (oyster) with good preservation in such shallower water setting must indicate an abrupt sea-level fall after accumulation. Whereas, decreasing accommodation space led to an increase in water energy. This caused the oyster bearing sediment to wash out in site by the high energy wave that winnowed away most of the lime mud in which the fossil shells were embedded.

f. Deep subtidal dolomitic foraminiferal grainstone: This lithofacies is present at different levels of the Duwi and Atshan members of the Duwi Formation (Figs. 2 & 3). Dolomitic foraminiferal grainstone consists of foraminiferal tests, phosphatic grains and dolomite rhombs, some shell fragments with iron-hydroxides. The recorded planktic species are *Globotruncana aegyptiaca*, *Pseudoguembelina deformis*, *Globotruncanella truncanella* and *Gansserina gansseri*. Some foraminiferal walls suffered from recrystallization while their chamberlets are filled with sparry calcite, dolomite rhombs and iron-hydroxides (Pl. 2/3). Dolomite rhombs are equigranular, hypidiomatic, and unzoned. The deposition of the grainstone has taken place subtidal regime under open marine circulation as the facies is grainstone and yields abundance and high diversity foraminifera.

g. Outer shelf foraminiferal packstone: This lithofacies marks the Duwi and Atshan members of the Duwi Formation (Figs. 2 & 3). Foraminiferal packstone contains a great amount of well preserved and diverse planktics beside some smaller benthics

(Pl. 2/4). The foraminifera are closely packed in a dense lime-mud matrix. The most common planktics are *Globotruncana aegyptiaca*, *Heterohelix globulosa*, *Pseudoguembelina excolata*, *Rugoglobigenina rugosa* and *Gansserina gansseri*. They are partially recrystallized into microsparite, while the chamberlets are filled with sparry calcite. The deposition of packstone has taken place in an outer shelf regime under open marine circulation.

h. Deep middle/outer shelf foraminiferal wackestone/packstone: This lithofacies is recorded from the Duwi and Atshan members of the Duwi Formation (Figs. 2 & 3). It contains abundant and high-diversity planktics with some benthics. The recorded planktic species *Globotruncana aegyptica*, *Heterohelix striata*, *Pseudoguembelina defomis*, *Globotruncanella petabidea* and *Gansserina gansseri* are embedded in a lime mud and highly recrystallized (Pl. 2/5 & 6). Some foraminiferal walls suffered from recrystallized while chamberlets are filled with granular sparite cement. This indicates deposition in a quiet-water, deep middle/outer shelf, and the ranges in depths from 120-200m. Under conditions of normal salinity, well oxygenated sea water, high nutrient supply and circulation with the open sea are occurred.

j. Lower intertidal lime-mudstone: The lime-mudstone marks at the top part of the Atshan Member of the Duwi Formation (Fig. 3). It consists of a dense lime mud matrix which may exhibit a clotted texture due to the patchy occurrence of microspar. Few silt and corroded fine sands may occur. It has been deposited in a protected, quiet water lower intertidal setting as indicated from its fine grained nature and low clastic content. This lithofacies designates a marked drop in a relative sea-level near the top of the Duwi Formation.

k. Intertidal chert: This microfacies is only encountered in the Duwi Member of the Duwi Formation (Figs. 2 & 3). It represents about 5% of the rock volume. These cherts are composed of authigenic silica usually in the form of finely crystalline quartz replacing the chalky limestone. Microscopically, microcrystalline silica associated with foraminiferal tests and some collophane, dolomite rhombs and glauconite grains, which embedded in microcrystalline silica and argillaceous matrix. Some shrinkage cracks appear and filled compalately or partially with chalcedony and/or euhedral megacrystalline silica (Pl. 2/ 7).

2. The Dakhla Formation (Upper Maastrichtian-Lower Paleocene):

The term Dakhla Shale was first introduced by Said (1961) exposed at Mut in the Dakhla Oasis, conformably overlying the Duwi Formation and underlying the Tarawan Chalk. It consists of a series of marls and shales which vary in thickness from one place to another. In the study area, the Dakhla Shale is differentiated into two members, the Hamama (Carbonate) Member at the base overlain by the Beida (Shale) Member at the top (Abdel Razik, 1972 and Awad et al. 1992).

Hamama Member is conformably underlain by the Atshan Member of the Duwi Formation (Quseir area) and overlain by the Beida Member. But, it is unconformably underlain by the Duwi Member of the Duwi Formation in Safaga area (Figs. 2 & 3). Hamama Member is usually composed of varicolored (yellowish to yellow), phosphatic, argillaceous fossiliferous limestone with some intercalated reddish brown shale beds in the upper part. The lower most part ~10m of Hamama Member consists of argillaceous limestone and dark gray silty shales. Its thickness increased toward Wasif and Atshan sections (Fig. 4). The Beida Member is conformably overlain by the Tarawan Chalk with a sharp contact, marked with an abrupt change from gray, green shale to white limestone or white chalky limestone. This member is easily recognized along the slope of the study area as it disconformably overlies the monotonous sediments of the Hamama Member. The lower contact was thick tan coloured sandy limestone sequence that is widespread in the region and marks the base of the Beida Member. Generally, it consists of dark gray silty shales intercalated with argillaceous limestones and heterogenous variety of phosphatic particles, fish debris, rare vertebrate remains and microfossils. The argillaceous limestones are decreased toward north and absent in Rabbah section (Fig. 4). The boundary between the two members (K/T) is marked by an erosive surface, reddish brown shale deposits, and faunal turnover event (Faris, 1997; Obaidalla, 2000).

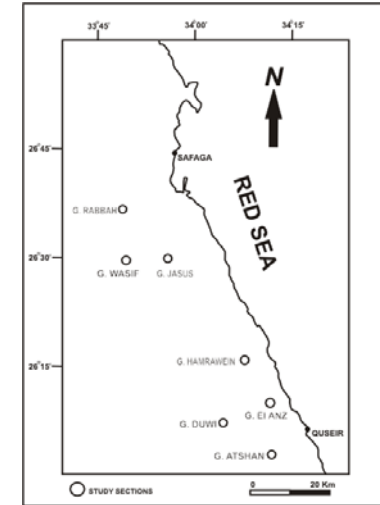
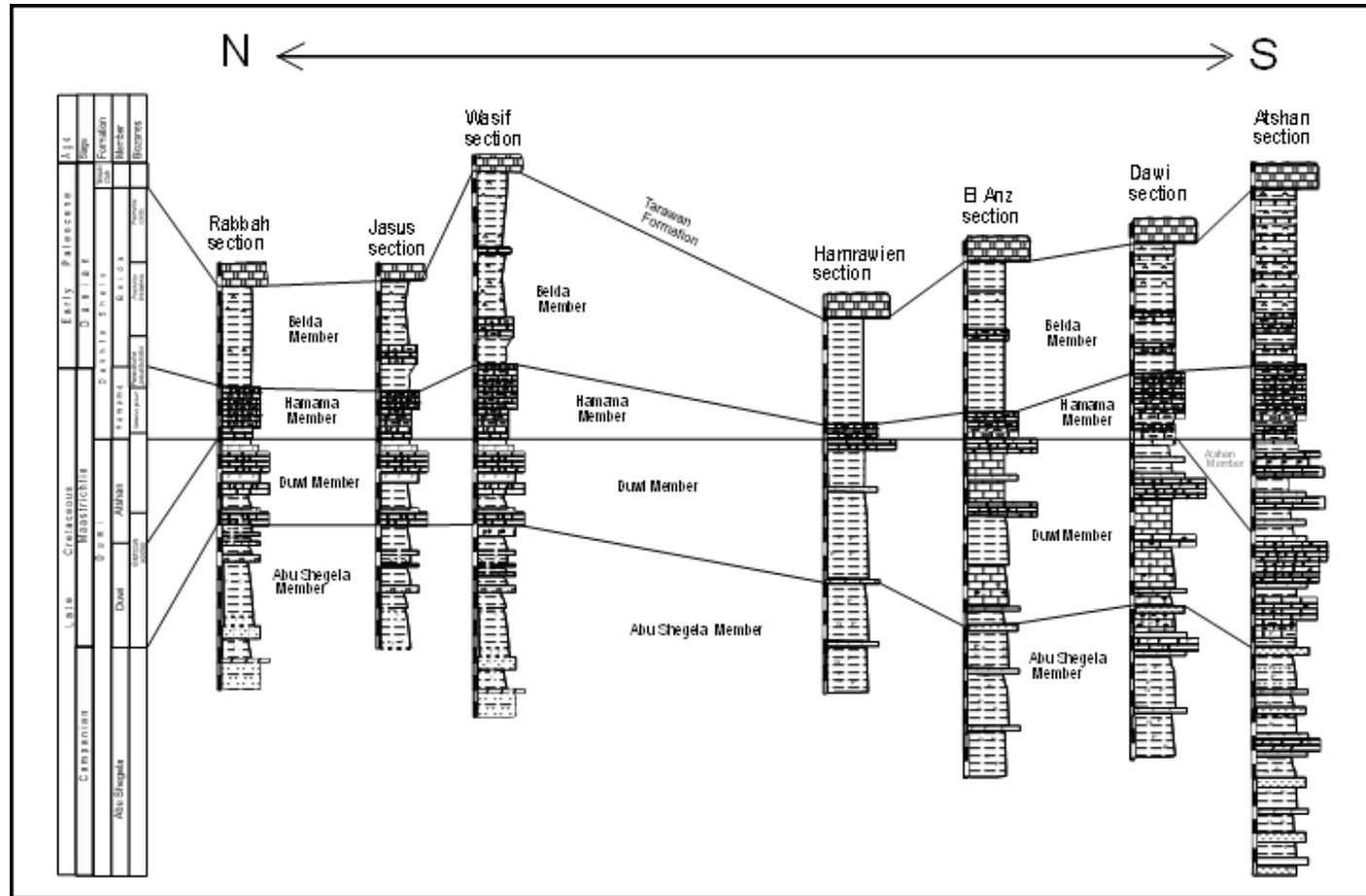


Fig. 4. Stratigraphic correlation of the studied sections in Safaga-Quseir area, foraminiferal zones after Obeidalla (2002), for legend see Figure 2.

PLATE 2

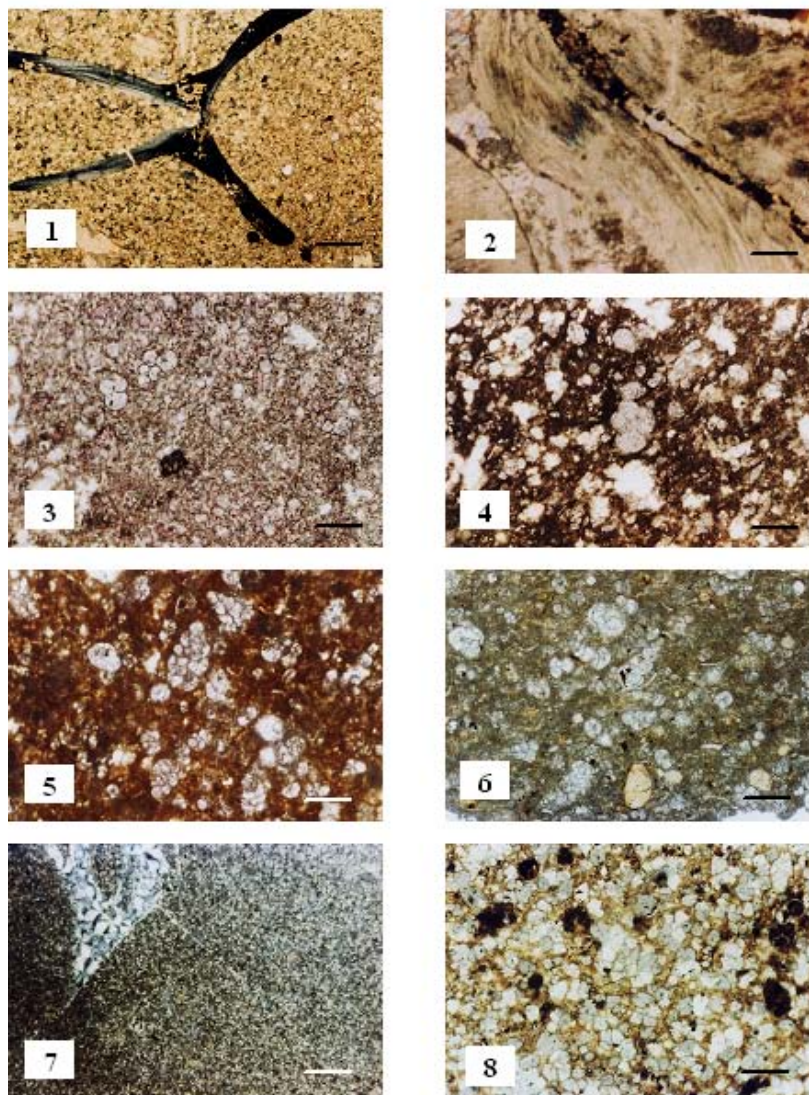


Plate 2/ 1: Phosphatic bioclastic dolostone, showing bone fragments (spine), chambers of tests replaced by microsparite mosaic interlocking, fine dolomite rhombs with many scattered fine to medium quartz grains and fish teeth, Abu Shegela Member, Duwi Formation, sample no. 12, p.l., Qusier area, bar scale= 75µm. **2:** Oyster rudstone, showing original foliated shell fragments (Oyster) with vein filled by zoned sparite cement, some reworked fossil of highly packed in lime-mud matrix, and fenestral fill by calcite cement, Duwi Member, Duwi Formation, sample no. 31, Qusier area, p.l., bar scale= 75µm. **3:** Dolomitic foraminiferal grainstone, showing different foraminiferal tests replaced by sparite and some stained with iron hydroxides, Duwi Member, Duwi Formation, sample no. 39, Qusier area p.l., bar scale= 180µm. **4:** Foraminiferal packstone, showing *Globiogerina* tests with small benthic foram replaced by microsparite, Abu Shegela Member, Duwi Formation, sample no. 30, Qusier area, p.l., bar scale= 75µm. **5:** Foraminiferal wackestone/packstone, showing abundant and high diversity planktonic species with highly glauconite grains. Atshan Member, Duwi Formation, sample no. 20, Qusier area, p.l., bar scale= 180 µm. **6:** Foraminiferal wackestone/packstone, showing abundant and high diversity recrystallized planktonic foraminiferal tests and stained partly by iron oxides with some glauconite grains scattered in framework. Abu Shegela Member, Duwi Formation, sample no. 41, Qusier area, p.l., bar scale= 180µm. **7:** Siliceous mudstone, showing shrinkage crack displaying two layers of fill, each composed of microcrystalline quartz and calcidony quartz, Duwi Member, Duwi Formation, Qusier area, sample no. 26, p.l., bar scale= 180µm. **8:** Phosphatic dolomitic foraminiferal wackestone showing skeletal grains (foraminiferal tests), phosphatic grains (collophan pellets, coprolites, francolite and bone fragments) with iron hydroxides and dolomite rhombs, Hamma Member, Dakhla Shale, sample no. 48, Qusier area, p.l., bar scale= 180µm.

Facies association

The lithofacies characteristics of the Dakhla sediments and faunal associations of planktonic foraminifera (according to Obaidalla, 2000) have been deposition in an open deep middle to outer shelf.

a. Deep middle/outer shelf plagic shale/claystone: The shale/claystone lithofacies is the most dominant lithology among the siliciclastics of the Beida Shale Member of

b. The Dakhla Shale (Figs. 2 & 3). It is varicolored, dark gray, green, brown, reddish brown, and black. The shale beds are fissile, papery, calcareous, partially glauconitic and fossiliferous with highly foraminiferal tests. They are mostly intercalated with lim-mudstone and interrupted by thin sharp-based sandstone streaks and sandstone lenses. Foraminiferal claystone is characterized by the presence of foraminifer's shells. This lithofacies rarely shows any sedimentary structure rather than bioturbation, and sometimes micro-lamination. The latter is usually flexed around foraminiferal tests. The foraminifer's shells are scattered throughout the matrix. The matrix is composed of argillaceous materials and organic matter. Detrital quartz is relatively uncommon and iron-hydroxides may be present within the foraminifer's tests and as pore fillings. The claystone is massive, calcareous, glauconitic, ferruginated and fossiliferous.

The shale /claystone facies appears to have been deposited in a quiet-water shelf lagoon below normal fair-weather wave base and mostly under restricted deep subtidal conditions. It was deposited during periods and in areas receiving appreciable influx of detrital clay size fractions. The thin sharp-based sandstone streaks reflect occasional periods of storm activity. The fossiliferous shale with planktic faunal assemblage has been formed in a moderately deep water of the deep subtidal regime, below storm wave base, under aerated calm water, normal marine salinity and open circulation. The depositional regime was interrupted by occasional periods of storms and sheet accumulation. Sometimes, shale facies is intersected by iron streaks. Most of the dark gray and black shale that enriched in organic matter is recorded. The dark color of this shale indicates deposition in reducing conditions (Temraz, 2005).

b. Deep/middle outer shelf dolomitic foraminiferal wackestone: This lithofacies is present in the Hamama Member, which has an average thickness of about 15m (Figs. 2 &3). Argillaceous limestones are yellowish-gray and black patches of manganese.

Dolomitic foraminiferal wackestone consists of skeletal grains, phosphatic grains, iron-hydroxides and dolomite rhombs (Pl. 2/8). The skeletal grains are shell fragments which are partially replaced with sparite and occasionally by chalcedony with highly foraminiferal tests. The phosphatic grains are isotropic as collophane, coprolites, and anisotropic grains as francolite and/or dahlite and bone fragments are replaced completely or partially with micrite, microsparite and sparite (phosphatic foraminiferal wackestone). Iron-hydroxides are staining some collophane. Sometime, the skeletal grains are mostly foraminiferal tests *Pseudoguembelina defomis*, *Globotruncanella petabidea* and *Gansserina gansseri* which filled partially with sparite and microsparite (foraminiferal bioclastic wackestone/packstone and dolomitized foraminiferal wackestone/packstone). Most of the foraminiferal tests are recrystallized and the collophane pellets are micritized.

This lithofacies reflects a marine environment with normal salinity, low energy and a considerable distance from the land mass (Dunham 1962). The high P/B, total absence of shallow water indicators and absence of bioturbation all point to deposition in a low energy, open deep middle shelf regime under rapid sediments accumulations and water depths of 100-150m.

c. Middle/deep shelf dolomitic lime-mudstone: This lithofacies marks the different parts of the Beida Member (Figs. 2 &3). Limestones are gray and semi-hard and dolomitic. Dolomitic lime-mudstone consists of few foraminiferal with dolomite rhombs. The recorded planktic species *Parasubbotha pseudobulloides*, *Globoconusa daubjergensis*, *Subbotina triloculinoides*, *Globanomallina compressa*, *Prraemurica trinidadensis* and *P. uncinata* are embedded in a lime mud, highly recrystallized Benthics also occur but in small numbers with calcareous species dominating over the agglutinating ones. They are loosely packed in a partially recrystallized and dolomitized lime mud with rare fish teeth and gluconitic pellets. The total absence of shallow-water indicators and absence of bioturbation all point to deposition in a low-energy, open deep middle shelf regime under rapid sediment accumulation and in water depths of 100-150m.

Age (biostratigraphy zones)

The macrofossils are recorded by previous work from the upper part of Duwi Formation (Duwi and Atshan members). They are represented by oysters, vertebrate remains, coprolites, fish teeth, and heteromorph ammonites. Biostromal oyster

limestone with *Ostrea vielli* is recorded from Duwi and Atshan members. The ammonites of Member Duwi are represented by *Bostrychoceras polypolocum* zone (Dominik & Schall, 1984) of Late Campanian age. The topmost part of Member Duwi is characterized by abundant fossils of *Trigonarca multidentata* and *Lybicoceras ismaeli*. The later fossil marks the advent of Maastrichtian (Youssef, 1957). Different microfossils studies (El Dawy, 1993 and Obaidalla, 2002) discussed the foraminiferal distribution. Obaidalla (2002) was discussed the hiatuses, faunal turnover and sea level changes. Five biostratigraphic biozones are defined by Obaidalla (2002) from the Campanian late Maastrichtian: *Globotruncana aegyptica*, *Gansserina gansseri* with a missed biozone *Abathomphalus mayaroensis*. Three biozones are defined from the Early Paleocene *Parasubbotina pseudobulloides*, *Praemurica trinidadensis*, and *Marzovella uncinata*. Two Paleocene biozones are missed as *Guembelitria Cretacea*, *Parvularugoglobigerina eugubina*. The missed biozones are attributed to the K/T mass extinction (Obaidalla, 2002). A detailed study of nannoplanktons by Faris (1997) mentioned *Nephrulitbus frequens* and *Micula murus* (Late Maastrichtian). The geographic distributions of *Micula murus* and *M. prinsii* and *Nephrulitbus frequens* are climatically controlled. *Micula murus* and *Micula prinsii*, tropical to temperate species, are relatively widely distributed, while *Nephrulitbus frequens*, a temperate to boreal species, has a limited geographic distribution in Egypt.

Mineralogy

Wasif and Atshan sections were selected to represent their mineralogical composition contents in the study area (Figs. 5-8).

1. Bulk rock

They are dominated by calcite (5-95%), quartz (5-75%), phosphorite (5-80%), phyllosilicates (5-60%) and iron hydroxides (5-40%). In the lowermost part ~5m of the Abu Shegela Member of the Duwi Formation, calcite-deposition ranges from 5-10%, quartz 15-45%, phosphorite 5-65%, phyllosilicates 10-45% and iron hydroxides 2-5%, suggesting relatively high energy conditions (Figs. 5 & 6). A significant change occurs in the lower part of the Abu Shegela Member, where calcite increases (15-60%), phyllosilicates (20-60%) and detritus quartz to 35%. At Duwi and Atshan members of the Duwi Formation and Beida Member of Dakhla Shale, calcite ratio shows a significant change between carbonate and shale beds.

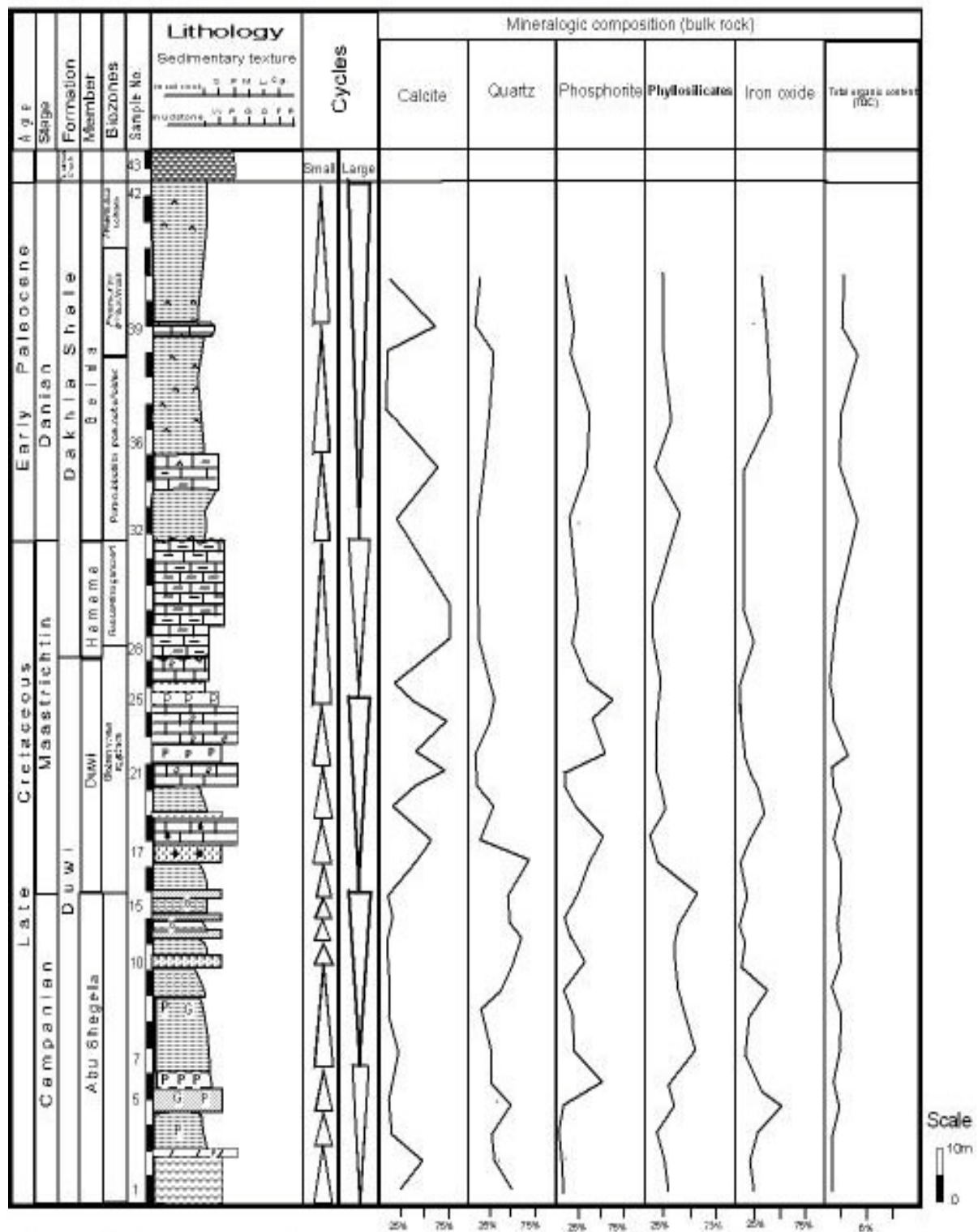


Fig.5: Bulk rock composition and total organic content (TOC) at Gabal Wasif (see legend Fig.2).

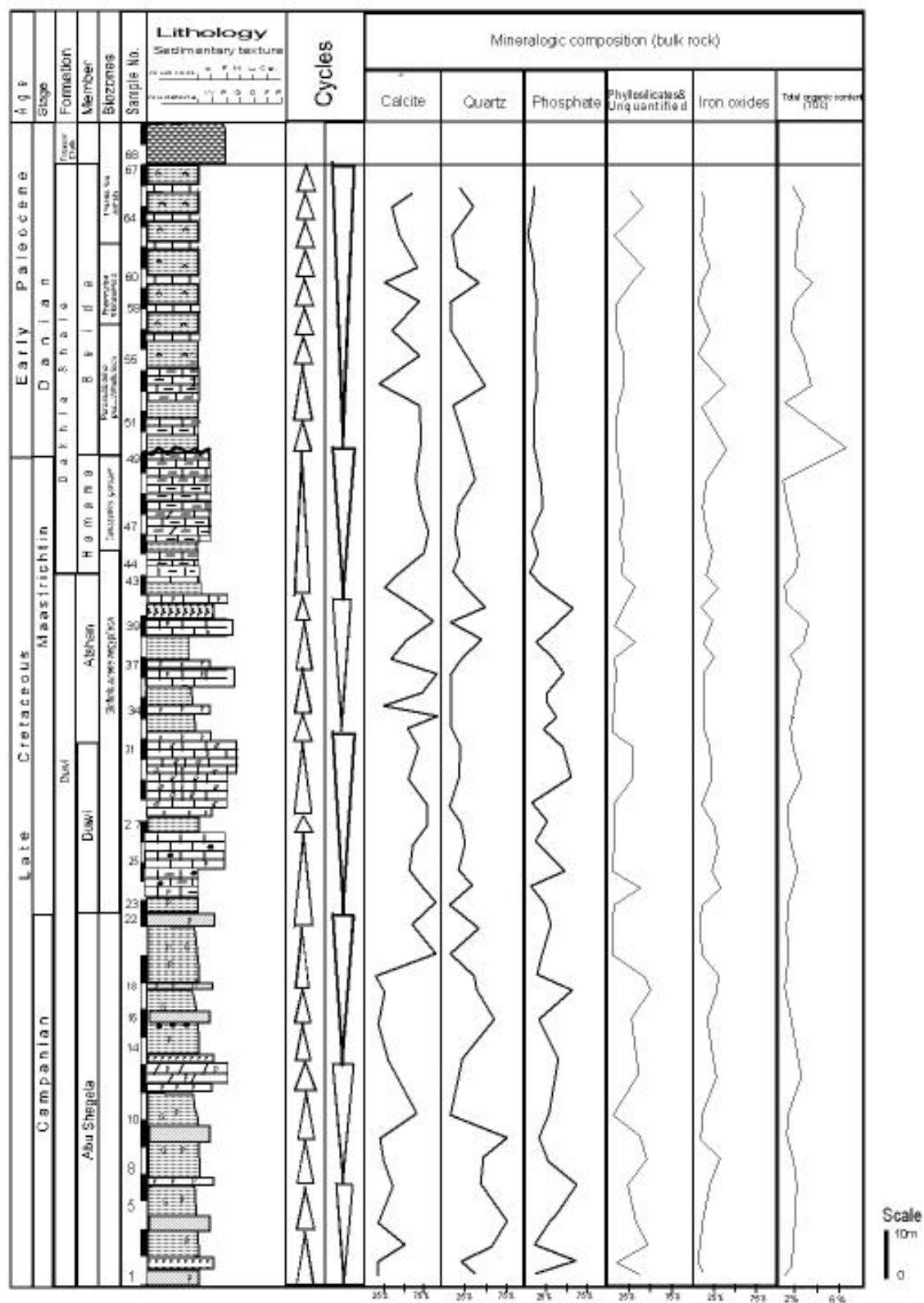


Fig.6: Bulk rock composition and total organic content (TOC) at Gabal Atshan (see legend Fig.2).

In carbonate beds calcite increases (80-95%), while in shale beds decreases (5-20%). Meanwhile, calcite decreases to 5% in claystone/shale beds due to its barren nature. Only at the Beida Member of Dakhla Shale at Gabal Atshan, calcite is fluctuating and ranges from 25-80%. Quartz enrichment observed at Abu Shegela Member and at the top most part of Duwi Member of the Duwi Formation. Maximum detrital influx occurred during the two members (Abu Shegela and Duwi members) at Gebel Wasif and suggesting uplift and subsequent erosions in the southeast area. The presence of the *Oyster villei* marker horizon in these sections indicates shallowing, possibly linked to uplift of Gabal Atshan and or falling sea level. Also, some quartz enrichment is present in some beds of the Atshan Member of Duwi Formation and fluctuates in Dakhla Shale at Gabal Atshan. It suggested a relatively low energy conditions, and indicated by a presence of the *Exogyra overuegi*. The boundary between the two members of Dakhla Shale K/T boundary (Obidalla, 2000), calcite is high again (70-95%), and low phyllosilicates and quartz (2-5%). These data suggest decreased detrital influx during a relatively higher sea level fluctuation. Also, phyllosilicates in shale beds (Beida Member) are gradually increased to a maximum value (~45%) as well as variable quartz percentages (15-55%), suggesting increased erosion and transportation as also indicated by several hiatuses.

At the same time, phosphorite minerals increase to a maximum (80%) at Duwi and Atshan members in Duwi Formation (Figs. 5 & 6). The presence of phosphorite beds suggesting slightly alkaline and slightly reducing marine environment (Eh-0.2m.v.) supported by organic matter (Mason, 1966). High levels of phosphorite minerals as in a Duwi and Atshan members of Duwi Formation are evidence for increased organic fluxes related to upwelling and to augmented continental runoff (Ernst et al., 2006). Phosphorite minerals decrease at the lower part of Dakhla Shale (Hamama Member) reaches to 20% and not recorded at Beida Member. Iron hydroxides are relatively low at both studied sections and a significant change occurs at the top most part of Abu Shegela Member at Duwi Formation and between the two members of Dakhla Shale, it increases (25-40%) at isolated bed (red and brown shale beds at Wasif and Atshan sections, respectively).

2. Clay minerals.

The main clay phase at the Gabal Wasif is kaolinite (15-95%), illite (2-30%), smectite (5-35%), vermiculite (5-15%) and smectite-vermiculite mixed layer (~35%).

While at the Gabal Atshan it consists of kaolinite (35-95%), illite (5-30%), smectite (~30%) and mixed layers smectite-vermiculite (3-35%) and illite-vermiculite (3-18%). In both sections kaolinite is fluctuating in the Abu Shegela Member at Duwi Formation (5-70%), Beida Member of Dakhla Shale (5-85%) at Gabal Atshan and Duwi Member (5-75%) at Gabal Wasif (Figs. 7 & 8). At the same time, increasing illite (5-30%) and mixed layer smectite-vermiculite (15-35%) and decreasing to smectite (0-5%). In Abu Shegela and Duwi members at both sections are marked by decreasing kaolinite (20-50%) and smectite (5-25%) and increasing of illite (25-30%) and mixed layers smectite-vermiculite and illite-vermiculite (20-35%), respectively. Upsection in the top of Atshan Member at Gabal Atshan kaolinite gradually increases (60-70%), where illite (5-15%) and smectite (3-10%) decrease. In clay mineral compositions remain relatively stable in the Hamama Member of Dakhla Shale with kaolinite dominant from 40-60%, illite (8-20%), smectite (5-10%) and mixed layers smectite-vermiculite (2-5%); illite-vermiculite (3-8%). Meanwhile, above the Hamama Member across the contact K/T boundary in reddish brown shale bed, kaolinite (17-25%) and vermiculite (0-7%) decrease while, illite (25-30%) and smectite (20-22%) increase. Near the top part Beida Member of at Gabal Wasif, dominant kaolinite (65-80%) increases where, illite (6-14%) and smectite (0-5%) decrease, with minor amounts of smectite-vermiculite mixed layer (3-15%). While the Beida Member at Atshan section, the kaolinite (70-85%) increases and reached a maximum abundance of 85% in the uppermost part, smectite (15-35%) increases, where illite (3-15%), illite-vermiculite (3-12%) and smectite-vermiculite mixed layers (3-18%) decrease.

Interpretation: Clay minerals in modern oceans seem to be controlled by contemporary climates; marine clays in ancient deposits have been widely used to reconstructed palaeoclimates. Also, it may provide integrated records of overall climatic impacts, whereas other techniques are more likely to reveal local or temporary climates (Thiry, 2000). Changes in clay mineralogy during diagenesis take place principally through rise in temperature accompanying increased depth of burial. Neoformed clays reflect the pore-fluid chemistry, degree of leaching and temperature that existed within the sample at some stage (Tucker, 1990). Also, transformed clays will carry a memory of inherited characteristics from the source area (Curtis, 1990)

During Late Campanian-Early Maastrichtian (Abu Shegela and Duwi members), both studied sections were located near the palaeoequator (Smith et al.,

1994), as also indicated by the high abundance of kaolinite, which is a weathering product in subtropical to tropical environments, where perennial rainfall and mean annual soil temperatures exceed 15°C (Gaucher, 1981). As well as, Late Maastrichtian-Early Danian (Atshan, Hamama and Beida members) warm/wet, tropical, and subtropical conditions are characterized by low seasonality contrasts (cool/dry) and predominantly chemical weatherings are suggested by high kaolinite, moderate illite and relatively low smectite. Meanwhile, a sudden increase in mixed clay layers (illite-vermiculite and smectite-vermiculite) suggests that warm and perennial humid conditions prevailed on the continent (Ernst et al., 2006). Furthermore, Robert & Chamley (1991) reported an increase in kaolinite during Campanian to Paleocene as a period of global warm and perennial humid conditions. Similar dominance of kaolinite was observed in Tunisia, Spain, Israel and Egypt, which considered pointing warm and perennial humid conditions (Li et al., 2000). The recorded smectite are variable, but gradually increase upsection (especially at Atshan section). The presence of smectite is suggested generally linked to transgressive seas and warm climate with alternating humid and arid seasons, but can also reflect volcanic activity where the volcanic activity supplied smectite dust (Deconinck & Ghamley, 1995). The coeval increases in smectite observed at the end of Hamama and Beida members may also indicate drier conditions (seasonality) in elevated areas (Qusier area). Deposition of illite and smectite clay minerals is interpreted as a result of arid conditions in the southern Tethys (Keller et al., 2002). While, the presence of smectite implies that the studied clay minerals are not transformed due to burial diagenesis and they are mostly of detrital origin as a result of local uplift and/or variations in weathering processes and soil formation in the bordering continental area (Chamley, 1989). Illite may be produced from abundance Kaolinite; it is easily transformed to illite (Weaver & Pollard, 1973). Smectite in shales generally transformed into smectite-vermiculite mixed layers (diagenetic alteration). The mixed layers are probably from incomplete adsorption of K^+ into these clay minerals (Milot, 1970). The clay degradation in black shales probably occurred close to the sediment-water interface. Under such conditions, smectite predominantly degrades into smectite mixed layer, or chlorite-like mineral, whereas the more resistant kaolinite is preferentially preserved.

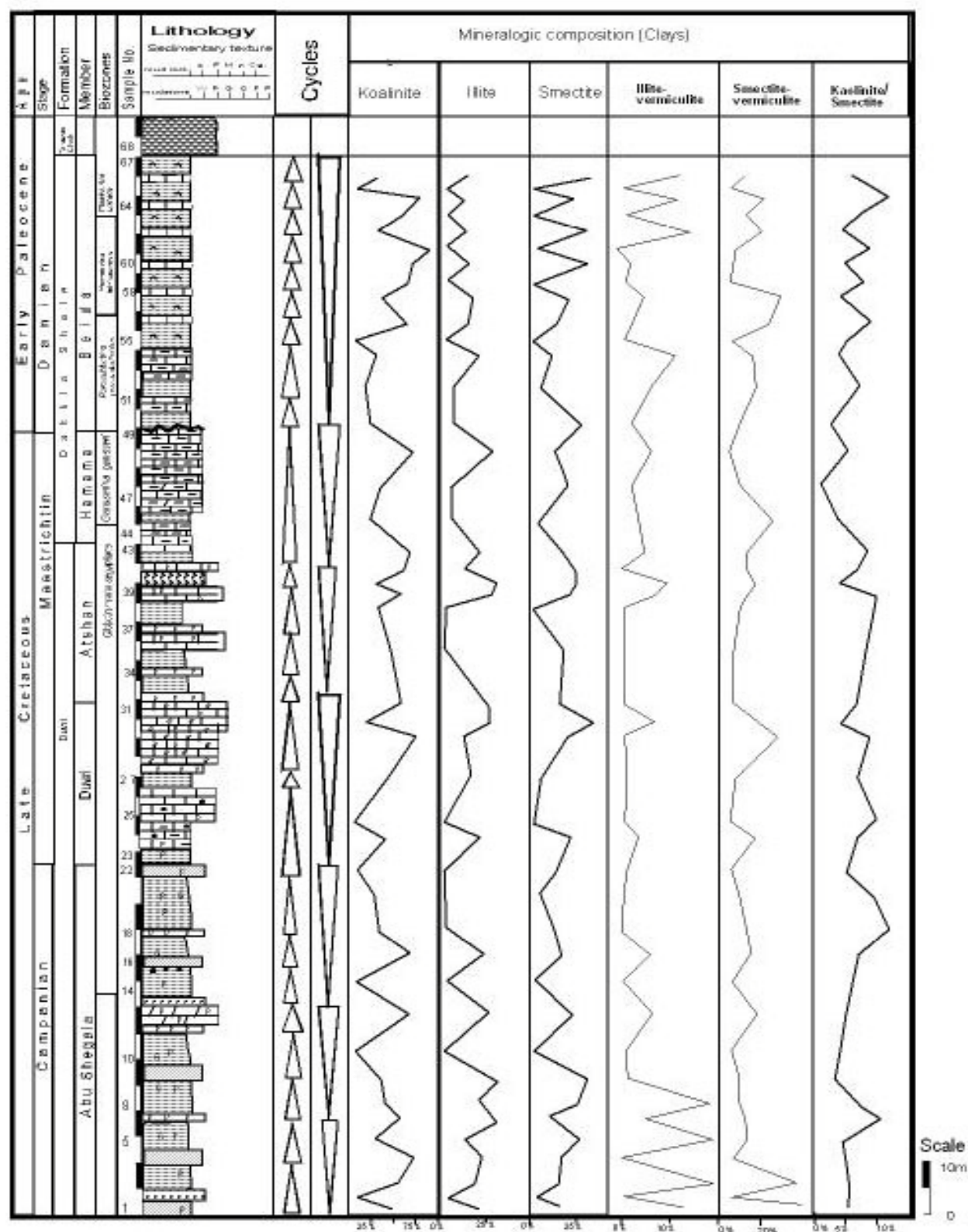


Fig. 8: Clay mineral composition at Gabal Atshan (see legend Fig.2)

The kaolinite/smectite ratio (K/SM) is a climate proxy that reflects humid/warm to more dry and seasonal climate variations and sea-level variations (e.g. Robert & Chamley, 1991; Robert & Kennett, 1992). The inverse trend in smectite in study area can be explained by degradation and partial destruction of smectite by organic acids (chemical weathering) under strongly reducing conditions (Chamley, 1989). Based on this proxy, perennially humid conditions prevailed in studied area during the Late Campanian and Early Danian except for two intervals with drier more seasonally humid climates during the latest Maastrichtian (at the boundary between two members of Dakhla Shale) and in the top of the section (at the end of Beida Shale Member) during the Early Danian (Figs. 7 & 8).

3. Total Organic Carbon (TOC)

The sediments have highest values of organic matter (2.1-6.8 %) occur in the middle part of the Dakhla Shale at Wasif and Atshan section respectively (Figs. 5 & 6). The high content of organic matter is probably responsible for metal enrichment. Although this peak value is anomalous within the other wise low TOC content, planktonic foraminiferal data may show a major increase in low oxygen-tolerant heterohellicids in Late Maastrichtian (Keller et al., 2002), which suggests an influx of nutrient-rich waters and expansion of oxygen minimum zone. In the lower and middle members Abu Shegela and Duwi members of Duwi Formation, values are low (0.1-0.31%), but increase to reach maximum of 2.94% within the upper Atshan Member only at Gabal Atshan. While, in Dakhla Shale (within Hamama Member), the organic matter values are relatively high (1.02-3.1%). This interval is bounded by two erosion surfaces. Also it contains shell fragments suggesting high erosion and transportation from shallower areas and it is indicated by bulk rock minerals. TOC values remain high (2-4.6%) at the top of this member. At the boundary between the two members of Dakhla shale very high TOC values 2.1-6.8% suggesting that the main part of the organic matter in shale could have been altered and partly destroyed. TOC values occurs decrease to 0.1-0.3 % in the K/T (reddish brown shale layers) but gradually increase to 2-3% in the Beida Member. Total Organic carbon (TOC) data indicate that values are generally low and rarely exceed 1.2-6.8 % at Wasif and Atshan sections respectively, (Figs. 5 & 6). Higher values are generally associated with hardground and hiatuses.

Depositional cycles

The stratigraphic successions of the Duwi Formation and Dakhla Shale (3-35m) are differentiated into 14-25 small depositional cycles (Figs. 2 & 3). The Duwi Formation consists of 10-15 repeated cycles, while, the Dakhla shale consists of 4-10 repeated cycles (deepening upward). Each cycle begins at the base with sandstone capped by limestone and such vertical sequences of beds in the cycles is referred to different terms, e.g. transgressive cycles (Mathews, 1974), deepening upward cycles (Grotzinger, 1986) and submergence cycles (Khalifa, 1996). The submergence cycles could be formed when the rate of sedimentation increases with subsidence. This causes an increase of water depth with time and the resulting sedimentary cycle is deepening upward (Figs. 2 & 3).

Cyclic deposition of alternating calcareous shales and argillaceous limestone has been analyzed into large seven shallowing upward cycles (17-70m) based on the interpreted environments and minerals associations. They are represented by a shallowing upward or warm/cold cycles. The first three cycles coincide with the Abu Shegela Member of the Dawi Formation which, show three warm/cold climatic cycles. Each cycle is represented by subtidal calcareous sandstone/shale and ended by intertidal phosphatic bioclastic dolostone. It is characterized by significant differences in clay minerals abundance, except for illite-vermiculite and smectite-vermiculite mixed layers, which show similar patterns in both lithologies. The other two (Duwi and Atshan members) are represented by two warm/cold climatic cycles. The first is oyster rudstone/packstone cycle and the second is represented by claystone interclated with foraminiferal grainstone and foraminiferal wackestone/packstone and both cycles ended by siliceous phosphorites. The Duwi Member at both sections is marked by decreasing kaolinite and smectite and increasing of illite and mixed layers smectite-vermiculite and illite-vermiculite, respectively. Upsection in the top of Atshan Member at Gabal Atshan kaolinite gradually increases, where illite and smectite decrease.

The Dakhla Shale is represented by two cycles. The lower one is corresponding to the Hamama Member and composed of a lime-mudstone and foraminiferal wackestone succession that ended by a reddish brown layer of foraminiferal shale at the K/T boundary. While, the upper one is represented by intercalations of foraminiferal shale and lime-mudstone interbeds. In general, kaolinite

is more abundant in shale intervals than in argillaceous lime-mudstone layers. The Hamama Member of Dakhla Shale is dominant with kaolinite, illite, smectite, and mixed layers as smectite-vermiculite, illite-vermiculite. Meanwhile, above the Hamama Member across the contact K/T boundary is represented by reddish brown foraminiferal shale beds (kaolinite and vermiculite decrease while, illite and smectite increase). The Beida Member of at Gabal Wasif is dominated by kaolinite increases where, illite and smectite decrease, with minor amounts of smectite-vermiculite mixed layer.

Phosphorus concentrations of the different phases reveal highly variable patterns throughout the seven cyclic intervals of the study area analyzed. Therefore, major variations in the P-concentrations are confined to the lithologic changes from phosphate-poor shale to the more P-concentrations phosphorites and bioclastic calcareous sandstone beds. The increases P-concentrations of bioclastic sandstone beds are derived main from the higher calcium whereas total organic shows no major changes, and iron-hydroxides values are highly irregular. These results are reflected from thin-section that reveals biogenic debris as the main source of phosphorite.

DISCUSSIONS

Hiatuses: Based on erosional surfaces, lithofacies, mineralogical changes, faunal assemblages and species abundance/variability, three hiatus can be recognized from the study area. The first hiatus (Late Campanian) is recognized at the middle part of the Duwi Formation (between Duwi and Abu Shegela members). It is indicated by a cold/arid climatic horizon and represented by a conglomeratic, shallow subtidal, sandy phosphatic bed (increase of smectites, and decreases in calcite and kaolinite). Also, this horizon is characterized by abrupt appearance of some foraminiferal species (Obaidalla, 2000). The Campanian-Maastrichtian hiatus is defined from Eastern Desert at the boundary between the Duwi and the Dakhla formations and lies at the biozone of *R. hexacamerata* (Tantawy et al., 2001). The second hiatus (Middle Maastrichtian) is indicated by missing of Atshan Member of the Duwi Formation at the northern sections (Safaga area) or the presence monotonous dark gray silty shales with foraminiferal wackestone/packstone and siliceous phosphorite intercalations (Quseir area).

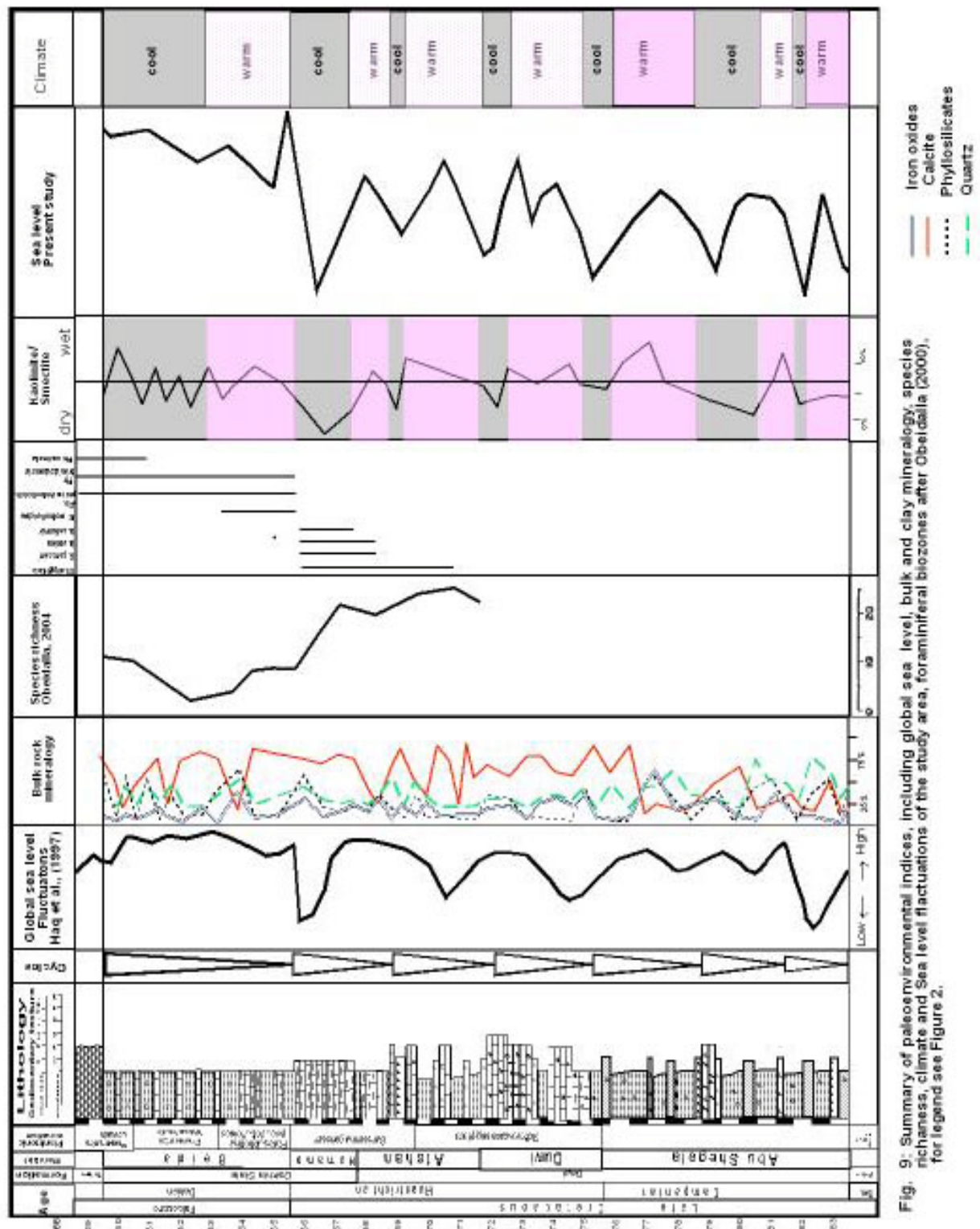


Fig. 9: Summary of paleoenvironmental indices, including global sea level, bulk and clay mineralogy, species richness, climate and Sea level fluctuations of the study area, foraminiferal biozones after Obeldia (2000), for legend see Figure 2.

High level of TOC and phosphorite concretions in Atshan Member of the Duwi Formation are evidence for increased organic fluxes to the sea floor related to upwelling and augmented continental runoff (Ernst et al. 2006). Clay mineralogy indicate cold climatic horizon by decreasing of Kaolinite/smectite ratio. The third hiatus (Late Maastrichtian-Early Paleocene), K/T boundary is represented by reddish to brown shale layer as a major hiatus. This hiatus is recorded biostratigraphy of the nannofossils across the K/T boundary interval in Gabal Duwi (Qusier area) and Gabal Wasif (Safaga area) as mentioned by Faris (1997). Also, Obaidalla (2000) pointed the missing of *Abathomphalus mayroensis* and *Guembelitria cretacea* and *Parvularugoglobigerina eugubina* biozones is recorded from the study area. Furthermore, in mineralogical changes show increased in smectite, quartz and phyllosilicates influxes, while kaolinite is decreased across contact K/T boundary (Fig. 9).

Sea level and climate: Late Campanian to Early Paleocene climate recorder is well known from studies in Tethys, equatorial Pacific and high latitudes (Kucera and Malmgren, 1998). Seven warm/cold climate cycles are recorded from the study area. The cold climates are accompanied by sea level regressions which could be traced from facies and mineralogical changes and the interpreted sea level regressions. Three cold climates are recorded from the Late Campanian sequence, and another four cold climates, one of them is recorded at each of the following horizons; Late Campanian-Early Maastrichtian, Late Maastrichtian, Early Danian, and finally at the Late Danian. Three of the studied climatic horizons coincide with three global regressions (Late Campanian, Campanian-Maastrichtian, and Late Maastrichtian) as mentioned by Haq et al. (1987) and shown in figure 9. These climatic cooling appear to be partly present and/or absence of some species, decreased calcite, kaolinite and increased phyllosilicates, quartz, smectite and illite and TOC. The cold events were intervening by rapid global warmings of sea surface temperatures. The warm events are easily marked by abundance in planktonic foraminifera (Obaidalla, 2000) with high calcite and kaolinite. A global increase in kaolinite is interpreted as a period of global warm and perennial humid condition (Ropert & Kennett 1992).

In the most complete sections at Quseir area, the K/T boundary is marked by reddish brown shale beds, decreased calcite and kaolinite and increased phyllosilicates, quartz, suggesting greatly reduced productivity and climate cooling.

Climate remained cool, though the K/T boundary (Gabal Atshan), where primary productivity did not recover until the Beida Member of Dakhla Formation at Gabal Wasif. Warm climate is recorded pre- and post- the K/T boundary. Early Paleocene sediments from Tunisia, Spain, Israel and Egypt contain abundant kaolinite, which are considered to be warm and perennial humid condition (Thiry, 2000). The kaolinite/smectite proxy reached maximum just before the K/T boundary.

Paleoenvironments

The Late Campanian-Early Paleocene succession displays shallow and deep marine lithofacies that have been deposited under varied paleoecologic condition. Alternatively, a lower sea level coupled with clastic influx and increased evaporation in southern Egypt may have significantly altered marine condition (increased salinity and decreased oxygen), leading to the observed low surface productivity (Schnack, 2000). The Egyptian Campanian-Maastrichtian phosphorites were deposited in very shallow restricted conditions just off an oyster bank (or reef) that developed on the highs created by the tilted blocks. They reflect a more or less static phase in a transgressing sea, interrupted by some marine inundations, and even local regression, before the main Maastrichtian transgression, which covered the whole area, took place (Philobos, 1996).

The study area, shallow subtidal non-fossiliferous shale, and phosphatic dolostones (10-100m) are recognized from the lithofacies study of Abu Shegela Member of Duwi Formation (Late Campanian) and increase in quartz and phyllosilicates. This environment is interrupted by intertidal environments (<10m) due to sea level fluctuations and the deposition of phosphorite, phosphatic glauconitic quartz arenite and wacke. The next two Duwi and Atshan members of Duwi Formation (Early Maastrichtian) are laid under deep subtidal (shallow and deep) and as also indicated by oyster rudstone, phosphatic foraminiferal wackestone/packstone and foraminiferal grainstone, fossiliferous shale intercalations (100-200m) and increases in calcite, Kaolinite. Both, sea-level regressions and coupled with local tectonic condition environment enhanced phosphorite which lay down under a shallow facies, semi-restricted conditions with sluggish circulation and high level of TOC.

During the Hamama member of the Dakhla Shale (Late Maastrichtian) the environment is represented by deep subtidal to deep marine where foraminiferal lime-mudstone and wackestone intercalations with foraminiferal shale are laid down under good circulated and normal salinity conditions (200-250m). This is indicated from

increases in calcite, Kaolinite and species diversity. On contrary, low species diversity, and low planktonic/benthonic foraminifera are recognized from the Hamama Member as indicated from low calcite and increases in smectite (cold climate). This condition reaches its maximum at the Late Maastrichtian/Early Paleocene boundary (end of Hamama Member). The critical high-stress conditions that is usually associated with the K/T boundary event. The condition that created this high-stress environment is likely related to local tectonic activity coupled with global climate, sea-level changes and high level of TOC. During the Beida Member of the Dakhla Shale the environment is represented by deep/middle outer shelf dolomitic foraminiferal wackestone and dolomitic foraminiferal lime mudstone with witnessed species recovery and retaining warm climates indicated from abundant of kaolinite and calcite. Upsection (Late Danian) data suggests gradually increased quartz and pylosilicates during a relatively higher sea level fluctuation, suggesting increased erosion and transportation.

CONCLUSIONS

- * Microfacies analyses, X-ray mineralogy and faunal contents are improved information on seven shallowing upward cycles coeval with cool/warm cycles.
- * Normal environmental conditions were established during the Late Campanian, whereas warm climate and surface productivity increased. At the end of Maastrichtian, the surface productivity is decreased significantly.
- * During the Early Danian the lithofacies data and faunal assemblage indicate that the similarity of fluctuating high-stress seasonality prevailed as elsewhere in the marginal eastern Tethys, with normal condition established in the Hamama member, or calcareous nannofossils. Mineralogical data indicate increased erosion and detrital influx due to local tectonic activity.
- * During the Late Campanian to Early Danian, the abundance of kaolinite suggests warm, wet, tropical and subtropical conditions characterized by low seasonality contrasts and predominantly chemical weathering (high kaolinite and low smectite).
- * Safage-Quseir area witnessed frequent short hiatuses due to local tectonic activities and global sea-level changes, as evident by the short and incomplete foraminiferal and calcareous nannofossils.

* Three hiatuses intervals are observed. Two hiatuses are recorded from Quseir area while three hiatuses are recorded from Safaga area corresponding to three stratigraphic horizons. The first hiatus (Late Campanian) is defined from the Quseir area as interpreted from the losing of Atshan Member. The second hiatus (Late Maastrichtian) is recognized at the uppermost part of the Duwi Formation as indicated by a conglomeratic phosphatic bed in the studied sections. The third hiatus (Late Maastrichtian-Early Paleocene) is recognized by the missing of *Abathomphalus mayroensis* and *Guembelitra cretacea* and *Parvularugoglobigerina eugubina* biozones.

* Based on Kaolinite/smectite proxy, perennially humid conditions prevailed in study area during the Late Campanian and Early Danian except for two intervals with drier more seasonally humid climates. The first is recorded during the latest Maastrichtian (at the boundary between two members of Dakhala Shale) and the second is founded at the top of the section during the Early Danian.

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الملخص العربى

سحنات وبيئات الترسيب لنتابع الكامبانى-الباليوسين الجانب الغربى من شاطئ البحر الاحمر، مصر

عليه زلط ، فاطمة رمضان وياسر الصافورى

تم دراسة سبع قطاعات استراتيجرافية لنتبغات متكونى الضوى و الداخلة التابعة لأعمار الكامبانى-الباليوسين بمنطقة سفاجا-القصير، الواقعة على الجانب الغربى من شاطئ البحر الأحمر بمصر. يمثل هذا التتابع رسوبيات بحرية مختلفة (شاطئية وعميقة)، تميزت باختلافات فى السحنات الصخرية وسمك الطبقات. وتم تحديد عديد من الدورات وبيئات الترسيب، بناء علىدراسة السحن الدقيقة المختلفة وحيود الاشعة السينية للعينات الصخرية والطينية و دلت نتائج الدراسة على تقدم البحر خلال متكون الضوى عند العضو ج (الكامبانى المتأخر). وتم الاستدلال على ذلك من زيادة معدنى الكالسيت والكاولينيت. وقد دل ذلك على وجود فترة مناخية حارة اثناء ترسيب نفس العضو وكذلك وجود فترتين متداخلتين لمناخ بارد، واستدل عليهما من وجود زيادة فى معدنى الفيلوسيلكات والكوارتز. مما ادى الى وجود ترسيبات من الفوسفات والحجر الرملى الكلسى الغنى بمعادن الجلوكونيت وترسيبات الطين الصفائى الخالية من الحفريات والتى تم ترسيبهم فى بيئات بحرية مختزلة (شاطئية ومستنقعات). بينما تميزت ترسيبات متكون الضوى عند عضويين ب،أ (الم استي يختى المبكر) بترسيبات ببيئة بحرية غنية بالحفريات والتى عكست دورتين مناخيتين حارة وباردة. وعاود المناخ البارد فى الظهور مرة أخرى عند متكون الداخلة عضو الجيرى (الم استي يختى المتأخر) مع زيادة الترسيبات النهرية (وتميزت تلك الفترة الزمنية بنقص فى الحفريات الدقيقة وزيادة فى السليكات والكوارتز والمادة العضوية). بينما تميز عضو الطين الصفائى لمتكون الداخلة فى الجزء السفلى (الم استي يختى المتأخر- والباليوسين المبكر) بوجود فترة مناخية حارة، أما الجزء العلوى لنفس العضو فيعكس ترسيبات متداخلة من الحجر الطينى الصفائى والحجر الجيرى لبيئات بحرية عميقة وكذلك وجود زيادة أنواع الحفريات الهائمة وتميزت بتذبذبات مناخية.

LAMINATED SI / SiC COMPOSITES

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ABSTRACT

Laminated Si/SiC ceramics were synthesized from porous preforms of biogenous carbon infiltrated with Si melt at a temperature of 1500°C for 2 hr. Due to the capillarity infiltration with liquid Si, intrinsic micro- and macrostructure in the carbon preform were retained within the final ceramics. The SEM micrographs indicate that the final material exhibit a distinguished laminar structure with successive Si/SiC layers. The produced bodies can be used as high temperature gas filters as indicated from the permeability results.

Key words: Laminated, Si/SiC composites, microstructure, permeability.

INTRODUCTION

Porous SiC ceramics have been a focus of attention in the field of porous ceramics due to their superior properties, such as low thermal expansion coefficient, high thermal conductivity and excellent mechanical strength, (Rojas et al., 1997 and Passalacqua et al.1998). However, their brittleness limits their use in most structural applications.

To improve fracture resistance in brittle-matrix composites, the use of a weak interface that promotes crack deflection is necessary (Evans and. Marshall, 1989). The earliest ceramics composites used as interfaces are boron nitride or carbon; however, these materials are prone to oxidation at high temperature. Porous-oxide layers seem to be an attractive alternative and have been successfully demonstrated as effective interface layers in laminated ceramic composites (Brien et al., 2000 and **Blanks** et al. 1998). Laminated system consisting of porous-Al₂O₃ interfaces between Al₂O₃ bars showed markedly improved fracture resistance for these composites as compared with monolithic Al₂O₃ (Brien et al., 2000).

Clagg et al. (1990) has produced laminated SiC with graphite interface layers. These multilayer SiC composites show apparent toughness and fracture energy 5 and 200 times respectively higher than the typical values of monolithic-SiC. Recently, it was shown that laminated composites without weak interfaces also exhibited damage-tolerant behaviors (Ebillat et al., 1998 and Kuoand & Kriven 1998).

Due to its internal sintering, SiC ceramics are usually fabricated at extremely high temperature (Omori and Takei, 1982). Thus the key problem is how to prepare porous SiC ceramics at relatively low temperature (Ding et al., 2008). Usually, oxide with low sintering temperature may be added into the starting materials to form oxide bonded porous SiC ceramics. These oxide bonded porous ceramics possess weak bonding interface between SiC and oxides which thus impairs the properties of porous SiC ceramics (She et al., 2002).

In the present study, a novel method of synthesizing laminated Si/SiC composite was described, by which SiC multilayer ceramic can be fabricated of biomorphic materials. The Physical and mechanical properties of the samples were investigated together with their microstructure and phase composition.

Experimental procedure:

Specimen preparation

A woven textile with square plates (about 15 plates of 10X10 cm²) were collected together forming a plate with dimensions of 10X10X1 cm³. About 10 pieces were pressed by two light weigh ceramic plates to obtain flat samples. They were heated under N₂ gas in vacuum furnace up to 350°C with a holding time of 2h, with heating rate of 1°C /min, up to 800°C for 1h and finally cooled down to room temperature. Carbon fibers like plates were produced. Si powder (Elken HQ from 1-10 μm) dispersed in isopropanol alcohol in the presence of a dispersing agent (Hypermer; polyalkylene amine derivative) was used as infiltrant. The pyrolyzed carbon specimens were impregnated with silicon slurry (4.5, 8 wt% silicon) under vacuum for 1 h to ensure complete impregnation. After drying at 90°C; heat treatment at 1500°C with a soaking time of 1 h at the peak temperature in a graphite reactor under vacuum resulted in the formation of a laminated porous Si/SiC composite. The obtained Si/SiC plates were cut in to bars of 5X0.5X0.5 cm³ for further investigation.

Characterization:

The bulk density and open porosity of the fabricated composites were evaluated using the Archimedes method (ASTM C-20) with water as the liquid medium. XRD analysis of powdered samples using monochromatic Cu K_α radiation (D 500, Siemens, and Mannheim, Germany) was used to identify crystalline reaction products. Microstructures were observed using SEM (Model XL 30, Philips, Eindhoven, Netherlands). Bending

strength was measured using a three point bending test on a universal testing machine (Model 4204, Instron Corp., Danvers, Mass.) at a crosshead speed of 1 mm/min. At least 10 specimens with the dimensions of 50 x 10 x 7 mm were measured. The permeability of air through specimens was measured at 1 atm. of gas pressures difference in a permeation test cell. Permeability was determined according to Darcy's law.

$$K = \frac{q \cdot L \cdot \eta}{\Delta p \cdot A}$$

Where: q , mass flow (m^3 / s), L , sample thickness (m), η : air dynamic viscosity (Pa s), Δp : back pressure (Pa), and A : sample area (m^2).

1 Darcy (D)* = $0.9869233 \times 10^{-12} \text{ m}^2$ SI-unit of $k = 1.02325 \times 10^{12} \text{ D}$

RESULTS DISCUSSION

Carbonization

The pyrolysis of the cellulosic fabrics has a great effect on the properties of the composite. It is important to choose a pyrolysis appropriate temperature for avoiding distortion and crack during the carbonization. Figure (1) shows the TG curve of the raw textile. It shows that the weight loss started below 100°C and was almost terminated at 500°C . The maximum decomposition rate was in the temperature range of 245°C - 400°C . Mechanisms involved in the conversion of cellulose to carbon are⁽¹³⁾: [1] desorption of adsorbed water up to 150°C , [2] splitting off of cellulose structure water between 150°C and 240°C , [3] chain scissions, or depolymerization, and breaking of C-O and C-C bonds within ring units evolving water, CO and CO_2 between 240°C and 400°C , [4] aromatization forming graphitic layers above 400°C and [5] completion of the decompositions and rearrangements, leaving a carbon template structure. Cellulose breaks down with a stepwise manner at $245\text{--}475^\circ\text{C}$, and the total weight loss of about 75% (Fig.1) occurred due to evolution of H_2O , CO_2 and volatile hydrocarbons from fragmentation reactions of the polyaromatic components. The mass loss of the textile preforms after carbonization up to 1000°C is about 80 wt% shrinkage in all directions.

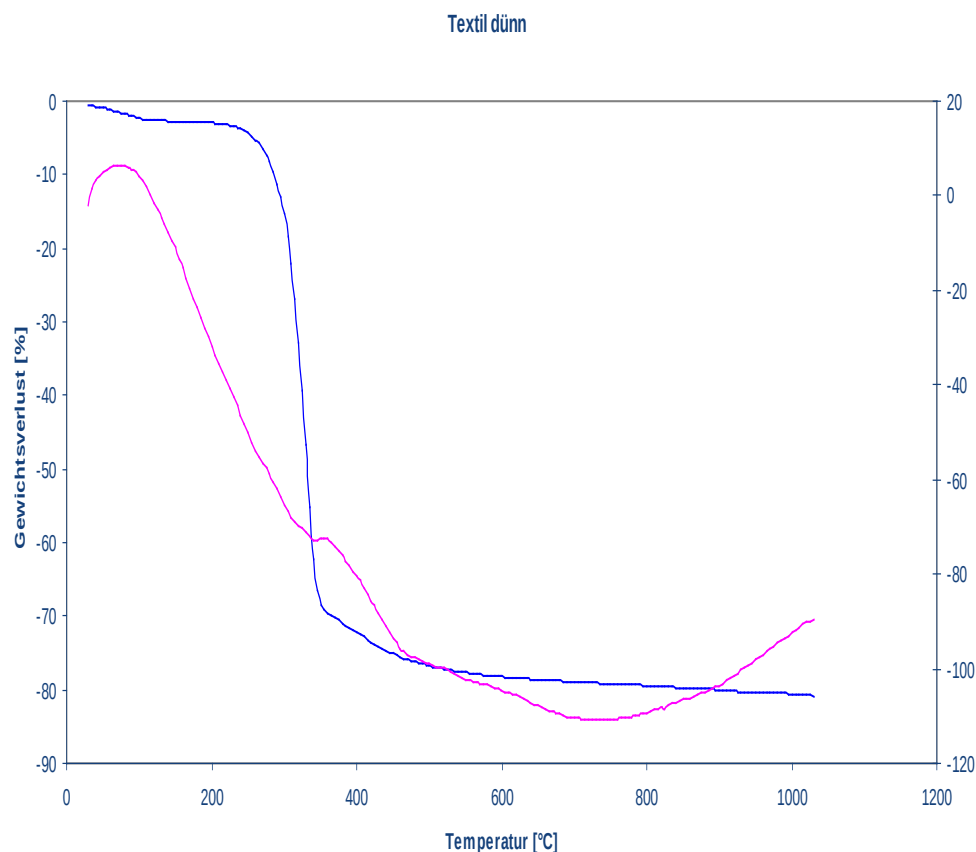


Figure (1) The TG curve of the raw textile.

Phase Composition

XRD patterns of the bodies fired at 1500°C, Fig. (2a, 2b) showed that the only phases present are β -SiC and Si. As expected bodies containing 8 wt % Si showed higher Si phase content. XRD pattern indicates that silicon reacted with carbon and converted into SiC. The residual free silicon formed the Si/SiC composites. In both compositions, there is no evidence for residual carbon or crystalline SiO₂ phase. The reaction mechanism of liquid Si with the porous carbon template can be divided into four stages:

- 1- Nucleation stage: heterogeneous nucleation of nano-scaled SiC grains on the inner surface of the carbon template by reaction with Si vapor below the melting point of Si ($T < 1400^\circ\text{C}$).
- 2- Initial phase: Simultaneous nucleation of nano-grained and coarse-grained β -SiC after Si melts infiltration ($T \geq 1400^\circ\text{C}$).

3- Intermediate stage: diffusion-controlled growth of the β -SiC layer into the carbon struts ($T > 1400^\circ\text{C}$).

4- Final stage: dissolution of the nano-grained SiC in the Si melt and re-crystallization on coarse-SiC grains resulting in a coarsening of the coarse-grained SiC phase ($T > 1400^\circ\text{C}$) (Zollfrank and Sieber, 2005)

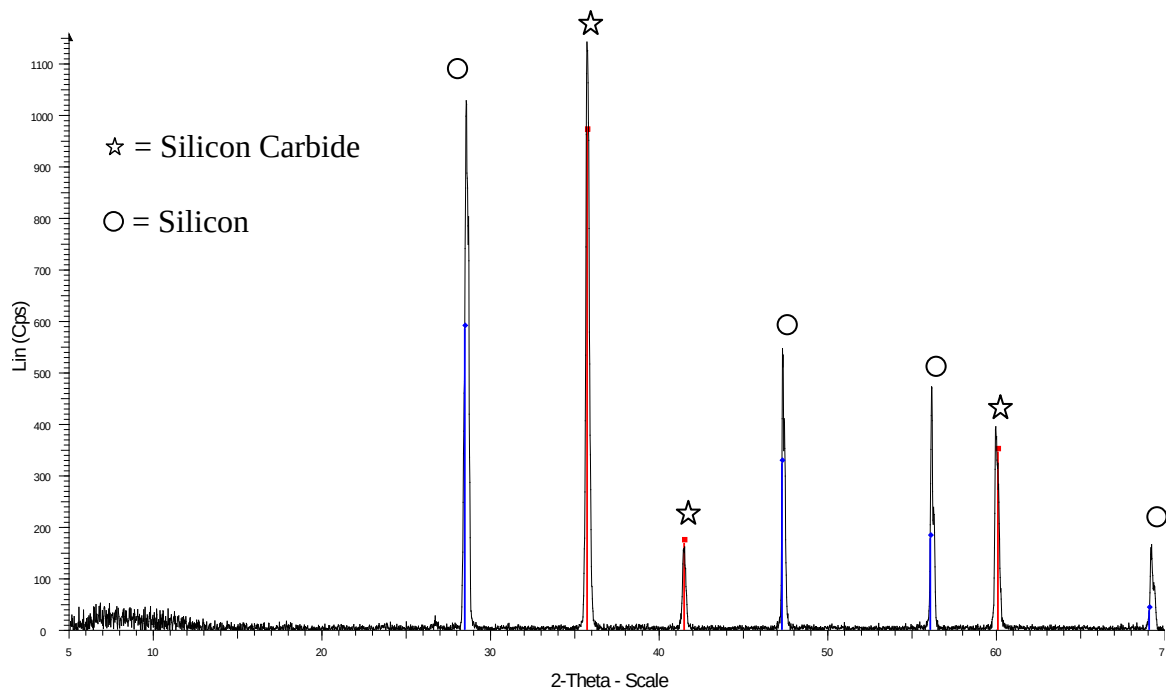


Fig. (2a): XRD patterns of the Si/SiC composites containing 8 wt % Si fired at 1500°C .

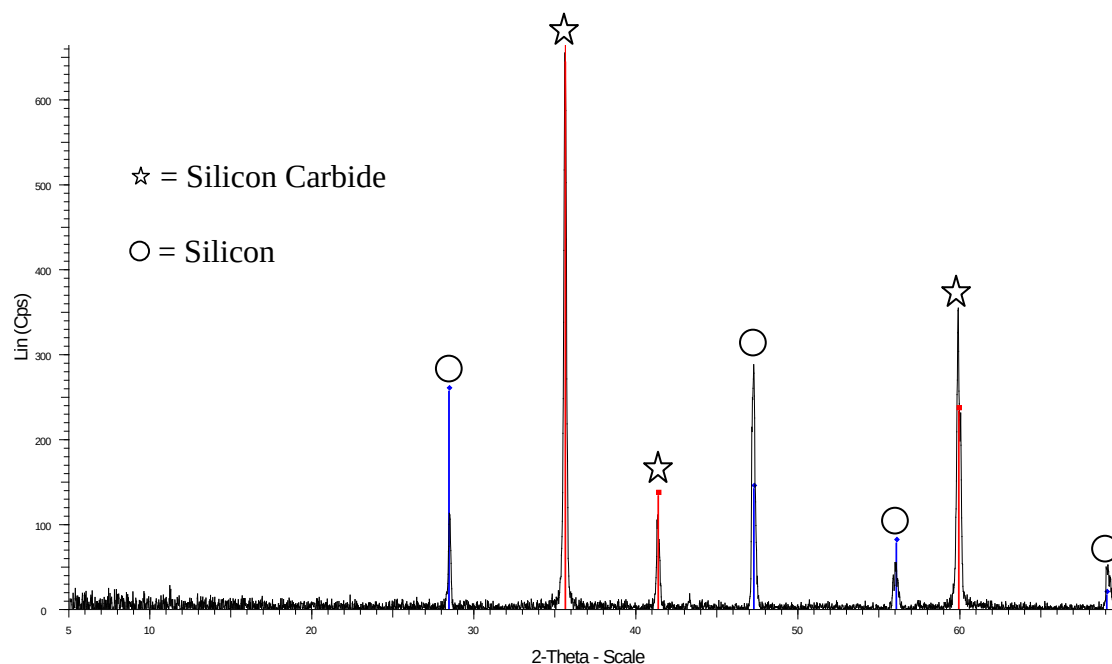


Fig. (2a): XRD patterns of the Si/SiC composites containing 4 wt % Si fired at 1500 °C.

Physical Properties

The density of Si/SiC composites containing 4.5 wt% is 0.46 g/cm³ and its apparent porosity is 48.13%. Increasing Si content to 8 wt% increases the composite bulk density to 2.10 g/cm³ and decreases its apparent porosity to 17.93%. Table (1) shows the densification of 8 wt% Si bodies resulted from the filling of preexisting pores with Si.

Microstructure:

A low temperature pyrolysis at 800°C of the cellulosic fabrics followed by infiltration of Si into the skeletal carbonaceous preforms under pressure and firing at 1500°C produces Si/SiC composites with structure of the native cellulosic fabrics, Fig. (3a). Low applied load, the laminated fabric layers resulted in gaps between carbon layers after carbonization. These gaps were filled with silicon and exhibited a distinguished laminated microstructure, Fig. (3b). Figure (3c) indicates the presence of SiC whiskers grown on the surface of the SiC fibers. Formation of SiC whiskers during the synthesis of biomorphic SiC ceramics from mineralized cellulosic preforms were reported in several recent publications⁽¹⁵⁻¹⁷⁾. Impurities from natural cellulose might be acted as nucleation sites.

Silica deposited on the carbon surface is known to promote the growth of SiC whiskers (Vyshnyakova et al. 2004).

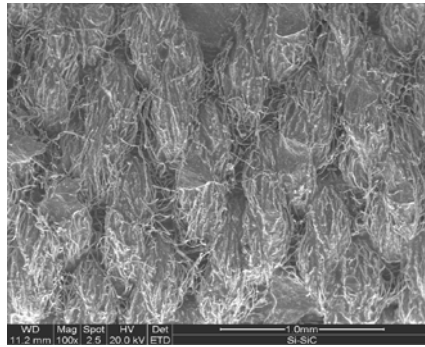


Fig. (3a) SEM micrograph of laminated Si/SiC composite fired at 1500°C showing retention of the native fabric architecture

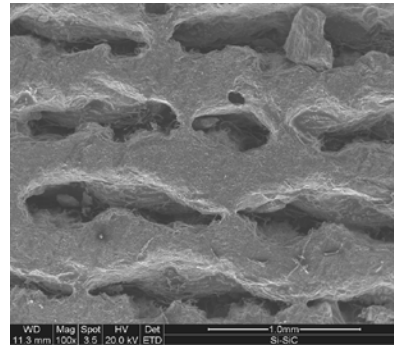


Fig. (3b) SEM micrograph of laminated Si/SiC composite fired at 1500°C showing necks between the stacked layers.

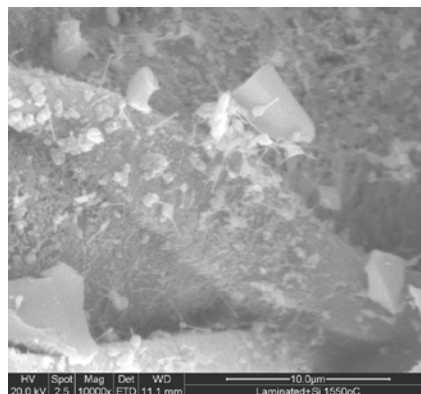


Fig. (3c): SEM micrograph of laminated Si/SiC composite fired at 1550°C showing very fine grains and whiskers of SiC.

Permeability Measurement

The fluid permeability is an important parameter for porous ceramics. Liu et al.(2001) indicated in their study that the gas permeability of the ceramics specimens is dependent on the average pore size than the open porosity. Plot of the gas flow rate vs. applied pressure gradient is given in Fig. (4), this plot is linear, showing that flow is in a good agreement with Darcy's law. Multiplying the slope of this line with the viscosity η of the fluid yields the permeability K . Accordingly; K results are illustrated in Table (1). It is suggested that the gas transportation in the studied bodies is affected by laminar flow when the bodies are fired at high temperature. The Darcian permeability of the produced

laminated Si/SiC composites was found to be $2.8 \times 10^{-10} \text{ m}^2$, which is in the order of magnitude of gas filter supports, and, therefore, also suitable for several technological applications.

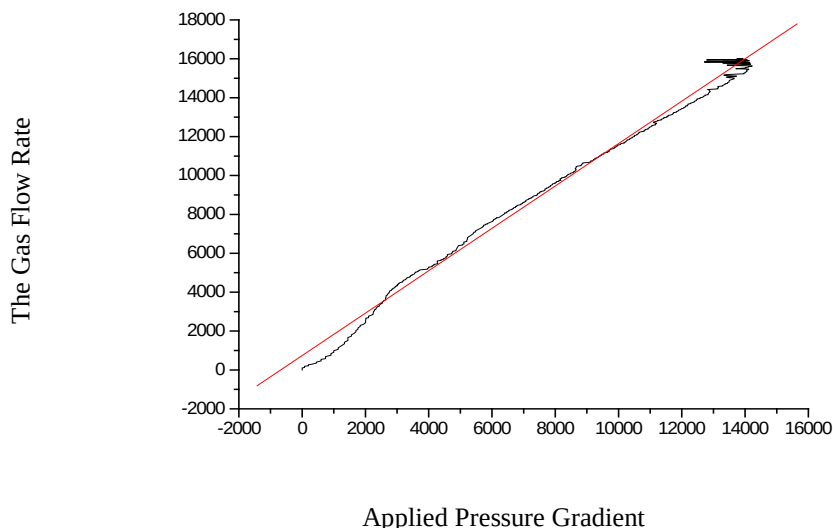


Fig. (4): The gas permeability of the Si/SiC composites containing 4 wt % Si.

Table (1): The permeability and physico- mechanical properties of Si/SiC

Si content, %	Physical Properties		Bending Strength	Permeability, K (D)
	Bulk Density	Apparent Porosity		
4.5	0.4637	48.13	34	9.72E+00
8	2.1	17.93	42	

Mechanical Properties:

Table (1) shows the bending strength of Si/SiC composites laminates. It is obvious that the bending strength of the composites infiltrated with 8 wt % Si is higher than that infiltrated with 4.5 wt % Si. The low porosity and high density of the Si/SiC composites infiltrated with 8 wt % Si played a significant role in increasing its bending strength. This suggested that the silicon content was the critical factor influencing the final bending strength.

CONCLUSION

- 1- Laminated Si/SiC composites derived from cellulosic fabrics were fabricated by process of lamination, carbonization, and infiltration with liquid silicon. The process is simple, low-cost and provides excellent shape-making capability.
- 2- The produced bodies exhibit a distinguished laminar structure with successive Si /SiC layers.
- 3- The biomorphous Si/SiC composites retained the features of the original native-cellulose fabrics. The produced porous materials possess a novel, directed pore morphology which cannot be reached conventional ceramic processing technologies.
- 4- The Si content of the composites has a significant effect on the mechanical properties.
- 5- The Darcian permeability of the produced composites is $2.8 \times 10^{-10} \text{ m}^2$

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INHIBITORY EFFECT OF GARLIC EXTRACT ON PENICILLIC ACID PRODUCTION AND GROWTH OF *PENICILLIUM HIRSUTUM*

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ABSTRACT

The inhibitory effects of aqueous garlic extract on growth and penicillic acid production of *Penicillium hirsutum* were established. Minimal inhibitory concentration (MIC) of the aqueous garlic extract was determined by the agar diffusion assay and it has been found to be 30 mg/ml. Growth of the fungus in broth containing higher concentration of garlic extract (18 and 24 mg/ml) showed that sporulation was completely inhibited after 7 days of incubation or become very slight after 10 days at these mentioned concentrations. The increase in garlic concentration induced a gradual increase in the average values of mycelial dry weights reaching a maximum at 24 mg/ml. In the contrary, the increase in garlic concentration induced a reduction in the levels of penicillic acid production. The amount of penicillic acid in presence of 24 mg/ml of garlic was approximately 44% of that present in control culture filtrate after 10 days of incubation, however penicillic acid was not detected completely at the same garlic concentration after 7 days of incubation. This study was also extended to analyze and evaluate the percentage of the main components in garlic extract that may responsible for these inhibitory effects by using GC-MS chromatogram analysis. This is the first report on inhibition of penicillic acid production by a natural substance like garlic extract.

Key words: Garlic; Mycotoxins; Penicillic acid; Antifungal activity; *Penicillium hirsutum*

INTRODUCTION

Over the years many efforts have been devoted to the search for new antifungal materials from natural sources for food preservation (Onyeagba *et al.*, 2004; Haciseferogullari *et al.*, 2005). Among the natural fungicidal substances, garlic extract has been found to be active in various trials (Yoshida *et al.*, 1987; Tariq & Magee, 1990; Ghahfarokhi *et al.*, 2003; Irkin & Korukluoglu, 2007). Moreover, aflatoxin production by *Aspergillus parasiticus* and keratinolytic activity of *Trichophyton mentagrophytes*, were reported to be inhibited by the use of garlic (Graham & Graham, 1987; Ghahfarokhi *et al.*, 2003).

Among the toxigenic fungi, *P. hirsutum*, is a frequent contaminant of different food products, flower and vegetable bulbs and seems to be common, widespread species occurring in storage and is known to produce a variety of secondary toxic metabolites (Frisvad, 1981; Frisvad & Samson, 2004; Satio *et al.*, 2004; Overy *et al.*,

2005) including penicillic acid (Frisvad & Filtenborg, 1983; Ezzat *et al.*, 2007; Ismaiel, 2007). The hazardous effects and carcinogenic nature of penicillic acid made it a cause of concern (Palmgren & Ciegler, 1983; Wannemacher *et al.*, 1991; Macri *et al.*, 2002; He *et al.*, 2004). The potential risk of penicillic acid for human health was suggested when penicillic acid had been isolated from agricultural products such as poultry feed, blue eye diseased corn, commercial corn, dried beans and tobacco products (Kurtzman & Ciegler, 1970; Pero *et al.*, 1972; Thrope & Johnson, 1974) and from mold-fermented sausage (Ciegler *et al.*, 1972).

In spite of reports on the antifungal activity of garlic (Graham & Graham, 1987; Ghahfarokhi *et al.*, 2003; Irkin & Korukluoglu, 2007) and inhibition of penicillic acid production by many chemicals (Lari & Thomas, 1980; Gourama & Bullerman, 1988; Garza *et al.*, 1993), no data have appeared on the effect of garlic on the production of this toxin.

MATERIAL AND METHODS

Preparation of the aqueous garlic extracts (Curtis *et al.*, 2004; Bakri & Douglas, 2005):

Garlic bulbs were purchased from the supermarket and peeled. The fresh garlic cloves (255 g) were blended in 255 ml distilled water, and homogenized in a domestic juicer (Braun Combimax 700 Vital, Germany) for 3 minutes at average speed to extract the juice. The juice was then centrifuged at 3000 rpm for 10 minutes. The precipitated material was weighted and the supernatant was filtered through Whatman no.1 filter paper and the resulting filtrate was then filter sterilized by passing through a 0.45 µm cellulose nitrate membrane filter. The garlic extract was used immediately or stored at 4 C° until used.

Microorganism:

P. hirsutum has been isolated from a local sample of paddy rice grains and was found to have the ability to produce the mycotoxin, penicillic acid. It was identified to the species level according to Frisvad & Samson (2004).

Culture media and general culture conditions:

Czapek-Dox's agar supplemented with yeast extract (0.5%) (Kurtzman & Ciegler, 1970) used for maintaining and culturing of the fungus during the determination of the antifungal activity tests and the MIC of garlic extract.

Modified Raulin-Thom broth, containing 0.07% Na_2HPO_4 in equimolecular weight to the original phosphorus source $(\text{NH}_4)_2\text{HPO}_4$ in the Raulin-Thom broth (Bentley & Keil, 1962; Lindenfelser & Ciegler, 1977), was the most preferable during a previous screening study for selection of the best fermentation medium for maximum penicillic acid production by *P. hirsutum* (Ismaiel, 2007).

Antifungal activity tests:

(a)- MIC determination:

Sensitivity of the fungus to garlic extract concentrations (30, 60, 120, 240, 360, 480, 540 and 600 mg/ml) was determined by inoculating sterile Petri-dishes with 0.2 ml fungal suspension contain approximately 10^6 spores/ml. Twenty five milliliters of agar medium was poured in each dish. Wells were punched in the agar and each filled with 50 μl of each tested garlic extract concentration. The plates were then incubated at 4 C° for 2 hours then incubated for 4 days at 30 C° and the resulted inhibition zones around the wells were then measured. The well containing the highest dilution (lowest concentration of garlic) that still showed a zone of inhibition around, it was seemed to be the MIC.

(b)- Effect of garlic extract on mycelial dry weights and penicillic acid levels of *P. hirsutum* :

The modified Raulin-Thom broth was adjusted to pH 3 using varying amounts of 1N of both HCl and NaOH, was transferred to 250 ml Erlenmeyer flasks at 50 ml aliquots and sterilized at 121 C° for 20 minutes. Different concentrations of aqueous garlic extracts (0.0, 0.60, 1.20, 3.00, 6.00, 12.0, 18.0, 24.0, and 30.0 mg/ml culture medium) were separately added to the culture medium. The flasks were inoculated with 6.0 mm of 7-days old *P. hirsutum* culture under aseptic conditions and incubated for 10 days at 25 C° which are the optimum conditions for maximum penicillic acid production by *P. hirsutum* (Ismaiel, 2007). The fungal cultures were observed visually after 2, 4, 7 and 10 days.

Mycelial dry weights:

At the end of the incubation period, the culture flasks were filtered through pre-weighted Whatman no.1 filter papers, oven-dried at 80 C° till a constant weight.

Determination of penicillic acid:

The amounts of penicillic acid in the culture filtrates were determined quantitatively by the colorimetric method described by Bentley & Keil (1962) and Wirth & Klosek (1972) with some modifications. A known volume of the culture filtrate was extracted with equal volume of ethyl acetate, shaken for about 10 minutes and allowed to stand for 10 minutes. The ethyl acetate layer was then separated, dried and evaporated in a water bath at 60 C°. The dried ethyl acetate extract was then dissolved in a known volume of hot water. After cooling, a 3-fold excess of concentrated ammonia was added. The resultant reddish purple complex was allowed to develop for 20 minutes and the absorbance was measured at 545 nm with a spectrophotometer (Spectronic Molton Roy Co., 20 D). Meanwhile, penicillic acid production by *P. hirsutum* was determined qualitatively after spotting on TIC plates with standard penicillic acid (Sigma Chemical Co., Louis St., USA) according to the method described by Ciegler & Kurtzman (1970) and Aziz & Moussa (2002).

Statistical analysis:

All experiments were conducted in triplicates and the mean $\pm$ standard deviation (SD) of these triplicates was calculated.

Chemical analysis of garlic extract:

A suspension of acetic acid garlic extract was prepared for the GC-MS analysis that was carried out in Mass Spectrometry Unit, Central Scientific Services Center, National Research Center, Dokki, Cairo, Egypt. 1.0 μ l of the suspension was injected into GC, model Thermo Trace GC 2000 equipped with MS equipment, model Finnigan SSQ 7000 instrument. The MS is coupled with a DBWAX capillary column (30 cm x 0.25 mm internal diameter, film thickness 0.25 μ m). The electron impact was 70 ev. The injector temperature was programmed at 220 C° and the column was held at 40 C° for 3 minutes and then programmed to 250 C° at 5 C°/2 minutes. The components were identified by comparison of retention indices and mass spectra with those of the standards in the library.

RESULTS AND DISCUSSION

In this work, a preliminary test was conducted to elucidate the antifungal effect of different concentrations of the aqueous garlic extract towards *P. hirsutum* by the agar

cup-plate method (Table 1). The size of the inhibition halo was clearly proportional to the concentration of garlic extract applied to each well (Figure 1).

It has been found that the MIC of aqueous garlic necessary for growth inhibition of *P. hirsutum*, was 30 mg/ml. Likely, Irkin & Korukluoglu (2007) found that the MIC of aqueous garlic extract against *Aspergillus niger* was 325 mg/ml. The MIC of aqueous garlic extract against *Candida albicans* was 8.9 mg/ml (Barki & Douglas, 2005). Many fungi have proven susceptible to the antifungal activity of garlic extract, including *Torulopsis*, *Trichophyton*, *Cryptococcus* (Fromtling & Bulmer, 1978), *Aspergillus* (Hitokoto *et al.*, 1980), *Trichosporon* and *Rhodotorula* (Tansey & Appleton, 1975).

The relationship between spore productions by *P. hirsutum* in Raulin-Thom broth containing varying concentration of garlic is summarized in Table (2). After 2 days of incubation, sporulation was very slight at 0.6 mg/ml and inhibited at all the higher concentrations. After 4 days of incubation, it was slight at 1.2 mg/ml and inhibited at 3 to 24 mg/ml. After the longer periods of incubation (7-10 days), it became intense at the lower concentrations (0.6 to 3 mg/ml), completely inhibited after 7 days of incubation at 18 to 24 mg/ml and may be also inhibited or very slight at these higher concentrations after 10 days of incubation. Graham & Graham (1987) showed that the higher concentration of garlic extract (0.25 - 0.4%) inhibited the sporulation of *A. parasiticus*. Allicin was assumed to be the main component responsible for the antifungal activity of garlic extract and was also reported to inhibit fungal spore's germination (Yamada & Azuma, 1997). Similarly, ajoene compound which is a derivative of allicin is very inhibitory against *A. niger*, *C. albicans* and *Paracoccidioides brasiliensis* (Naganawa *et al.*, 1996). Antifungal effects of garlic essential oils have also been investigated (Benkeblia, 2004).



Figure 1: Photographs of inhibition zones caused by different concentrations of garlic extract on growth medium seeded with spores of *P. hirsutum*. a- 30 mg/ml, b- 60 mg/ml, c- 120 mg/ml, d- 240 mg/ml, e- 360 mg/ml, f- 480 mg/ml, g- 540 mg/ml and h- 600 mg/ml.

Table 1: Relation between concentration of aqueous garlic extract (mg/ml) and the inhibition zones diameters (mm) of the tested *P. hirsutum*.

Concentration of garlic extract (mg/ml)	Diameter of inhibition zones (mm)
30	7.66 ± 1.15
60	8.66 ± 1.52
120	11.66 ± 1.15
240	14.0 ± 0.00
360	18.0 ± 1.00
480	18.6 ± 0.577
540	19.0 ± 0.00
600	21.0 ± 2.00

MIC = 30 mg/ml

Table 2: Influence of varying concentrations of aqueous garlic extract on spores production by *P. hirsutum* in Raulin-Thom broth incubated at 25 C° for different days

Concentrations of garlic extract (mg/ml)	Spores production			
	Days of incubation			
	2	4	7	10
0.00	+	++	++++	++++
0.60	+	++	++++	++++
1.20	-	+	+++	+++
3.00	-	-	+++	+++
6.00	-	-	++	++
12.0	-	-	-(+)	+
18.0	-	-	-	-(+)
24.0	-	-	-	-(+)

(+ = very slight), (++ = slight), (+++ = heavy), (++++ = very heavy)

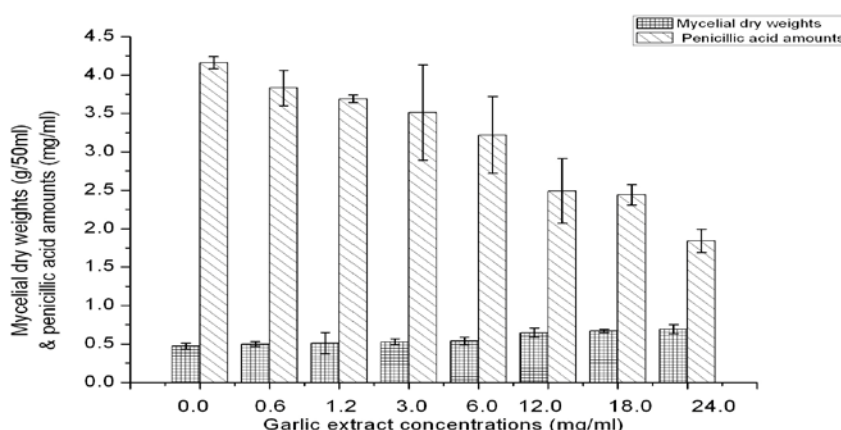


Figure 2: Influence of varying concentrations of aqueous garlic extract on mycelium dry weights and penicillic acid levels of *P. hirsutum* in Raulin-Thom broth incubated at 25 C° for 10 days.

Figure (2) summarizes the effect of different concentrations of garlic (the sublethal concentrations less than 30 mg/ml) on mycelial dry weights and penicillic acid production by *P. hirsutum*. It was obvious that the average values of mycelial dry weights were increased gradually with the increase of garlic concentrations reaching a maximum at 24 mg/ml. Inhibition of mycelial production was essentially complete at a garlic concentration of 30 mg/ml. Similar results were obtained by Graham & Graham (1987), who showed that apparent stimulation of mycelial production at the lower garlic concentrations was due to the presence of small amounts of nutrients such as minerals and vitamins in the added garlic. The stimulation of mycelial growth at the concentrations less than 30 mg/ml of garlic extract may be due to the presence of lower amounts of allicin and sulfide compounds in these concentrations which were insufficient to inhibit or decrease the mycelial growth. These components in garlic extracts are responsible for antifungal activity (Bianchi *et al.*, 1997; Ankri & Mirelman, 1999). Removal of allicin from the reaction by solvent extraction decreased the antifungal activity of garlic extract (Hughes & Lawson, 1991). The contrary was took place for penicillic acid production where any increase in the garlic concentration was accompanied with a decrease in the levels of penicillic acid. The amount of penicillic acid produced in the presence of 24 mg/ml of garlic was approximately 44% of that produced in the culture control filtrate (free from any garlic extract) after 10 days of incubation. It is also interest to mention that penicillic

acid was not detected completely at a garlic concentration of 24 mg/ml after 7 days of incubation. Moreover, the fungal mycelial growth and toxin production were inhibited by garlic concentration of 30 mg/ml. Garlic extracts were proved to have inhibitory effect on aflatoxins production by *A. parasiticus* (Graham & Graham, 1987; Lawson, 1996) and keratinase activity in *T. mentagrophytes* (Ghahfarokhi *et al.*, 2003). The antifungal mode of action of garlic extracts were recognized from their capability to decrease oxygen uptake (Szymona, 1952), inhibit the synthesis of lipids, proteins and nucleic acids (Adetumbi *et al.*, 1986), damage membranes (Ghannoum, 1988), collapse the fungal hyphae, increase the number and size of the vacuoles in cells and increase the cell wall thickening (Bianchi *et al.*, 1997).

The main components of acetic acid garlic extract were determined using GC-MS chromatogram analysis as represented in Table (3) and electronically photographed as shown in Figure (3). It is interest to mention that the garlic extract consists mainly of about 17 components. The major components were quinolinium 1-ethyl-iodide (CAS) (quinoline ethiodide), 60.83% ; 8, 9, 10, 11- tetrahydrocyclonona [de] naphthalene -7, 12-dione, 5.88% ; trisulfide, methyl-2-propenyl (CAS) (methylallyl trisulfide), 4.88% ; benzene,(1.3-daimethyl-3-butenyl)-(CAS) (1-pentene,2-methyl-4-phenyl-), 4.54%. The results also indicated that the garlic extract contains less percentages of 3,4-dihydro-3-vinyl-1,2dithiin, 3.61% ; 1,2-divinylbenzene, 3.24% and disulfide,di-2-propenyl (CAS) [1,2-bis(allyl)disulfane], 2.18%. In this connection, the volatile compounds of suspension of micronized garlic powder were determined using GC-MS analysis and were found mainly consisted of linear chain of aldehydes, allyl disulfides and disulfides (Bianchi *et al.*, 1997). A concentrated garlic extract containing 34% allicin, 44% total thiosulfinates and 20% vinyl dithiins possessed potent *in vitro* fungistatic and fungicidal activity against three different isolates of *C. neoformans* (Davis *et al.*, 1994). Allicin (diallyl thiosulfinate), is formed from allin by the action of allinase and gets metabolized rapidly into diallyl sulfide, diallyl disulfide, diallyl trisulfide, ajoene, S-allyl mercaptocysteine, S-allyl cysteine and dithiines (Dirsch *et al.*, 1998; Hirsch *et al.*, 2000; Sigounas *et al.*, 1997; Sundaram & Milner, 1993; Welch *et al.*, 1992). Most of the biological effects of allicin including its antimicrobial activity can be related to its strong SH-modifying capacity and antioxidant properties (Koch & Lawson, 1996; Lawson, 1998).

In conclusion, the results obtained indicate that, garlic had inhibitory effects on growth, spore formation and penicillic acid production by *P. hirsutum*. Its use in foods

and feeds products as a natural and safe substance, provides protection against food intoxication from penicillic acid. The future for using plant natural extracts and products is promising because they are less expensive and less hazardous to the environments.

Table 3: Main components of acetone garlic extract

Retention time (min)	Compounds	Area (%)
11.35	2- (3-hydroxy-1-oxopropyl) -1.1-1- trimethyl-hydrazinium hydroxide inner salt	2.20
15.54	Phenyl acetylene-2-d	0.33
16.41	1-hexanol, 2-ethyl-(CAS)	1.79
18.10	Disulfide, di-2-propenyl(CAS)	2.18
19.42	4-methoxy-1-(2-oxobut-3-enyl) azetidin-2-one	1.12
20.07	Trisulfide, methyl-2-propenyl (CAS)	4.88
21.90	3,4-Dihydro-3-vinyl-1,2 dithiin	3.61
22.65	[4S-(2E, 4R*, 5S*, 6R*, 7E)] -10-(benzoyloxy)-5-hydroxy-4,6-dimethoxy-2,8 dimethyl-2,7-decadienoic acid methyl ester	3.84
24.69	8-(Trimethylsilyl)-6-octenteneitrile	2.92
37.32	Benzene, [(Fluoromethyl) thiol]-(CAS)	0.73
39.43	8, 9, 10, 11- tetrahydrocyclonona [de] naphthalene- 7,12-dione	5.88
40.01	Tetradecanoic acid (CAS)	0.44
41.16	Quinolinium, 1-ethyl-, iodide (CAS)	28.69
41.52	Quinolinium, 1-ethyl-, iodide (CAS)	24.46
43.32	Quinolinium, 1-ethyl-, iodide (CAS)	7.68
43.96	1,2-Divinyl benzene	3.24
44.65	Hexadecanoic acid (CAS)	0.48
45.80	Benzene, (1,3- dimethyl-3-butenyl)-(CAS)	4.54
46.22	(E)- 3,3-diphenyl-4-hexenoic acid	0.97
Total		99.98%

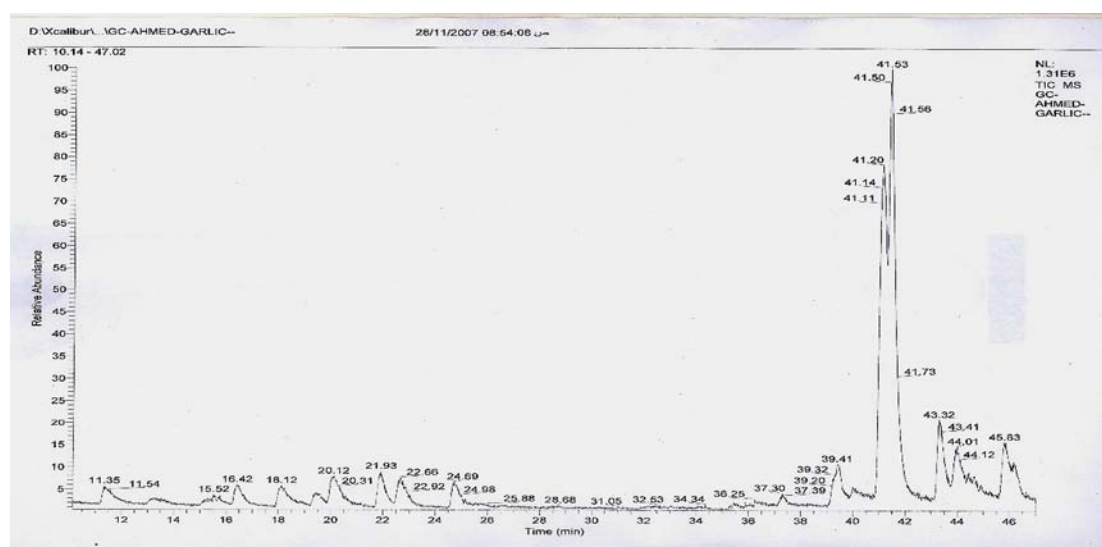


Figure 3: GC-MS chromatogram of acetone garlic extract.

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التأثير المثبط لمستخلص الثوم علي إنتاج السم الفطري حمض البنسيليك والنمو لفطرة البنسيليوم هيرسيوتم

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في هذا البحث ، تم دراسة التأثير الضدي لمحلول مستخلص الثوم المائي علي نمو فطرة البنسيليوم هيرسيوتم وإنتاجه من السم الفطري حمض البنسيليك و إتضح أن اقل تركيز مثبط لنمو الفطرة هو 30 ملليجرام لكل مليلتر وذلك بعد انتشار مستخلص الثوم داخل الوسط الصلب. وقد كان لمستخلص الثوم عند التركيزين 18 و 24 ملليجرام لكل مليلتر تأثيرا مثبطا علي إنتاج جراثيم الفطرة وذلك بعد 7 أيام من التحضين بينما لوحظ أن إنتاج الفطرة للجراثيم يكون خفيف جدا بعد 10 أيام من التحضين عند هذين التركيزين. إتضح

أيضا انه بزيادة تركيزات مستخلص الثوم يزداد الوزن الجاف للفطرة تدريجيا حتى الوصول إلي اعلي معدل عند التركيز 24 ملليجرام لكل مليلتر. وعلي النقيض ، انخفضت معدلات إنتاج حمض البنسيليك السام بزيادة تركيزات مستخلص الثوم فقد أدي التركيز 24 ملليجرام لكل مليلتر إلي انخفاض في كمية حمض البنسيليك المنتجة بنسبة 44% مقارنة بالكمية المنتجة من الحمض في الراشح المزروع للفطرة النامية علي وسط سائل غير معامل بمستخلص الثوم وذلك بعد 10 أيام من التحضين ، بينما فقدت الفطرة قدرتها تماما علي إنتاج حمض البنسيليك أثناء نموها علي وسط سائل يحتوي علي نفس التركيز من مستخلص الثوم بعد التحضين لمدة 7 أيام.

إمتد هذا البحث إلي عمل دراسة تحليلية لتعيين وتقويم نسب المركبات الأساسية المتواجدة في مستخلص الثوم والتي يمكن أن يكون لها هذا التأثير الضدي علي نمو فطرة البنسيليوم هيرسيوتم وذلك بإستخدام تحاليل الغاز الكروماتوجرافي و طيف الكتلة.