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Effect of Two Biofertilizers on Growth Parameters, Yield Characters, Nitrogenous Components, Nucleic Acids Content, Minerals, Oil Content, Protein Profiles and DNA Banding Pattern of Sunflower (*Helianthus annus* L. cv. Vedock) Yield

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Abstract: A field trial was conducted during 2000 and 2001 growing seasons at the green house of Botany Department, Faculty of Science, Ain Shams University to study the effect of biofertilization with different doses of two biofertilizers (biogien and microbien) on the vegetative growth, yield and its components as well as, seed oil content and quality, nitrogen components, total protein and protein profiles and DNA banding patterns in sunflower (*Helianthus annus* L. cv. Vedock)plant yield. Application of both biofertilizers either alone or in combined treatment leads to considerable improvement in sunflower yield characters as compared with their respective control. However, response differed according to the type of biofertilizers. The results revealed that biofertilization perform significant improvement in plant productivity and quality. The highest stimulatory effect and the maximum enhancement were exerted in plants treated with biogien at the recommended dose (5%). It seems reasonable to conclude that biofertilization significantly increased seed yield, nutrient contents of seeds, nitrogen and all nitrogenous compounds, mineral and seed oil contents. However, both biofertilizers decreased the main saturated fatty acids; palmitic and stearic, while increased the main unsaturated fatty acids (oleic, linoleic and linolinic). Thus the yield oil becomes more safer for human consumption. It is of interest to mention here that, The application of biofertilizers induced over or low gene expression of most mid and high molecular weight protein bands. They also induced two new proteins of low molecular weight (2.1 kDa for biogien and 14.9 kDa for microbien). These low molecular weights proteins may be used as an adaptive mechanism for biofertilizer application in plants to give the maximum yield. These results are supported by the finding that, biofertilization showed little changes in DNA banding patterns. These changes are either monomorphic quantitative or qualitative. Quantitative changes were recorded clearly using primer OPI-01, while quantitative changes with disappeared and many faint bands were obtained using primers OPI-02, -03, -04 and -05. Based on combined effect of both biofertilizers, protein and DNA profiles of sunflower showed the lowest degree of variability. In general, it could be concluded that, biofertilization is a good tool to improve the crop yield productivity and quantity, reduce the rate of chemical fertilizers and the cost of crop production and allow for better environment.

Key words: Sunflower, biofertilizers, growth, yield, oil, fatty acids, mineral, protein profile, DNA pattern

Introduction

In Egypt, soil fertility is diminishing gradually due to soil erosions, loss of nutrients, accumulation of salts and other toxic elements, water logging and unbalanced nutrient compensation. Organic wastes and biofertilizers are the alternate sources to meet the nutrient requirement of crops and to bridge the future gaps. Farming regions that emphasize heavy chemical application lead to adverse environmental, agricultural and health consequences. Numerous efforts are being exercised everywhere to combat the adverse consequences of chemical farming. The biofertilized farming system is emphasized, biofertilizer, organic manuring and biocontrol of agricultural pests (Saber, 1998). In recent years, biofertilizers have emerged as a promising component of integrating nutrient supply system in agriculture. Our whole system of agriculture depends in many important ways, on microbial activities and there appears to be a tremendous potential for making use of microorganisms in increasing crop production. Microbiological fertilizers are an important part of environment friendly sustainable agricultural practices (Cakmakci *et al*., 1999; Bloemberg *et al*., 2000). Biofertilizers include mainly the nitrogen fixing, phosphate solubilizing and plant growth-promoting microorganisms (Goel *et al*., 1999). Among biofertilizers benefitting the crop production are *Azotobacter*, *Azospirillium*, blue green algae, *Azolla*, P-solubilizing microorganisms, mycorrhizae and *Sinorhizobium* (Hegde *et al*., 1999). In this field, many experiments were conducted to study the effect of biofertilizers alone or in

combination with other chemical fertilizers (Patel *et al*., 1992; Chela *et al*., 1993; Prasad and Prasad, 1994; Fisinin *et al*., 1999; Ghosh *et al*., 2000; Seema *et al*., 2000).

Despite tremendous potential and proven benefits, wide acceptability of biofertilizers has been constrained because unpredictability and inconsistency in their performance under field condition (Hegde *et al*., 1999).The type of biofertilizer systems and their mode of action are nitrogen fixation, phosphate solubilizers and mobilizers, plant growth regulators, siderophores and antibiotics.

Sunflower (*Helianthus annus* L.) is one of the most widely cultivated oil crop in the world. Because of moderate cultivation requirements and high oil yield, planted area has increased in recent years (Skoric, 1992).

In this investigation a field experiment was carried out to determine the importance of biofertilizer application in order to improve the yield quality and productivity of sunflower.

Materials and Methods

The experimental plant used in this study was *Helianthus annus* L. cv. Vedock. The seeds were obtained from the Agricultural Research Center (ARC), Cairo, Egypt. The biofertilizers applied (biogien and microbien) were also obtained from the same source. The seeds were surface sterilized with 1% sodium hypochlorite for 20 min, then rinsed with water several times. Three concentrations of each biofertilizer were applied (10, 5 and 2.5%; 5%:recommended dose). The seeds were classified into 8 groups. Each group was treated with a specific concentration. The treatments were applied as in the pamphlet obtained from ARC. One of the rest two groups of seeds was treated with a combined recommended dose of the two biofertilizers, while the other was left as a control (not treated). The seeds were left to dry in shadow. Then, they were cultivated in the soil at the green house of Botany Department. After 30 days, samples of each treatment were taken for studying the growth parameters. Plants were left in the soil till the yield maturity. The yield was taken after 150 days from yield sowing. The morphological characters of yield were determined. Samples were collected for chemical analysis. All data were subjected to analysis of variance and the mean values compared on the basis of least significant difference (LSD) at 0.01 and 0.05 probability levels (SAS Program, 1982).

Extraction and estimation of nitrogen and minerals: Total nitrogen and phosphorus were determined in the acid digested sample (1ml 50% H_2SO_4 and 1ml 30% perchloric acid), using Berthelot reaction (Chaney and Marbach, 1962) and modified Fiske-Subborow method (Clark and Switzer, 1977). Potassium, calcium and magnesium were estimated in the same extract by the atomic absorption.

Extraction of oils: The air dried samples of sunflower seeds were milled twice using an experimental mill, the powdered samples were soaked in n-hexane 64-68°C, for 48 h with occasional shaking. The crude extracts were collected by decantation. The meal was soaked once more with the same solvent for another 24 h. The combined extracts were filtered over sufficient amounts of anhydrous sodium sulfate and free of solvent by distillation under vacuum. The produced oils were kept in dark bottles in the refrigerator for oil analysis.

Fatty acids composition of oils: The methyl esters were prepared using benzene: methanol: concentrated sulfuric acid (10:86:4) and the methylation process was carried out for one hour at 80-90 °C according to Stahl (1967). Identification of the fatty acid methyl esters were performed by GLC. Apye Uniacm Gas Liquid Chromatography equipped with a flame ionization detector and coiled glass column (1.6 m X 4 mm) packed with 10 % PEGA (polyethylene glycol adepate) supported on chromosorb W-AW 100-200 mesh was used. The samples (1.0-1.5 ml) were injected into the column using a Hamilton micro-syringe. Gas chromatography conditions used for isothermal analysis were column (190°C), detector (220°C) and injection (220°C). Flaw rates hydrogen, 33 ml/min, nitrogen 30 ml/min and air 330 ml / min, peak area were measured using spectrophysic integrator.

Total nucleic acids (DNA and RNA): Nucleic acids (DNA and RNA) were extracted according to Guinn (1966) and estimated using UV Spectrophotometer (Spectronic 601) as adopted by Ogur and Rosen (1950).

Protein analysis: Soluble proteins were determined in the seeds of the yield according to the method described by Bradford (1976).

Electrophoretic protein profile was analyzed by SDS-PAGE technique (Laemmli, 1970). Data were analyzed and identified by gel documentation system (GDS) which comparing polypeptide maps which include the use of band intensity, molecular weight and the rate of mobility of each polypeptide with standard markers using Gel Pro-analyzer Version 3 Media Cyberene Tice Imaging Experts Software.

RAPD-PCR analysis: The seeds obtained from the yield were germinated in pots until the vegetation stage. Young

Table 1: Oligonucleotide sequences of five OPI primers

Primer	Sequence (5` to 3`)	GC %
OPI-01	ACCTGGACAC	60
OPI-02	GGAGGAGAGG	70
OPI-03	CAGAAGCCCA	60
OPI-04	CCGCCTAGTC	70
OPI-05	TGTTCCACGG	60

leaves were used for the extraction of genomic DNA according Sambrook *et al.* (1988). DND was amplified by PCR using decamer primers (Operon Tech., Kits A, B, C, D, E, F, G, H and I, Inc., USA). Forty five decamer primers used, only five primers of Kit I yielded informative data (Table 1).

For RAPD-PCR reaction, the total reaction volume is 25 ul containing 20 ηg DNA, 16 ηg primer, 100 μM dNTPs mix (dATP, dGTP, dCTP and dTTP), 1.5 mM $MgCl_2$ and 1U *Taq* polymerase. Amplification was performed using DNA Thermal Cycler (Perkin Elmer, Model 480). The temperature profile was as follows: one cycle (1 min at 94°C), 40 cycles (1 min at 94°C, 1 min at 36°C and 1 min at 72°C) and one cycle (10 min at 72°C). The amplified products were analyzed using 1.4% agarose NA and then stained with ethidium bromide. Finally, the results were documented by photo using Polaroid Camera [R]. Data analysis was carried out by the Gel Scan Database Software.

Results and Discussion

Growth parameters and yield characters: The growth parameters of *Helianthus annus* L. cv. Vedock plants as shoot length, fresh and dry weight of shoots, number of nodes, number and mean area of leaves were significantly increased by all concentrations of both biofertilizers applied (Table 2). The highest stimulatory effect and the maximum enhancement were exerted in plants treated with biogien at the recommended dose (5%). The stimulatory effects of both biofertilizers used on growth parameters of *Helianthus* are in accordance with the results obtained by Rani and Sathiamoorthy, 1997; Mahmoud and Amara, 2000; Panwar *et al*., 2000 and Seema *et al*., 2000. In addition, in agreement with the obtained results, Ismail and Hasabo (2000) using new commercial Egyptian biofertilizers (one of them was microbien containing nitrogen-fixing bacteria and phosphate dissolving bacteria), found that all treatments significantly increased plant growth parameters compared with the untreated plants. In addition, similar trends were observed by Mekki *et al.* (1999a) and Galal *et al*. (2000). Such increases might encourage a limited application of chemical fertilizers and consequently reduce pollution and health hazard. These results are in harmony with those obtained by Verma (1996) on pearl millet.

The stimulatory effects might be attributed to the activation of the growth of microflora including many plant growth stimulators (Fisinin *et al*., 1999). The enhancement of the growth parameter attributes leads to improving the crop productivity (Ghosh and Mohiuddin, 2000). These results are in agreement with those obtained by Mehta *et al.* (1995), Snehal *et al*. (1998), Mahmoud and Amara (2000) and Das *et al*. (2001).

The yield characters expressed as head diameter, number of seeds per head, weight of seeds per head and weight of 1000 seeds are shown in Table 2. All the above characters were significantly increased due to the application of each of the two fertilizers either separately or in combination. Biogien showed more significant increases than micrbien. The most important character (weight of 100 seeds) increased by 126.9, 138.5 and 126.9% for 10, 5 and 2.5% of biogien compared to the untreated plants. However, such values were 105.8 and 126.9% for 10 and 5% of microbien. Moreover, the interaction of both the two biofertilizers showed an increase by 115.4%. From the above results, it is clearly shown that biogien application is more effective than microbene application.

The stimulatory effects of both biofertilizers used are in accordance with the results obtained by Chauhan *et al.* (1995) who found that inoculation of Azospirillum - as a biofertilizer - markedly increased pods number and length and seed yield of *Brassica juncea* L. plants over the non-inoculated plants. In addition, Buragohain (2000) found that sugar cane cv. COBLN 9103 yield was significantly higher in the cultivated crops with *Azotobacter* than uninoculated crops. Also, Cakmakci *et al.* (1999) showed that, in the green house trial bacterial inoculation of seeds increased sugar beet and barley seed yields compared with control. Moreover, Namdeo and Gupta (1999) using inoculants of *Rhizobium*, phosphate solubilizing bacteria (PSB) and *Rhizobium* + PSB with 100, 75 and 50 % levels of the recommended dose of fertilizer (RDF), found that *Rhizobium*, PSB and *Rhizobium* + PSB with 100% level of RDF produced 13.8, 9.9 and 20.4% higher grain yield of pigeon pea, respectively, than 100% of RDF alone. The treatment *Rhizobium* + PSB along with 75% level of RDF indicated a saving of 25% of the chemical fertilizer by way of producing yield equivalent to that of 100% RDF alone. Similar results were obtained by Tomar *et al.* (1996) using gram plants, Jeyabal and Kuppuswamy (1999) using rice, Chaudhary (1999) using fenugreek, Kumar *et al.* (1999) on sorghum and Panwar *et al.* (2000) using wheat under field condition.

In China, it was found that application of G-typed biofertilizer (GBF , which contain a large amounts of bacteria) could reduce the need for chemical fertilizers and improve yield. It could increase the organic content of soil, alleviate hard pan in soil profiles and increase the

Table 2: Changes in growth parameters of *Helianthus annus* L. cv. Vedock in response to biogien and microbien application after 30 days of sowing. Each value is a mean of 10 samples

Dose		Shoot length (cm)	No. of internodes	No. of leaves	Area of leaves (cm^2)	Fresh wt. of shoot system (g)	Dry wt. of shoot system (g)	Root length (cm)	Fresh wt. of root (g)	Dry wt. of root (g)
Control		32	10	11	182	10.40	1.19	45	1.90	0.11
Biogien	10%	46**	16**	17**	293**	15.20**	1.72**	67**	2.86**	0.16**
	5%	59**	20**	21**	360**	19.70**	2.87**	94**	4.12**	0.23**
	2.5%	44**	15**	16**	272**	14.60**	1.69**	62**	2.76**	0.15**
Biogien (5%) + Microbien (5%)		42**	14**	15**	253**	13.80**	1.59**	58**	2.50**	0.14**
Microbien	10%	38**	12*	13*	219*	12.30**	1.43**	50**	2.31**	0.13**
	5%	44**	15**	16**	270**	14.4**	1.68**	64**	2.80**	0.29**
	2.5%	34	11	12	195*	11.2*	1.30*	48*	1.93	0.10
L.S.D. at 5%		3.90	1.20	1.10	12.30	0.70	0.07	2.90	0.04	0.004
L.S.D. at 1%		5.10	1.90	1.90	18.90	1.60	0.13	5.00	0.09	0.008

Table 3: Effect of biogien and microbien application on yield and its components of *Helianthus annus* L. cv. Vedock. Each value is a mean of 10 samples

Dose		Diameter of head (cm)	No. of seeds Per head	Weight of seeds per head	Weight of 100 seeds (g)
Control		12.90	800.00	45.00	5.20
Biogien	10%	15.20**	940.00**	63.00**	6.60**
	5%	15.90**	1000.00**	74.00**	7.20**
	2.5%	14.70**	930.00**	61.00**	6.60**
Biogien (5%) + Microbien (5%)		14.00**	870.00**	53.40**	6.00**
Microbien	10%	13.60*	850.00*	48.00*	5.50*
	5%	14.80**	936.00**	62.60**	6.60**
	2.5%	13.50*	840.00	46.20	5.20
L.S.D. at 5%	0.40	42.00	2.70	0.20	
L.S.D. at 1%	1.00	63.00	5.20	0.70	

Table 4: Effect of biogien and microbien application on the total soluble nitrogen, total soluble protein and nucleic acid contents of seed yield of *Helianthus annus* L. cv. Vedock. Each value is a mean of 3 determinations

Dose		Total nitrogen (mg/100g dwt)	DNA (mg/100g dwt)	RNA (mg/100g dwt)	Total soluble Protein (ug/g dwt)
Control		312	120	126	0.86
Biogien	10%	411**	460**	257**	1.70**
	5%	424**	480**	271**	1.80**
	2.5%	407**	430**	165**	1.10**
Biogien (5%) + Microbien (5%)		397**	270**	136*	0.92**
Microbien	10%	367**	205**	129	0.89*
	5%	370**	240**	136*	0.92**
	2.5%	348**	160**	128	0.88*
L.S.D. at 5%	8.4	10.1	7.2	0.01	
L.S.D. at 1%	12.5	17.3	9.8	0.04	

Table 5: Changes in the mineral contents of *Helianthus annus* L. cv. Vedock seed yield in response to biogien and microbien application to their parents The values listed are expressed as mg/100 g d wt. Each value is a mean of 3 samples

Dose		Ca	K	P	Mg
Control		174	851.2	1224	198.9
Biogien	10%	255**	1211.3**	1799**	284.8**
	5%	304**	1508**	2141**	359.9**
	2.5%	244**	1124**	1716**	278**
Biogien (5%) + Microbien (5%)		225**	1121**	1589**	274.9**
Microbien	10%	213**	1061**	1504**	260**
	5%	221.7**	1117**	1555**	266**
	2.5%	170	1052**	1338**	253**
L.S.D. at 5%		9.2	107	112	8.5
L.S.D. at 1%		11.3	105	118	10.6

** Highly significant difference * Significant difference

disease resistance and drought resistance of crops. A number of specific plant diseases were prevented after application of GBF (Guo and Guo, 2000).

In addition, Goel *et al.* (1999) reported that, the inoculation with certain plant growth-promoting rhizobacteria (PGPR) may enhance crop productivity either by making the other nutrients available or protecting plants from pathogenic microorganisms (allelopathic effects). Zodape (2001), also concluded that, the increase in yield productivity with biofertilizer application is due to micro-element and plant growth regulator contained in the fertilizer.

Nitrogen, phosphorus and potassium (NPK) content: The beneficial effect of biofertilizers application is the improvement of nitrogen contents. The application of biofertilization technology to a coarse-textured soil with low fertility had a positive effect on plant growth, N-gained from the air and enhancement of fertilizer uptake (apparent recovery function) (Galal *et al*., 2000). Table 4 showed highly significant increases in the nitrogen contents of seed yield in response to the application of both the biofertilizers. These results are in agreement with those obtained by Sarawgi *et al.* (1999) and Gopal *et al.* (2000). The most significant increases were observed in response to biogien application. In addition, Sharma and

Table 6: Comparative analysis of relative concentrations (band %), molecular weight (M.wt.) and mobility rate (R_m) of the different types of protein bands of M2 seeds of *Helianthus annus* L. cv. Vedock treated with different concentrations of biogien and microbien biofertilizers

	Band %									
Band No.	1	2	3	4	5	6	7	8	R_m	M.wt. (kDa)
1	-	-	-	-	-	-	-	0.62	0.070	183.28
2	-	-	0.16	-	-	-	-	-	0.073	179.51
3	-	-	-	-	-	0.27	-	-	0.075	175.82
4	-	-	-	0.10	-	-	-	-	0.081	165.19
5	-	-	-	-	-	-	-	0.36	0.088	155.20
6	-	-	-	-	-	-	0.10	-	0.090	152.00
7	-	-	-	-	-	0.19	-	-	0.092	148.87
8	0.11	-	-	0.33	0.12	0.15	-	0.14	0.11	127.10
9	-	-	0.21	0.11	0.39	0.51	0.33	-	0.14	104.45
10	1.13	1.12	0.92	0.46	1.25	1.34	0.86	0.92	0.16	89.01
11	0.37	0.57	-	-	-	-	-	1.21	0.19	74.55
12	-	-	0.10	0.13	-	-	-	-	0.20	72.37
13	-	0.11	-	-	1.90	-	-	0.59	0.22	64.53
14	0.61	0.49	0.24	0.22	1.05	0.99	0.59	1.46	0.24	59.75
15	-	-	-	-	0.21	0.59	0.28	0.28	0.26	57.04
16	0.14	0.11	0.10	-	-	-	-	-	0.28	55.14
17	0.91	0.85	0.89	0.81	0.26	0.69	0.68	0.69	0.30	53.41
18	0.87	0.43	0.92	1.07	0.53	0.45	0.65	0.56	0.34	49.41
19	0.19	-	-	-	-	-	-	-	0.37	46.91
20	-	0.13	-	-	-	-	-	-	0.38	46.36
21	0.64	-	0.23	0.27	0.43	0.33	0.12	0.47	0.40	44.19
22	-	-	-	-	1.16	0.83	0.38	1.15	0.42	42.64
23	7.24	8.33	10.90	3.78	4.84	2.77	9.97	3.70	0.45	40.40
24	12.90	12.00	10.90	15.70	9.09	6.86	8.34	6.11	0.46	39.26
25	6.23	4.76	4.98	6.75	3.45	3.02	3.65	-	0.50	36.26
26	-	-	-	-	-	-	-	3.85	0.52	35.78
27	-	-	3.98	4.82	4.48	3.89	4.35	-	0.53	35.21
28	4.08	4.87	3.57	3.12	3.03	3.15	3.04	2.87	0.54	34.38
29	6.26	7.47	6.59	6.27	4.15	5.13	6.10	4.26	0.55	32.26
30	0.51	0.95	1.03	0.57	0.45	0.68	0.61	0.51	0.60	28.35
31	-	-	0.12	-	-	-	-	-	0.62	27.27
32	-	-	0.10	-	-	-	-	-	0.65	25.90
33	-	-	0.67	1.69	0.72	1.63	0.28	0.25	0.67	24.77
34	2.09	6.48	5.88	7.78	3.33	3.25	2.32	5.36	0.71	22.81
35	3.92	5.37	8.11	7.10	6.70	8.55	7.28	9.67	0.74	21.34
36	7.32	8.58	9.98	9.26	8.97	10.10	8.30	11.50	0.76	20.62
37	3.38	5.25	4.97	5.15	10.50	8.20	7.59	6.38	0.80	18.64
38	9.11	5.27	3.76	2.59	5.29	10.20	2.97	11.40	0.84	17.15
39	8.80	3.90	7.08	7.04	7.66	6.97	11.80	6.21	0.87	14.89
40	-	-	-	0.13	-	-	-	-	0.92	3.14
41	-	-	0.12	-	-	-	-	-	0.93	2.13
Total	21	20	26	24	24	25	23	25		

Lane 1 : Seeds obtained from control samples. Lane 2 : Seeds obtained from plants treated with 10% biogien.
Lane 3: Seeds obtained from plants treated with 5% biogien. Lane 4: Seeds obtained from plants treated with 2.5% biogien.
Lane 5: Seeds obtained from plants treated with 5% biogien in combination with 5 % microbien.
Lane 6: Seeds obtained from plants treated with 10% microbien. Lane 7: Seeds obtained from plants treated with 5% microbien.
Lane 8: Seeds obtained from plants treated with 2.5% microbien.

Namedeo (1999a) found that crop dressing or seed inoculation of soybean cv. HS-71-05 with phosphate solubilizing microorganism as biofertilizer increased the uptake of N. Also, Satapathy (1999) found that biofertilization using *Azolla* (a blue green alga) increased the soil N content and consequently the N absorbed by plants. In addition, inoculation of *Pennisetum americanum* with the biofertilizer microbien increased nitrogen content of the plant (Mekki *et al*., 1999b). Inoculation with *Azotobacter* increased yield uptake (Gopal *et al*., 2000).

It was found that uptake of N and P increased with application of phosphate-solubilizing bacteria (Mahendran and Chandramani, 1998 and Sarawgi *et al*., 1999). Biofertilizers increased NPK uptake values in soybean plants (Sharma and Namdeo, 1999b). In addition, Zodape (2001) using seaweeds as biofertilizers (since they contain micro-elements and plant growth regulators like cytokinins) found that they increased plant nutrient uptake. Moreover, Microbien (N-fixing and phosphate dissolving bacteria) increased the uptake of P in wheat and chickpeas plants (Mukherjie and Rai, 1999). Furthermore, Nitrogen and phosphorous contents in dry matter were increased due to organic manure or either singly or in combination with the biofertilizer microbien. Fe, Mn and Zn were also increased (Mekki *et al*., 1999a). The improvement of the growth and nitrogen contents in response of application of cyanobacteria as biofertilizers

Table 7: Effect of biogien and microbien application on the percentage of seed oil and fatty acid composition contents of *Helianthus annus* L. cv. Vedock

Dose		Seed oil content (%)	Fatty acid composition (%) C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	C24:0	% of decrease in saturated fatty acids (C16+C18)	% of increase in unsaturated fatty acids (C18:1,2&3)
Control		34.12	9.60	4.10	16.70	8.00	19.60	20.00	-	6.80	6.80	8.40	100.00	100.00
Biogien	10%	41.56**	2.20	3.00	8.00	7.00	30.00	16.00	12.60	8.00	6.20	7.00	39.27	148.00
	5%	42.42**	2.10	3.80	4.00	3.00	40.10	28.00	6.00	7.00	-	4.00	71.66	187.00
	2.5%	40.56**	6.70	5.70	12.80	5.00	30.30	20.90	5.00	10.40	-	10.20	27.90	141.90
Biogien (5%) + Microbien (5%)		40.74**	5.60	5.40	13.10	4.50	24.40	24.10	8.10	5.70	5.10	4.00	28.70	142.90
Microbien	10%	37.48**	3.40	7.00	9.40	9.40	29.40	15.70	6.00	6.20	7.00	6.50	23.90	129.00
	5%	39.64**	-	-	6.10	6.70	37.30	27.00	7.00	9.50	-	6.40	48.18	180.10
	2.5%	37.33**	7.00	4.00	12.00	8.00	26.00	16.70	5.00	6.00	7.00	8.30	19.02	120.50
L.S.D. at 5%	2.5													
L.S.D. at 1%	2.8													

** Highly significant difference * Significant difference C16:0 (Palmitic acid) C18:0 (Stearic acid) C18:1 (Oleic acid) C18:2 (Linoleic acid) C18:3 (Linolinic acid) C20:0 (Arachidic acid) C24:0 (Lignoceric acid)

Table 8: RPPD-PCR fragments and their molecular size in base pairs generated by five arbitrary primers in seedlings of M2 seeds of *Helianthus annus* L. cv. Vedock whose parents were previously treated with different concentrations of biogien and microbien

	OPI-01								OPI-02								OPI-03							
DNA fragment (bp)	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1800	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1200	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1100	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-
1000	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-
980	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
620	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+
500	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+
480	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
460	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
370	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	1	1	1	1	1	1	1	1	3	3	-	-	-	2	-	2	3	3	2	-	2	2	-	2

	OPI-04								OPI-05							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1800	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+
1200	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-
1100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
980	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
620	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
500	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	+
480	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
460	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+
370	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	2	2	1	-	-	-	-	2	3	3	-	-	-	-	-	3

Lane 1: Seedlings obtained from control samples Lane 5: Seedlings obtained from plants treated with 5% biogien and 5% microbien Lane 2: Seedlings obtained from plants treated with 10% biogien
Lane 6: Seedlings obtained from plants treated with 10% microbien Lane 3: Seedlings obtained from plants treated with 5% biogien Lane 7: Seedlings obtained from plants treated with 5% microbien
Lane 4: Seedlings obtained from plants treated with 2.5% biogien Lane 8: Seedlings obtained from plants treated with 2.5% microbien (+): Band present (-): Band absent

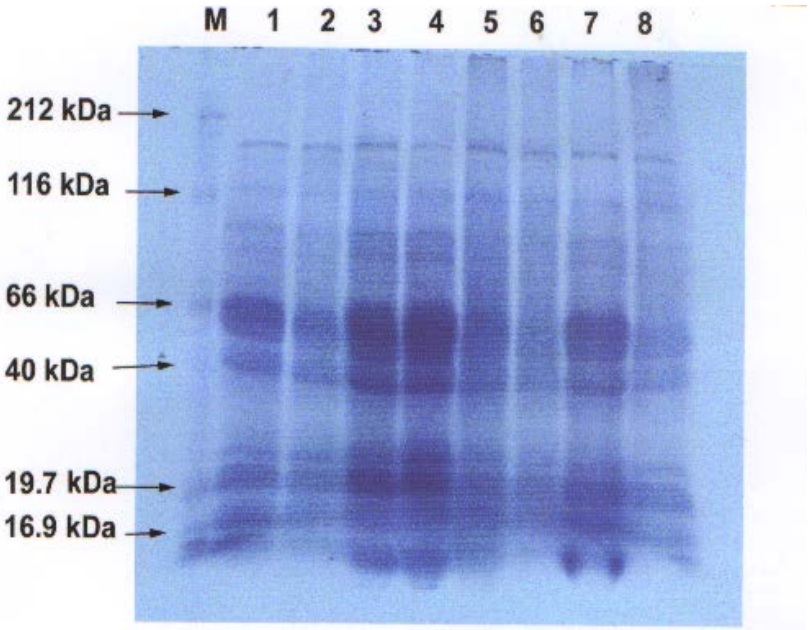

Fig. 1: Changes in protein banding patterns of M2 seeds of *Helianthus annus* L. cv. Vedock whose parents were previously treated with different concentrations of biogien and microbien. Lane M: protein molecular weight marker, lane 1: control samples, lanes 2, 3 and 4: samples treated with biogien (10, 5 and 2.5%, respectively), lane 5: samples treated with biogien (5%) in combination with microbien (5%), lanes 6, 7 and 8: samples treated with microbien (10, 5 and 2.5%, respectively)

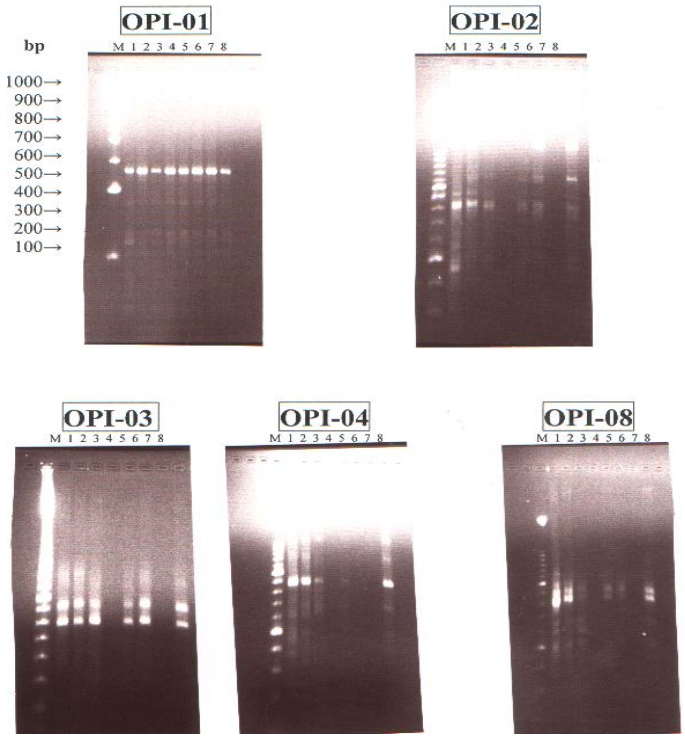

Fig. 2: RAPD-PCR profiles detected using five arbitrary primers (OPI-01, -02, -04, -06 and –07) in seedlings of M2 seeds of *Helianthus annus* L. cv. Vedock whose parents were previously treated with different concentrations of biogien and microbien. Lane M: protein molecular weight marker, lane 1: control samples, lanes 2, 3 and 4: samples treated with biogien (10, 5 and 2.5%, respectively), lane 5: samples treated with biogien (5%) in combination with microbien (5%), lanes 6, 7 and 8: samples treated with microbien (10, 5 and 2.5%, respectively)

on seed germination and related processes of wheat, sorghum, maize and lentil could be attributed to the nitrogen as well as nitrate reductase activities of the alga associated with the surface of plant or the amino acids and peptides produced in the algal filtrate and / or other compounds that stimulate growth of crop plant (Adam, 1999). Inferring use of biofertilizer may help to solve the energy crisis of nitrogen to some extent (Kumar, 1994). In general, biofertilizer application is a good tool to reduce the rate of chemical fertilizers, the cost of crops production and allow for a better environment.

Nucleic acid contents: The effects of various concentrations of biogien and microbene biofertilizers on nucleic acids (DNA and RNA) of *Helianthus annus* L. cv. Vedock seed yield are shown in Table (3), which clear that the concentration of DNA and RNA contents in seed yield of biofertilizer treated plants were markedly increased than those of the control plants. The values of increases in DNA contents were 383, 400 and 358% for biogien application in response to treatment with 10, 5 and 2.5%, respectively and 225% for the combined effect of both biogien and microbien application. The increasements in case of microbien application were 170.9, 200 and 133% in response to 10, 5 and 2.5%, respectively. The values of increases in RNA contents were 204, 215 and 131% in response to application of biogien at 10, 5 and 2.5%, respectively. The interaction of both biogien and microbien biofertilizers showed an increase by 108%. However, the increases in RNA content in response to microbien application were not significant at 10 and 2.5% doses, while that at 5% dose was significant.

From the above results, it can be concluded that the nucleic acids (DNA and RNA) contents were much higher in seed yield obtained from plants treated with biogien in all applied doses and the interaction of the tow biofertilizers. This may be due to the active synthesis of nucleic acids concurrently with decreasing the hydrolytic and oxidative enzyme activities. In this respect, Zodape (2001) using seaweeds (as biofertilizer), found that they contain micro-elements and plant growth regulators like cytokinins. Furthermore, it is known that auxins, gibberellins and cytokinins stimulate the synthesis of nucleic acids and inhibit the activities of RNase, DNase and 3-nucleotidase (Key *et al*., 1986; Steward, 1991).

Mineral contents: In the present investigation, calcium level markedly increased in the seed yield of treated plants (Table 5). Calcium may influence growth directly or indirectly through cell division and middle lamellar deposition, regulation of related osmotic responses, cell structures and membrane functions. It has been implicated in certain hormonal and environmental responses and regulates the activity of certain enzymes (Epstein, 1978 and Hopkins, 1995). Magnesium content showed similar pattern like calcium. Magnesium is required as an activator for numerous important enzymes. Concerning the changes in potassium content Table 5, it showed significant increases in response to biofertilizer application. The most pronounced increment was observed in seed yield resulted from treated samples. Sharma and Namdeo (1999a) obtained similar results. Such increases are attributed to the great uptake (Solankey *et al*., 1998). Potassium ions serve to activate certain enzymes especially those involved in photosynthesis, respiration and in starch and protein synthesis (Hopkins, 1995). Moreover, opening and closure of stomatal guard cells or daily changes in the orientation of leaves are affected by potassium concentration.

Protein content and protein profiles: Little investigations were carried out to study the effect of biofertilizer application on the protein content and protein profile. In this investigation, the total soluble protein content was significantly increased, the increase was more pronounced with fertilization with Biogien (Table 7). These results are in accordance with those obtained by Tiwana *et al*. (1992), Chela *et al*. (1993) and Sharma and Namdeo (1999b). Seed protein content was increased in response to biofertilizer application to soybean cv. MALS 13 (Sharma and Namdeo, 1999b). Bacterial culture (as a biofertilizer) alone or in combination with nitrogen fertilizer increased crude protein (Tiwana *et al*., 1992 and Chela *et al*., 1993). Isolate 103, Mutants Mac 27 and Mal 27 (as biofertilizers) could enhance the protein yield to the extent of 12.0, 43.10 and 36.2 percent, respectively, as compared to control. The increase in the crude protein yield is an expected result to the successive increase in nitrogen level in response to biofertilizer treatment (Patel *et al*., 1992).

The electrophoretic protein patterns of seed yield of biofertilizer treated *Helianthus annus* L. cv. Vedock are shown in Fig. 1 and analyzed and recorded in Table 6. Twenty one polypeptide bands of molecular weight ranged between 127.1 and 14.9 kDa were observed in the seed yield extract of the control plants. Fourteen polypeptides were common between the control extract and all the applied concentrations of the two biofertilizers. Their molecular weights were: 89.1, 58.9, 53.4, 40.4, 39.3, 34.4, 32.3, 28.4, 22.8, 21.4, 20.6, 18.6, 17.2 and 14.9 kDa. Concerning the most effective concentrations used of biogien and microbien (5% dose), 26 and 23 polypeptides were separated in each with regard to the biogien and microbien application, respectively. The separated

polypeptidesin case of biogien at 5% have molecular weights ranged between 179.5 and 2.1 kDa. This treatment is characteristic by the appearance of two new inducible proteins (179.5 and 2.1 kDa). However, in case of microbien, the separated ones have molecular weights ranged between 152 and 14.9 kDa, it also seen the appearance of one specific band of molecular weight 152 kDa.

From the above results, it is appearant that each of the recommended doses of the biofertilizers induce new proteins of low molecular weight (2.1kDa for biogien and 14.9 kDa for microbien), but the separated one in case of biogien has a lower molecular weight and this may be an adaptive mechanism for the biogien absorption in plants to give higher yield productivity.

Oil content and fatty acids composition: Oil seeds has attracted the interest of some research work to study the composition and quantitative changes in fatty acids during seed maturation and germination in response to fertilization (Teama and Mahmoud, 1994). In this investigation, it could be concluded that oil content of seed yield significantly increased in response to biofertilizer application as being compared to the control (Table 7). The maximum values were obtained to Biogien application. Sharma and Namdeo (1999a) obtained similar results using *Glycine max* L. Merrill plants fertilized with *Rhizobium* + FYM + PSB. These results are also in agreement with those obtained by Kumar (1994), who found that, using of *Azotobacter chroococcum* isolate 103 and its mutants Mac 27 and Mal 27 as biofertilizer could enhance the oil yield to the extent of 12.00, 43.10 and 36.20%, respectively, as compared to the control. Such increases may be attributed to the increases in 1000-seed weight and K content which has stimulatory effect on the storage capacity of assimilates.

Fats are classified into saturated and unsaturated fats. Saturated fats tend to increase blood cholesterol levels, while unsaturated ones show the reverse direction; they are mostly from plant sources. The most common saturated fatty acids found in plant lipids contain 16 or 18 carbon atoms. Usually only palmitic acid (C16) and stearic acid (C18) are present in significant amount, but the saturated fatty acids collectively account for only 20% of the total fatty acid content of most plants, while those with one or more double bonds (unsaturated fatty acids) account for the remaining 80%. In many fatty seeds, oleic {18:1(9C)} and linoleic {18:2 (9C,12C)} acids frequently account for more than 70% of the fatty acid content (>90% in sunflower) (Anderson and Beardall, 1999). Little work was done to study the role of fertilization in increasing and improving the oil quantity and quality of sunflower plants (Mekki *et al*., 1999a).

Concerning the changes in fatty acid composition, Gas Liquid Chromatographic analysis of the oils (using standard having 10 authentic fatty acids) showed the appearance of one fatty acid (18:3) in response to biofertilizer application as being compared to the control. However, both biofertilizers decreased the main saturated fatty acids; palmitic and stearic, while increased the main unsaturated fatty acids (oleic, linoleic and linolinic). Thus the yield oil becomes more safer for human consumption.

RAPD-PCR analysis: Of forty five decamer primers used, only five primers of Kit I yielded informative data. The RAPD profiles of these primers are shown in Fig. 2 and analyzed in Table 8. The numbers of amplified fragments generated by the primers OPI-01, -02, -03, -04 and –05 were 1, 3, 3, 2 and 3, respectively. The molecular size of these fragments ranges between 370 and 1800 bp.

In this investigation, RAPD analysis was effective in detecting the qualitative and quantitative changes of sunflower plants in response to biofertilization application. The amplification of nucleic acids with arbitrary primers is mainly driven by the interaction between primer, template annealing sites and enzyme and determined by complex kinetic and thermodynamic processes. The outcome is the generation of a population of polynucleotide products usually representing those genomic regions (amplicons) that have been predominantly amplified. Polymorphisms in nucleic acid scanning result from changes in DNA sequence, initially within primer-defined sites in the genome. However, they can also arise from the deletion, insertion or inversion of a priming site or segments between priming sites and from conformational changes in DNA that would alter the efficiency of amplification or priming. These DNA polymorphisms can become useful markers in general fingerprinting (Kawakami *et al*., 1999). Also, Yang and Quiros (1993) postulated that, quantitative changes could be explained on the basis of alterations of some DNA sequences.

Ionic components are crucial determinants of amplification. Magnesium is one important example. Consistent fingerprints can be obtained with relatively low levels of magnesium (1.5-4 mM) for plant and animal DNA and with high levels (4-8 mM) for bacteria and fungi (Caetano-Anolles *et al*., 1992). However, magnesium requirements are dependent on the counter ion and other buffer components. Its activity is also modulated by the concentrations of primer, template and deoxynucleoside triphosphates. An excess of any of these components can inhibit the amplification reaction due to the sequestration of free magnesium cation. In turn, an excess of magnesium

levels decrease amplification stringency and increase primer-template mismatching. Potassium, ammonium and detergents alter amplification efficiency and specificity. In contrast, pH had little effect (Caetano-Anolles, 1994).

A fingerprint pattern is only informative if it can be compared to other patterns. During electrophoresis , some bands are not well resolved as others. Absence of amplification is a common problem. It may indicate the presence of inhibitors in the DNA sample. It may also indicate the recalcitrant behavior of template DNA.

Different primers have different performances in detection of genetic changes. Quantitative changes were shown in all samples by one common band at a molecular size 480 bp using primer OPI-01 (Table 8 and Fig. 2). These changes may be due to slight changes of the nucleotide sequences recognized by primers as a result of biofertilizer application or due to the reduction of annealing efficiency between primers and DNA templates by masking the recognition sequences or due to the inhibition of *Taq* polymerase activity (Emam *et al*., 2000 and Flowers *et al*., 2000). The disappearance of some protein bands may be attributed to the alteration of their structural genes. Qualitative changes were recorded using primers OPI-02, -03, -04 and –05. There are two bands at molecular sizes 370 and 980 bp recorded in control and samples treated with 10% biogien and disappeared in all other treated samples. Using primer OPI-03, there are two bands with molecular sizes 500 and 620 bp not recorded in samples treated with 2.5% biogien and 5% microbien. On the other hand, there are two bands at molecular sizes 460 and 500 bp recorded in control and samples treated with 10% biogien and 2.5% microbien. These monomorphic qualitative changes were found to be reproducible when repeated at different times under the same amplification conditions. Also, the use of these primers resulted in a large number of faint bands which were not detected by video printing of the gel. The faint bands may be attributed to the low copy number of these fragments. These results are in agreement with that reported by Emam *et al*. (2000), who used five arbitrary primers to detect the effect of different salt concentrations on different cultivars of rice.

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