ORIGINAL ARTICLE JGEB Vol. 5, No. 1, 2007 ISSN:1687-157X

Effect of UV Treatments on the Activity of Delta-Endotoxin Protein Crystals in *Bacillus thuringiensis*

Fatma. N. Talkhan[1], H. H. Abo-assy[1], Kh. A. Soliman[2], A. S. Abdel-Razek[3], Marwa M. Azzam[1] and A.Z. E. Abdelsalam[4]

[1]Genetics and Cytology Department, National Research Centre, [2]Genetics Dept., Ain Shams Universiy, [3]Pests and Plant Protection Department, National Research Centre, Dokki, Cairo, Egypt. [4]Faculty of Biotechnology, Misr Universiy for Science and Technology,

ABSTRACT

The ability of some isolates of *B.thuringiensis* to produce dark brown pigment were measured as an indicator to UV resistant, M_5 isolate was used as wild type to improve it to be more resistant to UV, upon . It exposed to UV irradiation with different periods, ranged between 1 to 10 hrs. The induced mutants were examined morphologically by phase contrast microscope. 144 mutants were obtained, ten of them were selected and tested for their toxicity against *Spodoptera littorallis*. The results showed that mutants 62, 64 and 85 were the most toxic ones. These three mutants and wild type were examined by transmission electron microscope. Crystal proteins, with bipyramidial shape and active against Lepidoptera insects, were detected in all the selected mutants.

Key Words: *Bacillus thuringiensis*, mutation, melanin pigment, bioassay, mortality

Corresponding Author: Fatma. N. Talkhan
E-mail: fatma_talkhan@yahoo.com

Journal of Genetic Engineering and Biotechnology, 2007, 5(1-2): 33-42

INTRODUCTION

Recently, commercial bio-insecticides, especially the bacteria, have been used in insect biological control as an alternative to synthetic chemical insecticides.There is a problem when *B. thuringiensis* products used in the field. The parasporal crystal proteins and spores, which are the primary toxic substances to insects, are inactivated quickly after their exposure to sunlight. The proteins are cross-linked by hydroxyl radicals (.OH) and hydrogen peroxide (H_2O_2) when exposed to UV and /or ionizing radiation *(Wang et al., 1999)*, which leads to reduce dissolution in insect midgut. Since it was discovered in 1901, so many ways were used to improve the ability of *B. thuringiensis* in the application field. Despite all its advantages, acceptance of insecticides formulated with *B. thuringiensis* has been disappointing, primarily because they have not proven as effective under field conditions as laboratory tests have predicted *(Bulla et al., 1985)*. The use of conventional *B. thuringiensis* as insecticides is however limited because the spores and toxins are inactivated by solar radiation *(Griego and Spence, 1978; Ignoffo and Garcia, 1978; Pozsgay et al., 1987 and Pusztai et al. 1991)*. Various formulations are not sufficiently stable under field conditions and rapidly lose their biological activities *(Beegle et al., 1981 and Cohen et el., 1991)*. Attempts to protect *B. thuringiensis* toxicity from damaging by UV radiation under field conditions have yielded limited success. Different formulations were developed with addition of variety of screens *(Cohen et al., 2001; Dunkle and Shasha, 1989 and Morris, 1983)*, Some substances with different optical features were selected in order to test their protective quality against inactivation of *B. thuringiensis* spores by UV *(Krieg, 1975)*. In the last few years, there has been a growing interest in melanin research as some important functions have been recognized. Many reports focus on the ability of melanin to protect against radiation damage. Melanin absorbs light at all wavelengths and reaches its maximum absorbance in the UV range. In particular, melanin's ability to increase UV resistance and preserve the insecticidal activity of *B. thuringiensis* products has been reported with *(Hoti and Balaraman 1993; Patel et al., 1996 and Saxena et al., 2002)*. A novel mutant of *B. thuringiensis* which produces a dark-brown diffusible pigment, which is characterized as melanin, a natural UV screen was reported by *Patel et al. (1996)*. This mutant has increased UV resistance as well as increased toxicity against *Plutella xylostella* (diamondback moth). Another mutant of *B. thuringiensis* producing melanin was described by *Saxena et al. (2002)*. They mentioned that characterization of such mutant with increased UV resistance might contribute to develop stable formulations for field application. Being a natural product, melanin

is easily biodegradable and, thus, will not pose any threat to the environment. On the other hand, *Chen et al. (2004)* isolated a UV resistant wild type strain of *B. thuringiensis* subsp. dendrolimus L-7601 producing a dark brown pigment in a general nutrition-abundant medium, which had no L-tyrosine.These studies aim to they identified this pigment as melanin based on chemical testing.

MATERIALS AND METHODS

Materials:

10 isolates of *B. thuringiensis* were used in this study and isolate M5 was selected according to their ability to produce pigment (melanin) and used as a wild type.

Media:

LB medium (per 1 liter): Yeast extract 5gm, Tryptone 10gm, NaCl 10gm, PH7. NCM medium (per liter): Tryptone 5gm, Yeast extract 3gm, 0.7 mM CaCl2, 0.05 mM MnCl2, 1 mM MgCl2, pH7.2. This medium was solidified with 2 per cent agar.

UV experiments:

Samples preparations:

M_5 isolate was selected and used to enhance its ability to UV resistance. Under sterilized conditions, 7 ml of one week culture (spores and crystals) of the selected isolate were centrifuged at 4000 rpm, supernatant was discarded then the pellets were washed with 5 ml of water then centrifuged, 5 ml of saline (NaCl 0.85%) were added after discarding water then sprayed onto a 3 cm diameter sterilized Petri dish (with a magnetic bar) to be ready for the UV irradiation treatment. The volume of saline were increased to be 10 ml instead of 5 ml when UV irradiation was used for long times (4, 5, 6, 7, 8, 9 and 10 hrs) to equal the evaporation and heating of the suspension of spores and crystal.

In a dark room, a UV lamp (30 W. Philips T-UV lamp type No. 57413 P/40) was used with a 20 cm distance away from the opened Petri dish that was put on a magnetic stirrer for slow stirring during the irradiation period. Irradiation periods were 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 hrs. After irradiation, solutions were left under dark cover for at least an hour to prevent photo-reactivation repair. Then, under sterilized conditions, 500 µl of irradiated solution were added to 9 cm test tubes with 4.5 ml of sterilized distilled water and a serial of dilutions were carried out. After that, a 500 µl of diluted solution were added to 10 cm Petri dishes with LB media and incubated at 28°C for 24-48 hrs then counted and studied for its UV survival.

Mutants' characterization:

Mutants were studied as colony morphology(Cry^+ and Cry^-) that was described by *Sarrafzadeh et al. (2006)* where colonies were selected randomly. Then, these colonies were studied upon microscopic examination concerning the existence of spores and crystal proteins or not.

Screening for dark brown pigment:

To study the ability of wild type strain production of dark brown pigment and according to *Ruan et al.(2004)*, components of 1/4 liter of NCM medium were dissolved in 200 ml distilled water and 0.25 gm L-tyrosine were dissolved in 50 ml distilled water (it was milky and didn't dissolve in water), then autoclaved separately. Upon cooling both were mixed well then flowed into Petri dishes then inoculated with bacteria and incubated at 42°C for 48 hrs then incubated at 37°C for 48 hrs. The ability of the UV mutants to produce the dark brown pigment was detected using the NCM broth medium and L-tyrosine. Broth was inoculated with the mutants and incubated at 42°C for 48 hrs then incubated at 37°C for 5 days. Growth and pigment were noticed and registered after 3 days and after 7 days. After 7 days, broth were centrifuged and pigment was measured by Spectrophotometer (Model Shimadzu, Graphicord) at 400, 500, 600 nm according to *Chen et al. (2004)*.

RESULTS

Melanin experiments for UV resistance:

The tested 10 isolates of *B. thuringiensis* were varied in their ability to produce melanin (Figures 1 and 2) in spite of the suitable conditions for that (L-tyrosine and incubation at 42°C). Whereas some isolates produced dark brown pigment, like J, M_5, M_1 with different levels, other isolates did not (Table 1).

Figure 1: The ability of *B. thuringiensis* isolates to produce dark pigments

Figure 2: Pattern of melanin production of (J) isolate, the highest, on a NCM plate containing 0.1% L-tyrosine at 42°C and a plate without any growth as a control.

Formation of this pigment depended on the presence of L-tyrosine in the culture medium. Therefore, melanin is formed by the action of tyrosinase *(Riley, 1993)*. Furthermore, *Ruan et al. (2004)* observed the presence of L-DOPA, which is subsequently polymerized to melanin via a series of nonenzymatic reactions, in the supernatant of *B. thuringiensis* cultured at 42°C and confirmed a hypothesis that the black pigment produced by *B. thuringiensis* was synthesized via L-DOPA.

The results illustrated in (Table 1), the isolate (J) was gave a high production of dark pigment. Isolates M_5 and M_1 were lower than (J) in their pigment productivity, wheil other isolates, (M_6, M_8, C, ATCF, M_{12} and M_{13}) had no pigment at all.

Table 1: Ability of *B. thuringiensis* isolates , under study, to produce dark pigments.

Isolates	Pigment
J	+++++
M_5	++
M_1	++
M_6	---------
M_8	---------
C	---------
ATCF	---------
M_{13}	---------
M_{12}	---------

UV experiments:

UV radiation almost is used as a mutagenesis agent to enhance the productivity in some microorganisms of some important byproduct substances (enzymes, proteins, antibiotics, alcohols.....etc).

Spores of the selected isolate (M_5) that was used as a wild type strain were exposed to UV irradiation for different exposure periods (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 hrs). Previously, UV irradiation was used for long periods of time (1, 2 and 3 hours) to get mutants able to produce dark brown pigment and, subsequently, able to resist the damage caused by UV *(Ruan et al., 2004)*.

Results of the UV experiments were illustrated in (Table 2), with the dilution factor 10^{-10}; the average number of colonies in the control reached 243 ones. This number was subsequently decreased with increasing periods of exposure to UV irradiation. With the dilution factor 10^{-1}, the average number of colonies at 1 hr of irradiation was 300 ones, at 2 hrs was 45.6, at 3 hrs was 35, at 4 hrs was 29.3, at 5 hrs was 14.3, at 6 hrs was 11.3, at 7 hrs was 9.3, at 8 hrs was 8.6, at 9 hrs was 8 and at 10 hrs was 7.3 ones, respectively.

Table 2: Data of UV irradiation, against spores of *B. thuringiensis* wild type (M_5) for different periods (1-10 hrs) at 10^{-10} dilution factor.

Treatment period (hrs)	Dilution factor	Colonies No.			Average No. colonies
		Rep.1	Rep.2	Rep.3	
0 (control)	10^{-10}	285	223	222	243
1	10^{-1}	250	300	350	300
2		36	56	45	45.6
3		37	26	42	35
4		30	25	33	29.3
5		16	13	14	14.3
6		9	14	11	11.3
7		12	6	10	9.3
8		7	8	11	8.6
9		10	6	8	8
10		7	10	5	7.3

Most of grown mutants colony were examined according to *Sarrafzadeh et al. (2006),* who mentioned that colonies phenotype of *B. thuringiensis* strains that lost their plasmid and subsequently lost its ability to produce crystal proteins (Cry^-), would be small and smooth. While, colonies phenotype of *B. thuringiensis* strains that have its plasmid and subsequently has the ability to produce crystal proteins (Cry^+) would big and rough (Figure 3). After colonies examination, spores and crystal protein of mutants were examined after 72 hrs by microscope phase contrast.

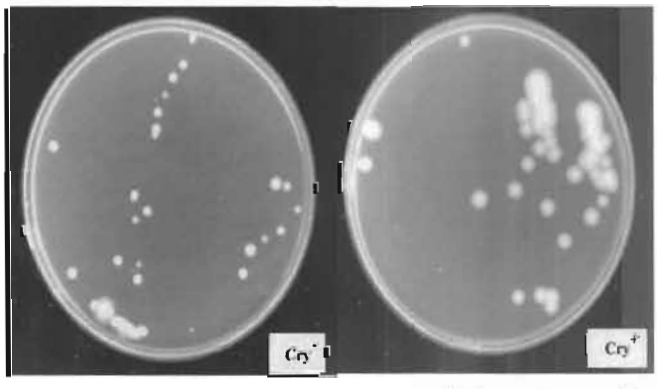

Figure 3: A comparison between colony morphology of two strains of *Bacillus thuringiensis*.The Cry+ strain formed rough (R) colonies with bigger size than smooth (S) colonies of the Cry− strain. Due to impurity of the Cry− sample, withdrawn directly from the culture broth, some R colonies were also observed *(Sarrafzadeh et al., 2006)*.

One hundred forty four mutants were selected according to colony morphology, where colonies were rough with big size and crystal protein, with bipyramidial shape. So, they were selected and tested for their ability to produce dark brown pigment.

Screening of melanin in UV mutants:

After UV experiments, selected mutants were grown on NCM broth medium with L-tyrosine, incubated at 30°C for 24 hrs, then incubated at 42°C *(Ruan et al., 2004)* for 7 days. Growth

and pigment was observed. Results were registered after 3 days and after 7days (Table 3). Some notes were observed; first, existence of L-tyrosine inhibited some mutants' growth in the first 3 days of the experiment (like mutants No. 14, 26, 33, 52, 58, 69, 74, 79, 82, 99, 103, 109, 116, 124 and 142) and after 7 days, growth and pigment were appeared in a slow way in these mutants. Second, the mutants' No. 11, 12, 14, 26 and 28 gave low pigment. while other mutants (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 15, 16, 17, 18, 19, 20, 21, 22, 24, 25, 27 and 29) were grown but didn't give any pigments. Finally, some other mutants, like 106, 119 and 130, did not grow at all for the 7 days with the presence of L-tyrosine.

Also, all UV mutants were measured by spectrophotometer at different wavelength, 400, 500 and 600 nm, according to *Chen et al., 2004.* The results, illustrated in (Table 4), showed variability between mutants with melanin productivity when compared with the wild type isolate (M_5).

Figure (4) showed the highest 10 UV mutants with melanin production and their numbers were 65, 62, 64, 32, 118, 131, 123, 132, 103 and 85 where the absorption at 400 nm by spectrophotometer were 2.322, 1.505, 1.303, 1.245, 1.242, 1.222, 1.212, 1.18 and 1.172, respectively.

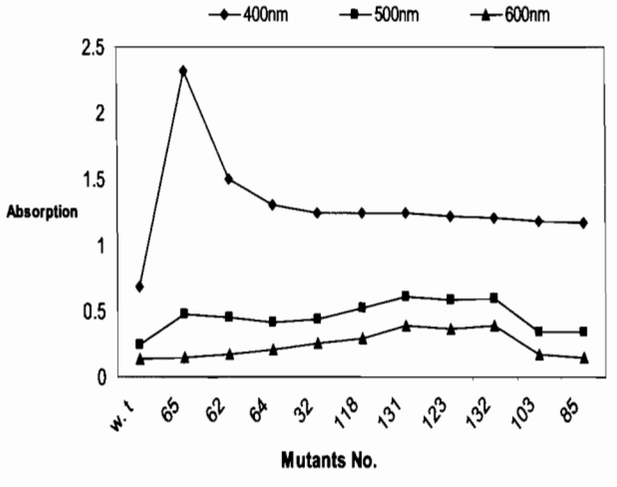

Figure 4: The highest ten UV mutants with dark brown pigment productivity compared with the wild type isolate at 400-500-

Relation between UV irradiation periods and pigment productivity:

Data of UV experiments, with different exposure periods and melanin productivity revealed that there is no relation between periods of UV irradiation and melanin productivity (Figure 5). Whereas, mutants No. 65, 62 and 64 which were irradiated for only 7 hours, were the highest melanin productivity (2.322, 1.505 and 1.303 at 400 nm). While, mutants No. 118, 131, 123 and 132 which were irradiated for 9 hours, were less in melanin productivity (1.242, 1.242, 1.222 and 1.212 at 400 nm).

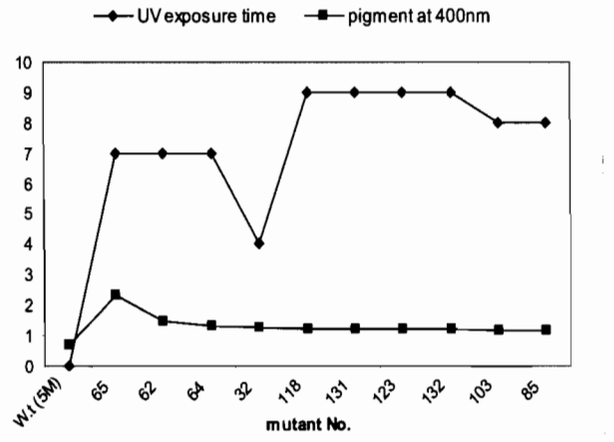

Figure 5: Relationship between UV irradiation periods and pigment productivity of UV mutants.

Mortality and LC_{50} values calculations of *B. thuringiensis* mutants:

Assessment of mutants' toxicity was expressed as mortality percentage of total larvae of each treatment, as well as in the control, after 4 days. A constant concentration (1500 µg/ml) from all the mutants was used with the screening experiments, to detect the most toxic mutants. Results of screening experiments have been shown in (Table 5) and (Figure 6). These results showed that from 10 mutants, used for screening their activities against 3rd instar larvae of the cotton leaf worm, *S. littoralis*, only 3 mutants gave mortality ranged between 90% to 63% where the mutants' No. 62, 64 and 85 gave 90, 77 and 63% mortalities, respectively and were the most toxic mutants.

On the other hand, the other mutants gave mortality ranged between 53 to 22% where the mutants' No. 123, 118, 32, 103, 65, 131 and 132 gave 53, 46, 40, 29, 28, 25 and 22% mortalities, respectively where that the control gave 7% of mortality. These results revealed that mutants 62, 64 and 85 were promising ones. So, these mutants were used for further studies with the bioassay and LC_{50} calculations .

Results of bioassay experiments, were shown in (Table 6), using the highest three active mutants (62, 64 and 85), in the screening mortality investigations. Five different concentrations (1500, 1000, 500, 250 and 125 µg/ml) have been tested for each mutant against 3rd instar larvae of *S. littoralis*. Corrected mortality was calculated according to Abbott's formula *(Abbott, 1925).*

The mutant No. 62 gave 89, 85, 78, 67 and 65% mortalities at 1500, 1000, 500, 250 and 125 µg/ml, respectively. The mutant No. 64 gave 72, 69, 61, 58 and 53% mortalities at 1500, 1000, 500, 250 and 125 µg/ml, respectively. The mutant No. 85 gave 67, 64, 56, 50 and 33% mortalities

at 1500, 1000, 500, 250 and 125 µg/ml, respectively. The bioassay results of these mutants were compared with the results of the wild type isolate (M_5).

Statistical analysis of the bioassay results of the UV mutants, were shown in (Table 7), reported that the LC_{50} values were, 52, 94 and 330 µg/ml for the 62, 64 and 85 mutants, respectively. Values of slope factors, confidence limits, variance and Chi^2 were also mentioned in (Table 7) for each mutant separately. The results of the variance and Chi^2 for the statistical analysis of the bioassay data, of the three mutants (62, 64 and 85), compared with the wild type isolate (M_5), showed that the percentage mortality at the diagnostic LC_{50} values in the susceptible population of *S. littoralis* were considered significantly different between the three mutants. Also, a significant difference between each of the three mutants and the wild type isolate (M_5) was resulted.

Examination of mutants and wild type isolate by transmission electron microscope:

After screening and bioassay experiments, the highly toxic 3 mutants, were selected for examination with transmission electron microscope and were compared with the wild type isolate (M_5). Crystal proteins, with bipyramidial shape and active against Lepidoptera insects, were detected in all the selected mutants 62, 64 and 85 (Figure7).

Electron microscope was used to state the regular and irregular shapes of *B. thuringiensis* isolates at the sporangium stage *(Dai and Wang, 1987)*. Also, *Bechtel and Bulla (1976)* described a comprehensive ultra structural analysis of sporulation and parasporal crystal development for *B. thuringiensis* using electron microscope.

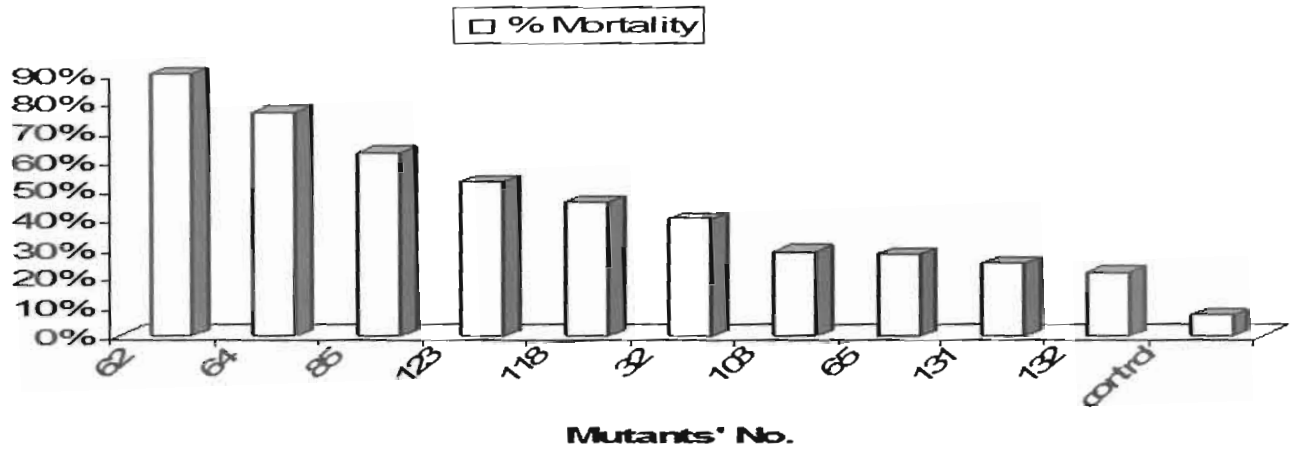

Figure 6: Mortality of the highest ten *B. thuringiensis* mutants with dark brown pigment against *S. littoralis*.

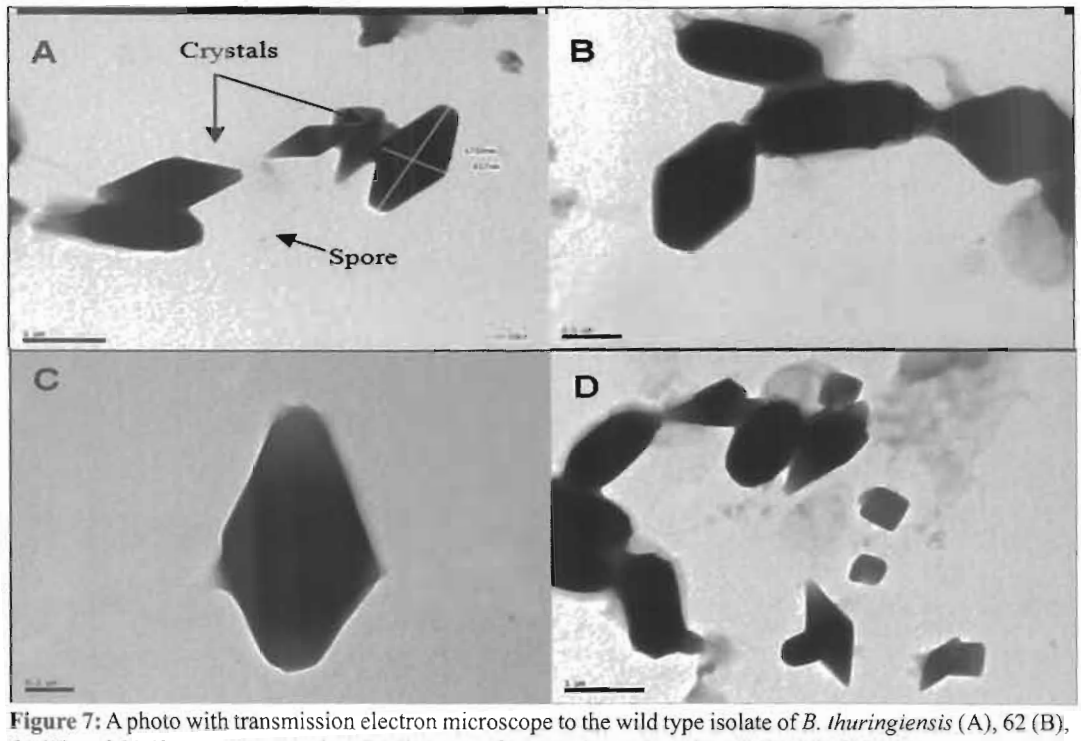

Figure 7: A photo with transmission electron microscope to the wild type isolate of *B. thuringiensis* (A), 62 (B), 64 (C) and 85 (D) mutants showing the diameter of a crystal protein, with bipyramidial shape.

Table 3: Capacity of *B. thuringiensis* mutants producing melanin following induction at 42°C.

Mutant No.	Growth		Pigment	Mutant No.	Growth		Pigment	Mutant No.	Growth		Pigment	Mutant No.	Growth		Pigment	Mutant No.	Growth		Pigment
	After 3 days	After 7 days			After 3 days	After 7 days			After 3 days	After 7 days			After 3 days	After 7 days			After 3 days	After 7 days	
11			+	49			++	75			+	98			+++	123			+++
12			++	50			++	76			+++	99	-		+	124	-		++
14	-		+	51			+	77			+++	100			+	125			+++
23			+	52	-		++++	78			++	101			+++	126			++
26	-		+	53			+	79	-		+	102			+	127			++
28			+	54			+	80			++	103	-		++	129			++
30			+	55			++	81			+	104			+	131			+++
31			++	56			+	82	-		+	105			+	132			+++
32			+	57			+	83			+	107			++	133			++
33	-		+++	58	-		++	84			+	108			++	134			++
34			++	59			+	85			++++	109	-		++	135			+++
35			+	61			++	86			+	110			++	136			++
36			++	62			+++++	87			+	111			++	137			++
37			+	63			+	88			+	112			++	138			++
38			+	64			+++	89			++	113			+	139			+++
39			+	65			++++++	90			+	114			+	140			++
42			++	66			++	91			+	115			+	141			+++
43			++	67			+	92			+	116	-		++	142	-		++
44			++	69	-		+	93			+	117			++	143			++
45			++	71			++	94			+++	118			+++	144			+++
46			++	72			++	95			++	120			++				
47			++	73			++	96			+	121			+				
48			+	74	-		+	97			+	122			++				

Note: Number of plus sign (+) represents the capacity of B. thuringiensis mutants to produce melanin..

Table 4: Light absorption in 400-500-600 nm wavelength of pigment produced by UV mutants of *B. thuringiensis*.

Mutant No.	400 nm	500 nm	600 nm	Mutant No.	400 nm	500 nm	600 nm	Mutant No.	400 nm	500 nm	600 nm	Mutant No.	400 nm	500 nm	600 nm
W.T.	0.678	0.249	0.131	109	0.96	0.247	0.111	56	0.861	0.284	0.15	105	0.692	0.2	0.09
65	2.322	0.473	0.146	81	0.958	0.256	0.12	66	0.851	0.325	0.177	84	0.681	0.248	0.141
62	1.505	0.446	0.175	44	0.953	0.277	0.04	54	0.85	0.288	0.158	72	0.679	0.23	0.127
64	1.303	0.413	0.212	59	0.952	0.316	0.167	110	0.842	0.364	0.225	39	0.639	0.058	0.19
32	1.245	0.443	0.254	53	0.951	0.377	0.222	116	0.842	0.336	0.179	82	0.593	0.197	0.099
118	1.242	0.526	0.297	124	0.95	0.405	0.236	138	0.832	0.301	0.164	36	0.576	0.165	0.076
131	1.242	0.61	0.387	107	0.942	0.372	0.2	74	0.83	0.346	0.203	102	0.574	0.209	0.123
123	1.222	0.587	0.36	133	0.942	0.398	0.226	11	0.827	0.389	0.219	38	0.548	0.038	0.165
132	1.212	0.598	0.388	62	0.932	0.44	0.272	37	0.825	0.26	0.135	96	0.544	0.224	0.133
103	1.18	0.338	0.165	127	0.932	0.368	0.204	43	0.805	0.252	0.131	90	0.531	0.212	0.134
85	1.172	0.34	0.146	137	0.93	0.389	0.234	50	0.804	0.244	0.125	14	0.527	0.137	0.059
125	1.162	0.466	0.27	48	0.926	0.288	0.145	104	0.782	0.287	0.158	80	0.518	0.146	0.063
135	1.142	0.472	0.265	51	0.922	0.346	0.201	142	0.782	0.251	0.13	94	0.489	0.195	0.114
139	1.142	0.433	0.226	35	0.915	0.319	0.178	114	0.781	0.28	0.158	99	0.482	0.117	0.065
101	1.126	0.376	0.192	23	0.911	0.298	0.161	52	0.772	0.272	0.153	88	0.48	0.194	0.127
122	1.112	0.456	0.265	34	0.902	0.313	0.173	55	0.77	0.172	0.064	58	0.478	0.111	0.045
61	1.095	0.308	0.147	47	0.893	0.305	0.169	121	0.762	0.278	0.154	98	0.469	0.173	0.092
141	1.092	0.51	0.311	117	0.892	0.319	0.173	28	0.757	0.329	0.177	100	0.435	0.146	0.087
77	1.072	0.253	0.103	75	0.889	0.369	0.226	49	0.753	0.236	0.125	86	0.42	0.177	0.12
31	1.037	0.259	0.101	111	0.886	0.364	0.22	57	0.753	0.236	0.122	87	0.402	0.139	0.09
126	1.032	0.449	0.276	33	0.883	0.222	0.105	113	0.752	0.281	0.162	79	0.38	0.114	0.033
67	1.013	0.251	0.106	71	0.88	0.231	0.101	52	0.747	0.138	0.037	93	0.36	0.098	0.054
144	1.012	0.401	0.243	42	0.879	0.295	0.156	45	0.746	0.199	0.092	26	0.357	0.083	0.031
129	1.002	0.425	0.243	120	0.878	0.35	0.198	143	0.722	0.287	0.17	69	0.345	0.093	0.031
136	1.002	0.379	0.211	112	0.872	0.316	0.163	115	0.721	0.246	0.134	91	0.342	0.073	0.036
12	0.977	0.465	0.231	30	0.869	0.282	0.153	78	0.714	0.244	0.126	92	0.324	0.101	0.062
134	0.972	0.366	0.201	46	0.869	0.291	0.158	63	0.712	0.208	0.103	89	0.296	0.063	0.03
108	0.962	0.379	0.218	140	0.862	0.32	0.176	83	0.697	0.249	0.139	97	0.279	0.037	0.011

Table 5: The mortality percentages of cotton leaf warm, *S.littoralis*, resulted after the treatment with *B. thuringiensis* mutants.

Mutants No.	Mean of dead larvae	% Mortality
Control	0.7	7%
62	9	90%
64	7.7	77%
85	6.3	63%
123	5.3	53%
118	4.6	46%
32	4	40%
103	2.9	29%
65	2.8	28%
131	2.5	25%
132	2.2	22%

Table 6: the bioassay data of mortality of *S. littoralis* larvae treated withdifferent concentrations from the best three *B. thuringiensis* mutants.

Mutants	Conc. (µg/ml)	% Mortality	% Corrected Mortality
w.t (M_5)	1500	95	94
	1000	87	83
	500	80	75
	250	60	50
	125	50	38
62	1500	90	89
	1000	87	85
	500	80	78
	250	70	67
	125	69	65
64	1500	75	72
	1000	76	69
	500	65	61
	250	63	58
	125	58	53
85	1500	70	67
	1000	68	64
	500	60	56
	250	57	50
	125	40	33

DISCUSSION

The main aim of this study is to improve the ability of the *B. thuringiensis* promising isolate, which is already producing a high and active crystal protein, to produce melanin pigment which led to resistance against UV radiation damage and subsequently to protect *B. thuringiensis* toxicity against the cotton leaf worm under field conditions.

The melanin pigment is well known as a protective agent from the damaging effects of UV radiation. Which means that, the highly dark pigment production of *B. thuringiensis*, the highly ability to UV damage resistance.

So, with the results illustrated in (Table 1), the isolate (J) was gave a high production of dark pigment and it was, subsequently, a UV resistant isolate. While, the isolates M_5 and M_1 were low with their pigment productivity and, subsequently, they had a low resistance to UV. While other isolates, (M_6, M_8, C, ATCF, M_{12} and M_{13}) had no pigment at all. So, they considered to be sensitive to UV radiation *(Riley, 1993; Patel et al., 1996; Saxena et al., 2002; Ruan et al., 2004 and Chen et al., 2004).*

The characteristics of UV inactivation of sporeforming and other bacteria phenotypes or shape were reported and discussed by *Burges et al. (1975) and Krieg, (1975).* Also, *Griego and Spence (1978)* described the inactivation of *B. thuringiensis* spores and spores treated with two protectants at wavelengths of the near-ultraviolet and visible spectra at 254 nm and mentioned that more than 50% of the spores were inactivated at 254 nm.

One hundred forty four mutants were identical with colony morphology, where colonies were rough with big size and crystal protein, with bipyramidial shape. So, they were selected to be tested for their ability to produce dark brown pigment, according to *Sarrafzadeh et al.(2006)* .

An observation was put in consider, with the mutants that were survived after exposure to UV radiation for 10 hours, that they were few, smooth colonies and most of them lost the ability to produce crystal protein when they were examined with phase contrast microscope. Loss of plasmids occurs both spontaneously and during exposure to curing condition in *B. thuringiensis* subsp. *thuringiensis (Gonzalez et al., 1981).*

Results in (Table 6) of the bioassay experiments revealed that the insecticidal activity of the *B. thuringiensis* UV resistance mutants, which had the ability to produce melanin pigment, were lower than the insecticidal activity of the wild type that was sensitive to UV radiation.

These results were agreed with some reports, whereas *Chen et al., (2004)* had a Bt L-7601 strain that was isolated as a wild type with a high UV resistance and it was shown to produce melanin, which is an excellent UV protective agent for Bt formulations. But they found that the formulations of Bt L-7601 strain had low toxicity to insects and suggested that, in the future, they can introduce the mel gene into

high toxic *B. thuringiensis* strains to solve the problem of *B. thuringiensis*'s susceptibility to UV, by constructing a melanin-producing transgenic bacterium.

The results of bioassay studies for the three mutants, compared with the wild type isolate, revealed that the LC_{50} value for the mutant 62 was lower than LC_{50} value of the wild type isolate and the other mutants (64 and 85) giving 52 µg/ml compared to 213, 94 and 330 µg/ml for the wild type isolate and the mutants 64 and 85, respectively.

These results are in a great agreement with some previous reports. Whereas, *Patel et al. (1996), Saxena et al. (2002) and Ruan et al. (2004)* have obtained brown pigment producing mutants of Bt-m-8, Bt-m and *B. thuringiensis* strain 94001, respectively by artificial-mutation of *B. thuringiensis* subsp. kurstaki after UV radiation. They assayed toxicity against *Plutella xylostella*, *S. littoralis* and *Heiothis armigera*, respectively. They suggested that the insecticidal activity of *B. thuringiensis* that produced melanin was significantly higher after UV irradiation than when melanin was not produced.

Electron microscope was used to state the regular and irregular shapes of *B. thuringiensis* isolates at the sporangium stage *(Dai and Wang, 1987)*. Also, *Bechtel and Bulla (1976)* described a comprehensive ultra structural analysis of sporulation and parasporal crystal development for *B. thuringiensis* using electron microscope.

REFERENCES

Abbott, W. S. 1925. A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18:265-267.

Bechtel, D. B. and Bulla, L. A.,Jr. 1976. Electron microscope study of sporulation and parasporal crystal formation in *Bacillus thuringiensis*. Journal of Bacteriology **127**(3):1472-1481.

Beegle, C. C., Dulmage, H. T., Wolfenbarger, D. A. and Martinez, E. 1981. Persistence of *Bacillus thuringiensis* Berliner insecticidal activity on cotton foliage. Environmental Entomology **10**(3):400-401.

Bulla, L. A.,Jr, Faust, R. M., Andrews, R. and Goodman, N. 1985. Insecticidal Bacilli. In The molecular biology of the Bacilli, edited by D. A. Dubnau. New York: Academic Press pp 187-209.

Burges, H. D., Hillyer, S. and Chanter, D. O. 1975. Effect of ultraviolet and gamma rays on the activity of delta-endotoxin protein crystals of *Bacillus thuringiensis*. Journal of Invertebrate Pathology **25**(1):5-9.

Chen, Y., Deng, Y., Wang, J., et al. 2004. Characterization of melanin produced by a wild-type strain of *Bacillus thuringiensis*. Journal of General and Applied Microbiology **50**(4):183-188.

Cohen, E., Joseph, T. and Wassermann-Golan, M. 2001. Photostabilization of biocontrol agents by berberine. International Journal of Pest Management **47**(1):63-67.

Cohen, E., Rozen, H., Joseph, T., et al. 1991. Photoprotection of *Bacillus thuringiensis* kurstaki from ultraviolet irradiation. Journal of Invertebrate Pathology **57**(3):343-351.

Dai, L. and Wang, X. 1988. A new subspecies of *Bacillus thuringiensis*. Microbiologica Sinica **28**(4):301-306.

Dunkle, R. L. and Shasha, B. S. 1989. Response of starch-encapsulated *Bacillus thuringiensis* containing ultraviolet screens to sunlight. Environmental Entomology **18**(6):1035-1041.

Gonzalez, J. M.,Jr, Dulmage, H. T. and Carlton, B. C. 1981. Correlation between specific plasmids and delta-endotoxin production in *Bacillus thuringiensis*. Plasmid **5**(3):352-365.

Griego, V. M. and Spence, K. D. 1978. Inactivation of *Bacillus thuringiensis* spores by ultraviolet and visible light. Applied and Environmental Microbiology **35**(5):906-910.

Hoti, S. L. and Balaraman, K. 1993. Formation of melanin pigment by a mutant of *Bacillus thuringiensis* H-14. Journal of General Microbiology **139**(10):2365-2369.

Ignoffo, C. M. and Garcia, C. 1978. UV-photoinactivation of cells and spores of *Bacillus thuringiensis* and effects of peroxidase on inactivation. Environmental Entomology **7**(2):270-272.

Krieg, A. 1975. Photoprotection against inactivation of *Bacillus thuringiensis* spores by ultraviolet rays. Journal of Invertebrate Pathology **25**(2):267-268.

Morris, O. N. 1983. Protection of *Bacillus thuringiensis* from inactivation by sunlight. Canadian Entomologist **115**(9) :1215-1227.

Patel, K. R., Wyman, J. A., Patel, K. A. and Burden, B. J. 1996. A mutant of *Bacillus thuringiensis* producing a dark-brown pigment with increased UV resistance and insecticidal activity. Journal of Invertebrate Pathology **67**(2):120-124.

Pozsgay, M., Fast, P., Kaplan, H. and Carey, P. R. 1987. The effect of sunlight on the protein crystals from *Bacillus thuringiensis* var. *kurstaki* HD1 and NRD12: A Raman spectroscopic study. Journal of Invertebrate Pathology **50**(3):246-253.

Pusztai, M., Fast, P., Gringorten, L., et al. 1991. The mechanism of sunlight-mediated inactivation of *Bacillus thuringiensis* crystals. Biochemical Journal **273**(1):43-47.

Riley, P. A. 1993. Mechanistic aspects of the control of tyrosinase activity. Pigment Cell Research / Sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society **6**(4 Pt 1):182-185.

Ruan, L. F., Yu, Z. N., Fang, B., et al. 2004. Melanin pigment formation and increased UV resistance in *Bacillus thuringiensis* following high temperature induction. Systematic and Applied Microbiology **27**(3):286-289.

Sarrafzadeh, M. H., Bigey, F., Capariccio, B., et al. 2007. Simple indicators of plasmid loss during fermentation of *Bacillus thuringiensis*. Enzyme and Microbial Technology **40**(5):1052-1058.

Saxena, D., Ben-Dov, E., Manasherob, R., et al. 2002. A UV tolerant mutant of *Bacillus thuringiensis* subsp. *kurstaki* producing melanin. Current Microbiology **44**(1):25-30.

Wang, W., Qian, C., Shen, J. and Yang, S. 1999. (The effects of reactive oxidants on *Bacillus thuringiensis* parasporal crystals). Wei Sheng Wu Xue Bao = Acta Microbiologica Sinica **39**(5):469-474.