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EFFECT OF AWILD TYPE AND THREE DIFFERENT MUTANTS OF CHITINOLYTIC BACTERIA, *Bacillus thuringiensis* BY U.V. IRRADIATION ON VIABILITY OF *Meloidogyne incognita* EGGS UNDER LABORATORY CONDITIONS

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ABSTRACT

The nematicidal activity of the wild type and three mutants namely (24, 10, 32) of the chitinolytic bacteria, *Bacillus thuringiensis* was evaluated against eggs of the root - knot nematode, *Meloidogyne incognita*, using U.V. irradiation as a safer and environment friendly control, alternate of chemical nematicides under laboratory conditions at 25 ± 5 ºC.

Results, generally indicated that the mutant no. 24 is more effective on the nematode eggs followed by mutant no.10, then mutant no. 32 as compared to the wild type which achieved the lowest mortality. These values ranged from (90.8 to 96.6 %), (79.8 to 89.9 %), (70.2 to 79.5 %), (46.3 to 68.8 %); respectively compared to 2.7% in untreated control after 96 he time. There were positive relation - ship between the percentage of mortality and the chitinase production from mutants, the production evaluated 385 %, 350 % and 300 %; respectively.

Keyword : Chitinolytic bacteria, *Bacillus thuringiensis*, mutantion, U.V. irradiation *Meloidogyne incognita*

INTRODUCTION

Plant-parasitic nematodes are considered as serious pests causing severe damage to a wide range of many commercially economic crops (Siddiqui *et al*., 2004). The chitinolytic enzymes, chitinases promise to be safer pesticides (than chemical ones) and microbial biocontrol agents due to the importance of chitinolytic enzymes in insects, nematodes and fungal growth (Shig *et al*., 2002). Root – knot nematodes, *Meloidogyne* spp. are the main contributors to these damages. In the life cycle of root – knot nematodes, *Meloidogyne* spp., two stages are susceptible to soil – borne antagonistic: the infective juveniles, moving through soil, and the eggs deposited in a gelatinous matrix, often on the root surface of host plant (Stirling, 1991). Reid and Ogrydziak (1981) succeeded in isolating a chitinase-over producing mutant, *Serratia marcescens* which produced two to three times more endochitinase activity than the wild type. Miller and Sands (1977) mentioned that chitinases are hydrolytic enzymes which are responsible for degrading chitin. They did investigated their effects on plant -

parasitic nematodes Bird and McClure (1976) showed that chitin, a polymer of unbranched chains of B-(1→ 4)-linked 2- acetamido-2-deoxy-D-glucose is considered a permanent component of the middle layer of egg shells of plant –parasitic nematodes. Deleterious effects of the thermostable toxins of *B. thuringiensis* on different species of nematodes were studied by many workers (Prasad *et al.,* 1972 , Ignoffo 1973, Ignoffo and Dropkin, 1977 and Wei *et al*., 2003). However, no information about a chitinase - over producing mutant by different methods of mutagenicity for the chitinolytic bacteria, *Bacillus thuringiensis* against egg shells and gelatinous matrix of the root – knot nematodes. The aim of this study is to investigate the effect of using U.V. irradiation on a wild type of chitinolytic bacteria, *B.thuringiensis* for increasing its activity in producing the chitinase enzyme and its activity on the root knot nematode,*M.incognita* eggs under laboratory conditions at25 ± 5 ºC.

MATERIALS AND METHODS

Nematode treatments:

Six dilutions (S, S/10, S/50, S/100, S/500 and S /1000) from cell suspension of a wild type (parental or pre-genetic improvement by U.V. irradiation) of *Bacillus thuringiensis* and three mutants namely 24, 10 and 32 from this bacteria were used. Eggs of *Meloidogyne incognita* were extracted from the egg-masses. The egg-masses were shaken in 0.5% sodium hypochlorite for 1.5 min; then washed through a 500- mesh sieve and decanted into a large volume of distilled water from which the eggs were removed by pouring through a 200 mesh sieve according to Bird and McClure (1976). This technique permitted the isolation of non-sticky eggs with no changes in shell ultrastructure or egg physiology. About 100 of *M. incognita* eggs were added to small vial containing 1 ml of each previous dilutions of the bacteria containing 7.5 × 109, 7.5 × 108, 1.5 × 108, 7.5 × 107 , 1.5 × 107 and 7.5 ×10 6 cfu. Distilled water was used instead of bacterial cell as untreated treatment. Each treatment was replicated four times. The numbers of viable and non viable eggs and dead abnormal juveniles as well as egg shells were recorded after 24, 48, 72 and 96 hrs of exposure periods by counting the viable and non viable eggs microscopically. Eggs were judged non viable when they became spherical instead of the normal ovoid shape, or when large light refractive vacuoles were visible. Percentages of mortality and of abnormal developmental stages were estimated.

Mutagenic treatments:

An isolate of chitinolytic bacteria, *Bacillus thuringiensis* as a wild type used in this study is obtained from Faculty of Agriculture, Ain Shams Univ. Cells of 24 hours age of this isolate were resuspended in a saline solution (0.9 % Na2Cl) and transfered to petri dishes of 70 mm diameter, then left on magnetic stirrer on a distance of 20 cm. between the dishes and the irradiation source. The source of U.V. irradiation was T-UV- 30 W Phillips lamp type No. 57413 P/40. and variable times of irradiation 0, 1, 2 and 3 minutes were applied. After each treatment, suitable dilutions were made

from which 1 ml was mixed with soft agar (6 g of Agar and 1000 ml of distilled water). The isolates obtained from U.V. treatment were transfered into complete media (15 g of agar agar, 5g of peptone , 3 g of meat extract and 1000 ml distilled water) and then to minimal media [8 g of ammonium sulphate, 56 g of K2HPO4 (di-Kalium hydrogen phosphate) , 4 g of sodium citrate (2H2O), 24 g KH2PO4 (Kalium dihydrogen phosphate), 0.8 g of MgSO4 7H2O and 1000 ml of distilled water] to obtain the mutants.

In the present U.V. treatment, the strain of *B.t.* was exposed to different periods viz. 1, 2, 3 minutes; respectively. Every period has three dilutions 4, 5, 6 and in the first minute for every dilution were 31, 25, 8 isolates while in the second minute were 12, 11, 5 isolates and in the third minute were 7, 4, 3 isolates; respectively. These results indicated that the more exposure U.V. time, the less isolates were obtained (Table 1).

Table (1): Number of *B.t.* isolates after U.V. treatment with different exposure periods comparing with the dilutions.

Dilutions	Time (Minutes)		
	1	**2**	**3**
4	31	12	7
5	25	11	4
6	8	5	3

The total number of *B.t.* isolates obtained from UV. treatment were 106 isolates which are the production measurements compared to the wild type production by using the diameter of analysis zone and the diameter of the growth zone, then, the difference between them is divided on the diameter of the growth zone as the following formula:

$$\frac{\text{Analysed zone diameter} - \text{Growth zone diameter}}{\text{Growth zone diameter}} \times 100$$

According Cronin, *et al.* (1997)

As cleared table (2) the mutanted *B. thuringiensis* isolates numbers 24, 10 and 32, their chitinolytic enzyme, chitinase production were 385, 350 and 300; respectively this means that the production improvement between three folds to four folds on comparing with the wild type. On the other hand, the isolates numbers 54, 61, 2, 15, 43 were between two and three folds and the isolate no. 29 was on the level of the production of the wild type. So, in this investigation, the isolates numbers 24, 10, 32 were selected to the present studies on the root – knot nematode, *M. incognita eggs.*

Table (2): Production of chitinolytic enzyme, chitinase by *B.t.* isolates which were obtained from U.V. treatment

Serial No.	Code No.	G	A	Chitinase production%**	Chitinase production based on wild type% #
1	603	10	10.53	5.30	29.15
2	113	8	9.27	15.88	87.35
3	600	2	2.00	0.00	0.00
4	507	9	9.42	4.67	25.67
5	500	6	6.80	13.33	73.34
6	504	4	4.00	0.00	0.00
7	106	4	4.00	0.00	0.00
8	109	5	5.00	0.00	0.00
9	111	3	3.00	0.00	0.00
10	118	4	4.00	0.00	0.00
11	24	10	17.00	70.00	385.00
12	32	11	17.00	54.54	300.00
13	602	5	5.84	16.80	92.41
14	104	8	8.42	5.25	28.88
15	108	5	6.69	33.80	185.92
16	107	8	8.00	0.00	0.00
17	41	11	14.00	27.27	150.00
18	112	5	5.84	16.80	92.41
19	56	5	5.00	0.00	0.00
20	35	4	4.00	0.00	0.00
21	100	9	10.69	18.78	103.29
22	31	17	20.00	17.65	97.07
23	45	12	14.00	16.67	91.68
24	23	11	13.00	18.18	100.00
25	46	9	9.00	0.00	0.00
26	54	17	25.00	47.06	258.85
27	33	10	12.00	20.00	110.01
28	30	12	14.00	16.67	91.68
29	55	9	11.00	22.22	122.22
30	47	9	10.0	11.11	61.12
31	51	17	21.00	23.53	129.43
32	53	16	20.00	25.00	137.51
33	29	9	11.00	22.22	122.22
34	43	8	11.00	37.50	206.27
35	44	9	9.00	0.00	0.00
36	63	0	0.00	0.00	0.00
37	60	0	0.00	0.00	0.00
38	40	14	15.00	7.14	39.27
39	50	5	5.00	0.00	0.00
40	48	5	5.00	0.00	0.00
41	61	8	11.00	37.50	206.27
42	52	22	24.00	9.09	50.00
43	64	2	2.00	0.00	0.00
44	49	5	5.00	0.00	0.00
45	62	4	4.00	0.00	0.00
46	65	2	2.00	0.00	0.00
47	66	5	5.00	0.00	0.00
48	67	8	8.00	0.00	0.00
49	57	1	1.00	0.00	0.00
50	59	3	3.00	0.00	0.00
51	58	2	2.00	0.00	0.00
52	101	12	12.84	7.00	38.50
53	506	12	12.84	7.00	38.50
54	116	9	10.27	14.11	77.61
55	117	6	6.00	0.00	0.00
56	502	5	5.00	0.00	0.00
57	510	6	6.00	0.00	0.00
58	105	6	6.42	7.00	38.50
59	603	5	5.00	0.00	0.00
60	604	5	5.00	0.00	0.00

Continued (2)

Serial No.	Code No.	G	A	Chitinase production%**	Chitinase production based on wild type % #
61	114	2	2.00	0.00	0.00
62	4	3	3.00	0.00	0.00
63	102	1	1.00	0.00	0.00
64	11	2	2.00	0.00	0.00
65	605	2	2.00	0.00	0.00
66	14	11	14.00	27.27	150.00
67	115	5	5.60	12.00	66.01
68	21	7	9.00	28.57	157.15
69	37	11	14.00	27.27	150.00
70	34	6	8.00	33.33	183.35
71	511	9	9.42	4.67	25.69
72	501	10	10.84	8.40	46.20
73	505	7	8.27	18.14	99.80
74	110	20	21.69	8.45	46.48
75	1	15	17.00	13.33	73.32
76	10	11	18.00	63.63	350.00
77	3	15	15.00	0.00	0.00
78	22	25	30.00	20.00	110.01
79	2	10	15.00	50.0	275.03
80	509	10	10.00	0.00	0.00
81	6	12	15.00	25.00	137.51
82	18	8	10.00	25.00	137.51
83	508	9	9.84	9.33	51.34
84	601	8	8.84	10.50	57.76
85	19	10	12.00	20.00	110.00
86	9	2	2.00	0.00	0.00
87	12	3	3.00	0.00	0.00
88	38	4	4.00	0.00	0.00
89	5	5	5.00	0.00	0.00
90	26	2	2.00	0.00	0.00
91	17	6	8.00	33.33	183.33
92	16	7	9.00	28.57	157.15
93	15	7	10.00	42.86	235.74
94	25	10	12.00	20.00	110.01
95	13	8	10.00	25.00	137.51
96	27	8	10.00	25.00	137.51
97	39	10	12.00	20.00	110.01
98	20	7	9.00	28.57	157.15
99	36	13	15.00	15.38	84.60
100	7	14	15.00	7.14	39.27
101	A	5	5.00	0.00	0.00
102	B	4	4.84	21.00	115.51
103	C	4	4.84	21.00	115.51
104	8	5	7.00	40.00	220.02
105	42	10	12.00	20.00	110.01
106	103	8	8.84	10.50	57.76
W.T.	*	11	13.00	18.18	100.00

** Chitinase production = $\frac{A - G}{G} \times 100$ where,

A = Diameter of analysed zone around the growth.
G = Diameter of the colony.

RESULTS

Effect of a wild type of *Bacillus thuringiensis* and three mutants by U.V. irradiation on percentage mortality of *M. incognita* eggs :

As illustrated in Table (3) all evaluated chitinolytic bacteria mutants by U.V. irradiation viz. 24, 10 and 32 and the wild type of *Bacillus thuringiensis* affected the percentage viability of *M. incognita* eggs throughout the experimental period (4 days). The viability percentages of unviability eggs were dependent on bacterial concentrations and the tested

mutants. Data showed that the average percentage of the nematode eggs viability after 24 hours for *B.t.* mutant No. 24 whose chitinase production was 385 %, viability ranged from 88.3 % to 96.0 % followed by *B.t.* mutant 10 which produced about 350 % from chitinase, mortality ranged from 76.8 to 85.5 %, *B.t.* mutant 32 (its chitinase production was 300 %) effect on viability ranged from 65.5 to 76.5 %. On the other hand, the lowest mortality in the nematode eggs was noticed after treatment with the wild type which mortality ranged from 42.3 % to 61.5 % when compared to untreated control (distilled water) which attained zero mortality.

Table (3): Effect of a wild type of *B. thuringiensis* and its mutants by U.V. irradiation on viability mortality of *M. incognita* eggs under laboratory conditions.

Treatments	Bacterial Conc.	%Nematode un viability after different exposure periods**				%Abnormal developmental stages #			
		24 hr	48 hr	72 hr	96 hr	24 hr	48 hr	72 hr	96 hr
B.t.	S*	96.0	98.2	97.0	96.6	96.0	93.0	93.5	93.3
Mutant 24	S/10	95.8	97.7	95.8	96.3	93.8	92.8	88.3	89.8
	S/50	93.8	97.2	94.3	94.5	82.0	75.5	66.5	69.0
	S/100	93.5	96.9	94.0	92.8	76.8	65.8	58.8	72.8
	S/500	92.8	96.3	93.4	91.8	66.8	60.0	57.2	61.0
	S/1000	88.3	95.3	93.3	90.8	59.8	61.8	58.8	55.5
B.t.	S	85.5	94.3	92.8	89.9	95.8	93.8	92.5	90.8
Mutant 10	S/10	82.0	88.8	89.2	88.3	92.8	91.5	85.8	88.8
	S/50	82.0	80.0	75.3	82.2	77.5	74.8	71.5	68.3
	S/100	77.5	78.9	68.9	80.8	75.8	64.0	60.5	70.5
	S/500	77.5	76.6	67.5	80.3	65.5	60.8	60.6	65.5
	S/1000	76.8	75.3	66.6	79.8	55.5	54.0	58.8	56.5
B.t.	S	76.5	71.9	64.0	79.5	93.5	93.3	90.3	90.3
Mutant 32	S/10	75.8	69.6	60.6	79.1	88.3	91.5	90.3	89.3
	S/50	73.5	69.3	58.9	75.0	77.5	73.0	65.0	73.3
	S/100	71.8	66.7	57.5	72.3	73.5	62.0	58.8	69.5
	S/500	66.8	64.9	57.2	71.2	61.5	60.0	47.9	61.5
	S/1000	65.5	64.9	56.3	70.2	55.0	53.8	52.8	55.0
B.t.	S	61.5	63.6	56.3	68.8	85.5	85.0	90.3	89.5
Wild type	S/10	59.8	58.9	51.2	66.8	82.0	86.8	87.0	89.3
	S/50	59.8	57.3	48.8	60.6	76.5	70.8	63.3	71.5
	S/100	55.5	56.3	47.9	51.9	71.8	55.0	55.0	68.5
	S/500	55.0	56.2	45.2	49.9	59.8	56.3	47.5	60.8
	S/1000	42.3	45.6	44.3	46.3	42.3	55.3	44.3	51.8
Distilled water (untreated treatmenr)		0.0	1.0	1.0	2.3	0.0	1.0	1.0	2.3

Values are average of four replicates. * S = the crude *Bacillus* isolate expressed as a standard concentration or 100 % concentration (=7.5×109 cfu).
**** Percentage un viability = [(mean number of alive eggs in check – mean number of alive eggs in treatment) / mean number of alive nematode in check)] × 100.**
 # Abnormal developmental stages including spherical eggs, egg shell and dead juveniles.

Also, after 48 hr as in the percentage mortality, of the nematode eggs treated with *B.t.* mutant 24, ranged from 95.3 to 98.2 % followed by *B.t.* mutant 10 which ranged from 75.3% to 94.3 % after that the mutant no. 32 which ranged from 64.9 to 71.9 %, however the lowest % mortality occured in case of the wild type which ranged from 63.6 to 45.6 % compared to 1.0 % in untreated control (distilled water).It is worth to notice that, the same trend was observed on the efficiencies of different mutants and the wild type of *B.t.* on % mortalities of *M. incognita* eggs after 72 and 96 hrs. These values ranged from 93.3 – 97 %, 66.6 - 92.8 %, 56.3 - 64 % and 44.3 – 56.3 % after treatment by *B.t.* mutants 24, 10, 32 and the wild type; respectively after 72 hr. But, the previous values scored 90.8 - 96.6 %, 79.8 - 89.9 %, 70.2 - 79.5 % and the wild type 46.3 - 68.8 %; respectively after 96 hr. There were positive relationships between the nematode eggs mortality and each of bacterial concentration and the mutant.

Moreover, the obtained data in Table (3) revealed that all the tested chitinolytic bacteria mutants and the wild type of *Bacillus thuringiensis* caused abnormality effect on the eggs in form of developmental stages which includes spherical eggs, egg shells and dead juveniles after 24, 48, 72 and 96 hr. There were positive relationships between the abnormal developmental stages and each of bacterial concentration and the enzyme production from the mutants

DISCUSSION

The present in- vitro bioassay experiment dealing with effect of a wild type of *B.t.* and its mutants by U.V. irradiation on viability of *M. incognita* eggs after different exposure periods declared that the tested treatments have an antagonistic action and a higher nematicidal activity against eggs of *M. incognita* as compared to distilled water (untreated treatment). The present results also, showed that the highest effect of the previous treatments on the nematode eggs in form of abnormal developmental stages including spherical eggs, egg shell and dead abnormal juveniles. Generally, using *B.t.* mutant no. 24 against *M. incognita* eggs was the most effective, the highest percentages of the egg nematode un viability including achieving the highest percentages of spherical eggs, egg shell and dead juveniles, followed by *B.t.* mutant no. 10, *B.t.* mutant no. 32 as compared to untreated treatment. Contrarily, the least effective treatment was observed with using the wild type of *B.t.*. It can be excepted that this effect may be attributed to the higher percentage activity of chitinase enzyme which is produced at a high amount by the mutant no. 24 (385 %, equal about four folds over the wild type) after U.V. irradiation followed by the mutant no. 10 (350 %, equal about 3.5 times over the wild type) and the mutant 32 which produced about 300% from chitinase enzyme which is equal about 3 times over the wild type production. However, the tested wild type of *B.t.* produced the least percentage of chitinolytic enzyme, chitinase. Such present results are in agreement with those of Osman *et al.* (1988), Sharma and Gomes (1996) and Cronin *et al.* (1997) also who showed that using some strains of *B.t.* suppressed populations and

reduced egg hatchability of *M. javanica Heterodera glycines* and *Globodera rostochiensis*; respectively. A high proportion of the spherical eggs rather than the normal ovoid eggs occurring following treatment with the different dilutions of the wild type and three mutants by using U.V. irradiation, suggest that structural changes occurred to the egg shell. Similar findings were reported by Mercer *et al.* (1992) who indicated that *M. hapla* eggs treated with chitinase produced by *Serratia marcescens* were changed in shape to spherical shape than ovoid. Seine the chitin is known to be a structural element in the egg shell of nematodes this result agree with Wharton (1980) who reported that chitin in nematode eggs is localised in the central core of the egg shell where it forms a 1 µm thick layer, and it is conceivable that the chitinases degraded egg shell chitin to the extent that juveniles were released prematurely. Miller and Sands (1977) and Wharton (1986) they reported that chitinases are hydrolytic enzymes and have been investigated already for their effects on plant – parasitic nematodes. Many of these juveniles were dead, probably because their development had been interrupted and they could not survive in the environment outside the egg. As a result of this study we can explained that using of the wild type of chitinolytic bacteria, *B.t.* and its mutants (namely; 24, 10 and 32) after treating with U.V. irradiation ended chitinase enzyme could have interrupted juvenile development within the egg during embryogenesis, J1 or J2. While, the eggs used in the present experiment were almost entirely embryonated, but in further work, a distinction should be made between released J1 and J2 juveniles. These data are seemingly in agreement with the results obtained by Mercer *et al.* (1992). Moreover, scanning electron micrographs of juvenile cuticle were done by Miller and Sands (1977) showing changes to cuticle morphology. Also, there was discrepancy may be attributable to a difference in the age of the eggs used, i.e. whether embryonic, J1 or J2.

The chitinase effect on the egg shell in the present work may have been lethally damaging to the cells of immature eggs and also, may have damaged juveniles, many of which hatched prematurely. These results agree with those of Mercer *et al.* (1992). As result of this study the worker suggested that using of U.V. irradiation on a wild type of chitinolytic bacteria, *B .thuringiensis* for increasing its activity in producing the chitinase enzyme and its activity against eggs of on the root knot nematode, *M. incognita* is promising as a know aspect of safe control.

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تقييم الطرز البري لبكتيريا باسيلس ثورينجينسيس المحللة للكيتين و ثلاث طفرات منتجة منها بواسطة الأشعة فوق البنفسجية كمبيدات نيماتودية آمنة - علي حيوية بيض نيماتودا تعقد االجذور تحت ظروف المعمل0

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تحت ظروف المعمل (25± 5) تم أستخدم بكتيريا باسيلس ثورينجينسيس المحللة للكيتين (الطرز البري والثلاث طفرات24، 10و 32 بتركيزاتهم المختلفة والمنتجة بأستخدام الأشعة فوق البنفسيجية) علي حيوية بيض نيماتودا تعقد الجذور حيث أظهرت تاثيرًاشديدًاعلى نسبة فقس بيض النيماتودا فتبين أن الطفرة 24 كانت أفضلهم فى تحقيق أعلى نسبة مئوية لموت البيض و تحليل محتوياتة مع تغييرة الى الشكل الكروى دون الشكل البيضاوى الطبيعى يليها فى التأثير الطفرة رقم 10 ثم الطفرة رقم 32 بينما أقلهم فى التأثير على البيض كان الطرز البرى الأصلى للبكتيريا مع ملاحظة إرتباط الزيادة المئوية لموت البيض مع زيادة المعلق البكتيرى حيث كانت نسبة التأثير على موت بيض النيماتودا تتراوح من (90.8 الى 96.6%) , (79.8 الى 89.9%) , (70.2الى 79.5%),(46.3الى 68.8%) وذلك للطفرات الثلاثة والطرز البري على التوالى للطرز البرى والطفرات الثلاثة مقارنة بنسبة 2.3% فى الكنترول الغير معامل (ماء مقطر فقط) بعد 96 ساعة من المعاملة ومن هذا يتضح وجود علاقة طردية بين نسبة الموت لبيض النيماتودا وكلاً من نوع الطفرة ودرجة إنتاجها من إنزيم الكيتنيز وتركيز البكتيريا المستخدمة حيث كان انتاجها من الانزيم 385 %، 350 % و 300% علي التوالي 0