The Use of UV Irradiation to Induce Mutants of *Bacillus thuringiensis* KS3 Spores to Increase Their Activity Against the Larvae of Insect *Ephestia cautella* (Walker) (Pyralidae: Lepidoptera)

Samira Auda Khlaywi Khlood Abid-alelah Mohammed Alkhafaji
Saffa Abid Al- hadi Saleh Safaa Abid Al-rahem Mahmood
Ahmed Fawzi Abass
Ministry of Science and Technology / Directorate of Agricultural Research
Baghdad – Iraq
E_ mail: samira_odaa@yahoo.com

Abstract

This research aimed to produce UV resist mutant of *Bacillus thuringiensis* KS3 as biological agent against important date larvae of insect *Ephestia cautella* (Walker). UV irradiation was used at 30, 60, 90 and 120 second treatment. The UV resists mutants evaluated as bioagents activity against 2nd instars larvae of *Ephestia cautella*. Number of spores reduced twice logarithmic cycles after 30 sec of treatment, another one log cycle reduced at 60 sec treatment. Number of treated bacteria becomes constant after 90 and 120 second. There is no change of the appearance of bacterial mutant colonies that examine at 30 and 60 sec, while differences of bacterial colony shape recognized for 90 and 120 mutants with reduction of crystal toxin protein to 0.6 mg ml^{-1} compared with 2 mg ml^{-1} produced by native bacteria. Mutant isolates produced alpha amylase, protease and hemolysin as well as native bacteria. Mutant isolates had the biological activity even after prolonged UV treatment without any photoprotactant additives.

Key Words: Mutation, UV Irradiation, Crystal Protein and *Bacillus thuringiensis.*

استعمال الأشعة فوق البنفسجية لاستحداث طافرات في سبورات بكتريا *Bacillus thuringiensis* لزيادة فعاليتها ضد يرقات حشرة عثة التين *Ephestia cautella*

سميرة عودة خليوي خلود عبد الاله محمد الخفاجي صفاء عبد الهادي صالح
صفاء عبد الرحيم محمود احمد فوزي عباس
وزارة العلوم والتكنولوجيا / دائرة البحوث الزراعية
بغداد – العراق

الخلاصة

يهدف البحث الحالي الى انتاج طافرات مقاومة باستعمال اللاشعة فوق البنفسجية من العزلة العراقية KS3 *Bacillus thuringiensis* ذات فعالية بايولوجية عالية ضد يرقات حشرات التمور مثل حشرة عثة التين. استخدم التطفير بأشعة فوق البنفسجية وللاوقات 30، 60، 90 و120 ثانية للحصول على طافرات مقاومة، قيمت الطافرات الناتجة من حيث كفاءتها كمبيد حيوي ضد يرقات الطور الثاني لحشرة عثة التين *E. cautella* . انخفض العدد الكلي للسبورات دورتين لوغارتميتين عند التعريض للاشعة فوق البنفسجية و لفترة 30 ثانية وانخفض عدد السبورات الحية لدورة لوغارتمية اخرى عند التعريض لفترة 60 ثانية ولوحظ ثبات العدد البكتيري عند التعريض للفترتين 90 و120 ثانية. لوحظ عدم وجود تغيرات في الشكل المظهري للمستعمرات الطافرة وخلاياها المنتخبة من فترتي التعريض 30 و60 ثانية مقارنة مع مستعمرات ذات شكل مختلف عن البكتريا غير الطافرة وظهور الخلايا بشكل غير طبيعي وانخفاض في تركيز بروتين التوكسين البلوري الى 0.6 ملغم/ مل مقارنة مع 2 ملغم/ مل للخلايا الام. انتجت عزلات الطافرات انزيمات الاميليز الفا والبروتييز والهيمولايسين وهو ماتم ملاحظته بالخلايا غير الطافرة. أظهرت الطافرات بعد تعريضها لفترات أطول للأشعة فوق البنفسجية ودون اضافة مواد حافظة فعالية بايولوجية ضد يرقات الحشرات.

الكلمات المفتاحية: طفرات، التشعيع بالاشعة فوق البنفسجية، البروتين البلوري و *Bacillus thuringiensis*

Introduction

One of the main strategies for nourishing crop production is applying pesticide at field. Many type of traditional chemical pesticide or their residues are very hazardous pollutant for environment, soil microorganisms, water supplier and crop themselves. For overcome this environment impact, biological control is considered as a best alternative to treat pest at field. The insecticidal property of *Bacillus thuringiensis* has been well known to control many important economic insects (Glazer and Nikaido, 1995). Beside their low hazard potential for mammalians makes them highly desirable for application at field. Many biovar of *B. thuringiensis* is well documented to form different types of intracellular parasporal crystal proteins during the sporulation period at the stationary phase of its growth cycle (Quesada- moraga, *et al*., 2004). The crystal structure is composed of three different domains, domain I is responsible for pore formation in the insect gut epithelial membrane, domain II may be for receptor recognition such as amylase receptor or protease receptor and the third domain III may be protect toxin protein from further degradation by endogenous and exogenous protease (Vontersch, *et al*., 1994).

Crystal toxins are very specific to their target insects beside they are non-harmful for human, vertebrate and birds, are easily biodegradable (IPCS/ WHO, 1999). Insects from Lepidoptera, Diptera and Coleoptera had been described to be susceptible for *B. thuringiensis* treatment (Aboussaid, *et al*., 2010; Palma, *et al*., 2012; Fakruddin, *et al*., 2012). More than 95% of biological control agents sales come from the bacterium *B. thuringiensis* with 100 million dollars at year (Kaur, 2002; Snahuja, *et al*., 2011). However, the most drawbacks of *B. thuringiensis* in the field is its short period of effectiveness, which is due to in part, to UV and heat inactivation (Griego and Spence, 1978) that results in shortening its persistence and activity, therefore limits its application at field (Myasnik, *et al*., 2001). UV mutagenesis of Bt may solve the problem of UV susceptibility through using spores that resist to UV light to improve the formulation of Bt (Zhang, *et al*., 2016; Sansinenea and Ortiz, 2015; Zhang, *et al*., 2008). The main scope of this research is to gain UV resist spores of a local isolate of *B. thuringiensis* KS3 through mutagenesis of the bacterium spores by UV without lost the bioactivity against the larvae of *Ephestia cautella* (Walker).

Materials and Methods

The Spore Formation of *Bacillus thuringiensis* KS3

The bacterium isolate of *B. thuringiensis* KS3 was isolated from Iraqi soil, identified for crystal toxin production and its bioactivity was determined against the 2nd instar larvae of *Ephestia cautella* (Al-khafaji, *et al*., 2017). Bt KS3 was maintained on nutrient agar plate at 4℃; this isolate was used in crystal toxin production and spore formation for UV irradiation experiments.

Crystal toxin and spores were produced from *B. thuringiensis* KS3 grown at LB broth (1% tryptone, 0.5% yeast extract and 0.5% NaCl pH 7.2) at 30℃ for 72h using shaker incubator (120 rpm/min). Culture was centrifuged at 10000 rpm for 10 min and bacterial pellet contains crystal/ spores mixture was applied in bioactivity against larvae.

Spore suspension was prepared by centrifugation of bacterial culture at 5000 rpm for 10 min, sediment biomass was washed twice time by sterilized distilled water with repeat the centrifugation step. Spores sediment was suspended and heat treated for 15 min at 80 ℃ to activate spores, killed the residual vegetative cell then spore suspension was ready for UV treatment (Federici, *et al*., 2006; Somerville and Hazel, 1975). While, cell free

supernatant was centrifuged at 10000 rpm /min for 10 min and precipitated crystal protein was suspended using 0.5 N NaOH. Crystal protein concentration was estimated by adopting the equation 1OD at 280 is equivalent to 1 mg /ml protein using nanodrop 2000 (Thermo Scientific).

Rearing of *Ephestia cautella* (Walker) Larvae and Toxicity Test

Second instar larvae of *E. cautella* (Walker) were used in bioactivity tests, they maintained at a laboratory of (Integrate Pest Management Center/ Biological Control Center) Agricultural Research Directorate/ Ministry of Science and Technology. Insect larvae were reared at artificial diet composed of: Crushed wheat 81%; 12% glycerin; 6% date syrup and 1% dry yeast, and were kept at identical condition of temperature and relative humidity (26°C±2; 50-60% r. h.) (AL-taweel, *et al*., 1989).

All treatments were done in three replicates and each replicate contains 10 larvae in 2nd instar of *E. cautella* . Sterile distilled water was used for control treatment.

Spores/ crystal protein mixtures were collected from 4 ml culture as mentioned above, mixed well with only 5 gram of larval medium then 10 of 2nd instar larvae were placed on the surface of the medium, incubated for 72 h with observation. The numbers of dead larvae were recorded and then the percentages of mortalities were calculated. Mortality percentages were modified according to Abbott (1925) for calculates the percentage of pesticide efficacy.

Ultra Violet Irradiation Experiment

Traditional UV irradiation protocol was modified using spectrophotometer to mutate *B. thuringiensis* KS3 spores. Briefly, One ml of spore suspension (4×10^7) was UV irradiated using quartz cuvate 1cm; UV was applied at 254 nm wave length through the visible/ UV spectrophotometer (Specord 250, Analytikjena). Each sample was put in the pre-sterilized quartz cuvate that placed in its position in the spectrophotometer. After permanence of surrounding environment, UV irradiation was used for 30, 60, 90 and 120 second using time mode program; precaution was taken to avoid visible light exposure. Living spores that resist to UV 254nm were counted using serial dilution methods, three replica were plated from dilution 10^4,10^5, 10^6 and 10^7 over nutrient agar and incubated at 30 ℃ for 18 h. Colonies forming unit were counted and recorded, ten out UV resist mutants were randomized picked up and maintained on nutrient agar for further analysis (Griego and Spence, 1978).

Characteristics of UV Resist *Bacillus thuringiensis* KS3

Several characteristics of UV resist mutants colonies were examined and compared with non- mutant bacterium. Their shape, appearance on nutrient agar, microscopic examination for gram stained smear of bacteria and spore formation were studied. Also, UV resisted bacteria were screened for alfa-amylase, hemolysin and protease enzymes production.

Crystal protein was extracted and its concentration was determined from mutant isolates as well as native bacterium grown in LB broth as mentioned above.

Bioactivity of UV resist mutant of *B. thuringiensis* KS3 against 2nd instar larvae of *E. cautella* was tested and compared with non- irradiated bacterium.

The Persistant of Mutants *Bacillus thuringiensis* KS3 at Elevated UV Exposure

Spores / crystal mixtures prepared from UV resist and the native bacterium were exposed to elevated doses of UV with time reached to 10, 20 and 30 min using the standard protocol as described by (Benoit, *et al*., 1990) Briefly, spores/ crystal protein mixtures was collected by centrifugation at 10000 rpm for 10

min then suspended with distal water, only 1 ml of suspension was withdrawn in open disposable Petri dish resting below a germicidal lamp from Raytech industries , Inc with a constant output at a wave length emitted at 250 nm for different periods (10, 20 and 30 min). Immediately after treatment, spores/ crystal mixtures were applied for bioactivity against 2nd instar larvae of *E. cautella* as described above and compared with native bacterium.

Statistical Analysis

The experiment was designed using Complete Random Design (CRD) and means were separated using least significant difference (LSD) (P = 0.05) (Steel and Torrie, 1960). Data were analyzed using genestat statistical analysis Software.

Results and Discussions

The percentage of spore survival when irradiated at elevated dose of UV 245nm was presented in Figure (1) which showed that more than 50% of the spores were inactivated at the first exposure (30 sec) while, elevated UV dose had a little effects on the spore survival in which the count decreased one logarithmic cycle from 12 $\times 10^5$ when treated for 30 sec to reach 4×10^4 after treated for 60, 90 and 120 sec.

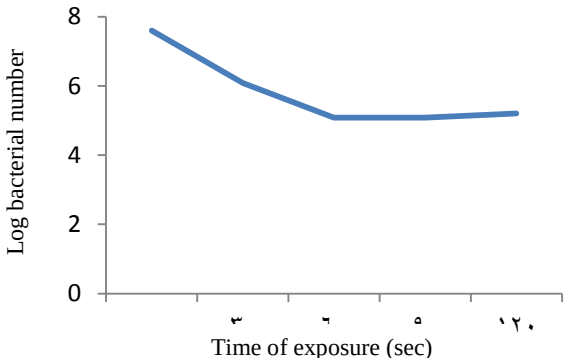

Figure (1) Survival Curve of *Bacillus thuringiensis* KS3 Spores at Different Dose of UV 245nm.

The evolution of UV resist mutant isolates at 30 and 60 sec treatment showed no changes in their shape, color or margin nutrient agar on medium, while colonies with two shapes (rough and grey) and (ordinary and white) would developed from spores treated for 90 and 120 sec. Microscopic examination showed no changes in the shape of cells except for rough grey mutant that gave smaller bacilli under light microscope

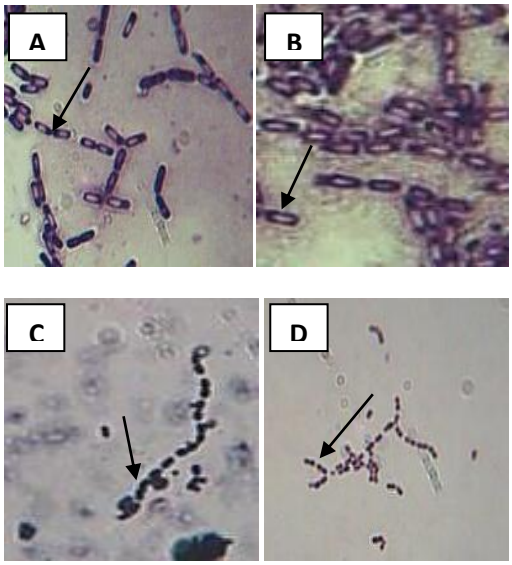

Figure (2) Microscopic Examination for UV Mutant of *Bacillus thuringiensis* KS3. A= non- mutant Cell, B= UV Mutant Cell from 30 Sec Treatment, C= UV Mutant Cell from Grey Colony (90 Sec Treatment), D= UV Mutant Cell from Grey Colony (120 Sec Treatment).

Despite that dormant spores are relatively more resist to UV light exposure between 200-400 nm than the vegetative bacterial cells, *B. thuringiensis* spores are more sensitive to UV treatment comparing with those produced by related species *B. cereus* as mentioned by (Griego and Spence, 1978). UV susceptibility of *B. thuringiensis* spores might due to an alter conformation or strand break of the DNA that dependent on many factors in which the most important factor is the species that spores come from Slieman and Nicholson (2000); (Veligura *et al*., 2005) or even among subspecies as mentioned by

(Myasnik , *et al*., 2001) who found that isolates of *B. thuringiensis* kurstaki were more susceptible to UV- b range than Bt israelensis. On the other hand, (Benoit, *et al*., 1990) explained that the

existence of many plasmids DNA in *B. thuringiensis* cells may had express codes for pro- toxin, that influence UV resistance. Also, the plasmid DNA may possibly express for a variety of small acid soluble proteins that interfere with UV resistant, also the quantity of dipicolonic acid deposited in the coat of spore which may contributed to UV resist mechanisms. Another interpretation advised that a special kind of prophage may induce during UV irradiation, the transduction of DNA may become lethal to spores of *B. thuringiensis* aizawi (Inal, *et al*., 1990). Other external factors play a critical role in the inactivation of spores by UV exposure such as photoprotectant material, time of exposure and intensity of UV irradiation (Cohen, *et al*., 1991). The nutritional requirement of UV irradiated spores would alter in which the spores become very fastidious in their nutritional demands and more susceptible to changes in environmental factors such as oxygene, temperature and pH. Results showed that hydrolytic enzymes (Amylase, Protease and Hemolysin Enzymes) were all secreted from UV resist mutant of *B. thuringiensis* KS3 as well as the native isolate (Figure 3).

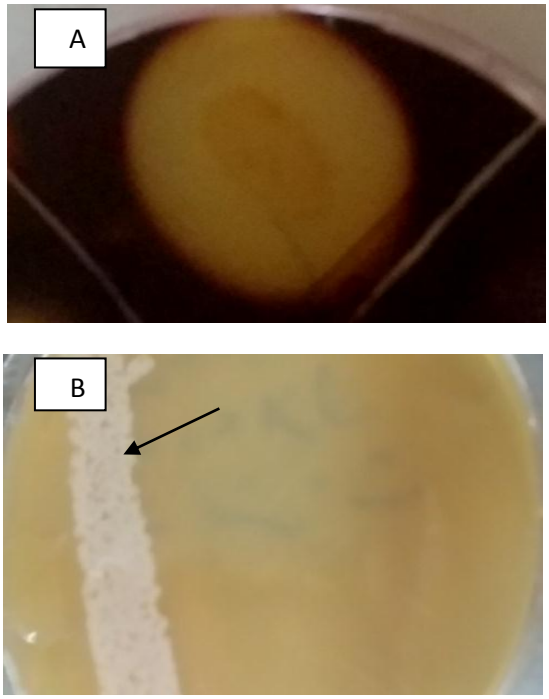

Figure (3) The Production of Hydrolytic Enzymes from KS3 Exposed for 90 Sec UV Mutant Isolate. A= Amylase Enzyme; B= Protease Enzyme.

Hydrolytic enzymes have distinctive importance in bacterial persistence in their environment in which amylase enzyme play a vital role in carbohydrate hydrolysis to produce glucose as the main source of carbon. While, proteases produced by *B. thuringiensis* kurstaki strains have many characteristic efficacy. They possess a role in proteolysis of different protein to get nitrogenous for their survival, sporulation, protein turnover and crystal toxin activation. Other reports recorded the secretion of protease and amylase from *B. thuringiensis* (Smitha, *et al*., 2013; Smitha, *et al*., 2015).Present estimation of crystal protein indicated that mutant isolates picked up from 30, 60 and 90 sec produced 3, 3.5 and 3.7 mg ml^{-1} of crystal protein respectively in comparison with 2.5 mg ml^{-1} produced from the native *B. thuringiensis* KS3. On the other hand, rough grey mutant selected from culture treated for 90 and 120 sec produced less crystal protein concentration 1.7 and 0.5 mg ml^{-1} respectively. This may due to the deleterious effect of UV on the DNA of cry genes themselves or the

genes responsible for regulation systems; UV irradiation might cause many breaks in DNA strand implication in A_A dimer and many strand super coiling (Rastogi, *et al.*, 2010).

Bioactivity experiment of UV resist mutants against the 2nd instar larvae of *E. cautella* revealed that single dose of spores/ crystal protein mixture collected from different white color UV resist mutants had high lethal capability reached to 93% for UV resist mutant designed as KS3 exposed for 90 sec. Statistical analysis showed significant differences in percentage of mortality between the native *B. thuringiensis* KS3 and selected white color UV resist mutants (Table 1).

While, insecticidal activity of grey color UV resist mutants decreased in comparison with native bacterium with no statistical differences. Only one UV resist mutant designed as KS3B exposed for 120 sec didn't give any lethal activity against larvae.

Table (1) The Bioagents Activity of UV Resist Mutants of *Bacillus thuringiensis* KS3 Against Insect Larvae of *Ephestia cautella*

Treatment	Crystal Protein Concentration mg ml^{-1}	Mortality %	Percentage of Isolate Efficacy
Control	-	10	-
Non-irradiated KS3	2.5	66.7	63
KS3: 30 sec	3	83.3	81.44
KS3: 60 sec	3.5	80	77.7
KS3: 90 sec	3.7	93	92.2
KS3A: 90 sec	1.7	56.7	51.4
KS3A: 120 sec	1.5	56.7	51.4
KS3B: 120 sec	0.5	Nil	Nil
		LSD= 15.93	

From the above result, the mutant isolate designed as Bt KS3 exposed for 90 sec chose for the experiment of resistance at elevated exposure dose of UV. Results outlined in (Table 2) indicated that mixture of spores/ crystal protein produced from Bt KS3 exposed for 90 sec resisted elevate exposure of UV 254nm for 1, 10, 20 and 30 min without detectable loss of their insecticidal activity aginst 2nd instar larvae of *E. cautella*. Decreased in bioactivity of KS3 exposed for 90 sec mutant reached to 16.7% after 30 minute of UV exposure in comparison with 20% decreased in bioactivity of native isolate. Also data revealed that extensive exposure to UV irradiation did not cause further loss of bioactivity for both native bacteria and the mutant isolate KS3 exposed for 90 sec. This results are in accordance with the recent survival curve of UV irradiated Bt KS3 in which the decline of logarithmic number of viable count become constant after 60 second of UV treatment with development of mutated colony that lacking crystal protein or decreased in the production value as previously mentioned (Table 1). This may due to the repair system which leads to repair DNA breaks and to obviate extra mutations in DNA.

Table (2) Percentage of Decreased Bioagents Activity for *Bacillus thuringiensis* KS3 at UV Irradiation.

Treatment	Exposure Time (Minute)	Mortality %	Percentage of Isolate Efficacy
Control	0	9.67	-
	1	10.00	-
	10	10.00	-
	20	8.33	-
	30	8.67	-
Native *B. thuringiensis* KS3	0	80.00	77.85
	1	75.00	72.22
	10	67.00	63.33
	20	60.00	56.36
	30	60.00	56.20
UV resist mutant *B. thuringiensis* KS3:90	0	76.70	74.17
	1	70.00	66.70
	10	60.00	55.60
	20	60.00	56.36
	30	60.00	56.20

LSD Value Below the 5% for the Interaction Between Treatment and Exposure Time = 5.299

The blackening appearance of the larvae as a result of treatment with the UV resist KS3 exposed for 90 sec was an indicative for UV bacterial mutant bioactivity, blackening of the larvae after ingestion of spore/ crystal mixture of *B. thuringiensis* is considered as the first insight of insecticidal activity of this bacterium (Figure4).

Figure (4) Bioagents Activity of Spore/ Crystal Mixture of UV Resist *Bacillus* Against *thuringiensis* KS3 Exposed for 90 Sec Larvae of *Ephestia cautella*.

A- Larvae treated with spore/ crystal mixture of UV resist *Bacillus thuringiensis* KS3 exposed for 90 sec

B- non- treated larvae

Conclusions

A simple and reproducible technique could adopt in bacterial UV irradiation experiment without the need for extra equipment or requirements to induce UV resist mutation in *B. thuringiensis* KS3. The resist mutant isolate designed as KS3 exposed for 90 sec had high insecticidal activity as well as the production of hydrolytic enzymes which may contribute to the insecticidal activity and the bacterial persistence in environment. The improvement of UV resist spores of insecticidal bacterium *B. thuringiensis* KS3 is of a big value due to the high susceptibility of bacterium to UV light exposure which resulted in high degradation of spores and crystal protein.

Recommendations

The use of UV resists *B. thuringiensis* isolate in formulation of pesticide after many experiments in formulation of *B. thuringiensis* KS3, production, shelf life of the product and the way of application at field will have a high interest to assess the product economic impact and persist in field after application.

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