J. of Plant Protection and Pathology, Vol. 1 (4): 179 - 187, 2010

ASYNCHRONISM EFFECT OF *Bacillus thuringiensis* CRY1AC ON GROWTH AND DEVELOPMENT OF *Helicoverpa armigera* (HUBNER) STRAINS

Moussa, S. M. and A. F. E. Afsah
Plant Protection Research Institute, ARC, Dokki, Giza, Egypt

ABSTRACT

American bollworm, *Helicoverpa armigera* (Hubner) was selected for eleven subsequent generations with purified toxin Cry1Ac *Bacillus thuringiensis* under laboratory condition. The resistance ratio (RR) reached 56.73 fold-resistances. The larval development of resistant, susceptible and their F_1 generation were significantly varied while reared on normal artificial diet. The growth rate of resistant strain was slower compared with susceptible and F_1 strains. The present study shows asynchronism effect for Cry1Ac resistant strain in relation with susceptible and F1 strains. The overlapping distribution between the adult peak of resistant and the adult peak of susceptible were investigated and found to be apart from each other. Subsequently, the probability of random mating among resistant individuals would be more likely to be happened that helps insect resistance development within the population. Thus, the current study would recommend reviewing the implementation of refuge strategy (non Bt cultivated area) while adopting Bt modified crops.

INTRODUCTION

The cotton bollworm, *H. armigera* (Hübner) (Lepidoptera: Noctuidae) is considered a key pest among field crops. It has become a major threat pest to cotton and many other vegetable crops in many parts of the world. Bt cotton plants are genetically modified to confer insecticidal toxins from the *B. thuringiensis* (Bt) Berliner bacterium against several Lepidopteran pests (Jenkins *et al.*, 1993 and Shelton *et al.*, 2002). The worldwide area of Bt cotton has increased to more than five million hectares from 2002 to 2008. China, India and Australia had the major Bt cotton cultivated area. Resistance to Cry1Ac in *H. armigera* is controlled by one or a few loci with partially dominant inheritance with non maternal or sex-linkage; Moussa and Gujar (2005). Selection for resistance to Bt does not always cause the capability of resistant insects to survive on their natural host plants; Tabashnik *et al.*, (2003), indicating that selection to formulated Bt toxins differ from that on modified plants and that further factors may contribute to insect mortality. Selection pressure of *Pictinophora gossypiella* larvae to Bt Cry1Ac under laboratory condition did not produce Bt resistant larvae that survive on Bt cotton bolls. On the other hand, Emergence, mating, and egg hatch were not significantly affected by selection, but fecundity was significantly reduced; Henneberry *et al.*, (2001). This would suggest a cost of resistance similar to those reported for other *P. gossypiella* selected strains; Carrière *et al.*,

(2001). Recently, several studies have been shown the importance of refuge strategy tactic as the most appropriate approach in order to delay Bt Cry1Ac resistance development under the field condition (Tabashnik *et al.*, 2003). Theoretically, if the growth rate of the resistant strain is slower than the growth rate of susceptible strain, the probability of random mating between the resistant individuals and susceptible individuals could reduce which may cause resistance evolution among resistant population (Liu *et al.*, 1999 and Tabashnik *et al.*, 2001). The current study is therefore deals with asynchrony effect of Bt Cry1Ac toxin on growth and development of *H. armigera* resistant, susceptible strains and their F_1 generation.

MATERIALS AND METHODS

Insects

H. armigera strains were reared at 27 ± 1°C with 70 - 80% relative humidity under a L14:D10 photoperiod in the laboratory. The susceptible strain was reared for more than 10 subsequent generations under the above mentioned laboratory condition using a normal chickpea semi synthetic diet described by Rajagopal *et al.*, (2009). In order to generate a resistant strain, the larvae were brought from field and was subjected to selection pressure for further generations by feeding the neonates of each generation (12hr old) on the earlier mentioned diet incorporated Cry1Ac.

***B. thuringiensis* toxin preparation**

Cry1Ac toxin was produced from the HD-73 strain of *B. thuringiensis spp*. *Kurstaki* provided by Dr. Donald Dean laboratory, Ohio State University, USA. The HD-73 strain was grown in LB medium (tryptone 1%, yeast extract 0.5%, NaCl 1%, pH 7–7.2) for 3–4 days at 28°C, at which time the separation of crystals from spores was observed under a microscope. The sporulation was centrifuged at 4°C at 150 rpm for 10 min. The bacterial cells were harvested by centrifugation. The pellet was resuspended in lysis buffer (50mM Tris, PH 8.0; 50mM EDTA; 15% sucrose; 10 µg /ml of lysozyme). Cry1Ac protoxin was purified according to methods previously described (Audtho *et al.*, 1999). The purified toxin samples were determined by 10% SDS-PAGE and were pooled, quantified by the method described by Bradford, (1976) and stored at ‒20°C.

Selection pressure protocol and bioassay:

In order to develop the resistance in *H. armigera* to Cry1Ac toxin, the field population larvae were brought to the lab and were fed on semi artificial diet incorporating sub lethal dose of Cry1Ac (0.035µg/g diet). This dose was used as a selection pressure concentration that decided from our previous experiments; Moussa and Gujar, (2005). The Cry1Ac purified toxin was mixed well with diet using sterilized mortar plate. Three to four grams of the mixed diet were kept in a single plastic plate (10 ml). A number of 10-15 neonates were released into each plate. Every generation, a total number of 500 of *H. armigera* neonates were exposed to the above semi synthetic diet incorporated Cry1Ac toxin for four days selection pressure. The survived individuals were transferred into a fresh and normal diet. The selection pressure was continued for eleven subsequent generations. Bioassay

experiments were performed to determine the level of resistance to Cry1Ac in the Cry1Ac selected strain every generation. The LC_{50}, slope, and standard error were estimated and recorded 96h after treatment.

Development of *H. armigera* strains

In order to study the various aspects in development of *H. armigera* strains, the newly emerged adults were paired in a plastic cup box with 10% honey solution as a source of food. Each cup was covered with one layer of tissue paper between the lid and the cup. The sheets of tissue paper carrying eggs in each strain were transferred into a cleaned plastic cup (100ml). Eggs were counted and the egg sheets were placed in an environmental chamber. A number of 90 eggs laid by each strain were taken and divided into three replicates. Each replicates was contained 30 eggs. This number was used as a starter culture for further experiments done under the following heads:

Hatchability and viability measurements:

In order to compare the average number and percentage of hatchability and viability of all the strains. The hatchability of eggs was monitoring every 0-6 days from egg's laid. When all viable eggs had hatched, the neonates were counted and recorded to estimate the average number and hatching rate.

Larval and pupal development:

The newly hatched of the larvae of susceptible, resistant and their F_1 generation were taken and fed with artificial diet without Cry1Ac toxin until they either died or pupated. The larvae were considered dead if they were unable to move in a coordinated manner when prodded with a blunt probe. The dead larvae and pupae were recorded and analyzed. The average numbers and percentage of larval mortality, pupation and adult's emergencies were all recorded. In addition to that, the emergence of adults was recorded daily.

Life history study:

In order to study their life history, the same number of the above mentioned eggs of each strain was incubated. The eggs were observed daily fin order to calculate the incubation time. The newly hatched neonates were reared individually. The average of larval and pupation period and larval and pupal body weight were recorded. Throughout the experiments, larval weights were individually recorded on the 8^{th} day and then, each larva was transferred into one cleaned plastic cup till its pupation time. Within 24h of pupation, pupae were weighed and sorted by gender. The longevity of adult (males and females) was all measured and recorded. The observations were taken 24h intervals throughout the experiments.

Sex ratio: Survival that had already pupated was sexed and the pupae were kept individually till emergence and the adult longivity was recorded. On the other hand, the larvae that had not pupated were put in plastic cups with little and fresh diet to allow larvae to pupate. The cups were checked daily and pupae were removed, sexed and weighed. The sex ratio percentage was calculated on the basis of emerged males and females.

Statistical analysis

The LC_{50}s were estimated using EPA probit analysis program (version 1.5). The slope, Standard error and 95% confidence limits were estimated. The resistance ratio was determined by dividing the LC_{50} of resistant strain by the LC_{50} of susceptible strain. The eggs laid, larval weights, larval duration period, pupal duration in all the strain were analyzed using analysis of variance (ANOVA). Larval surviving percentage, eggs hatchability percentage, pupation and emergency percentage were also analyzed by ANOVA. The variation between the means were investigated and analyzed using the least significant difference test (LSD) Snedecor and Cochran (1967). The relative growth rate (%) of each strain (Resistant and F_1 population) was calculated as a mean weight on resistant strain divided by the mean weight in susceptible strain X 100%. The relative growth rate in susceptible strain was considered as 100%.

RESULTS AND DISCUSSION

After 11 generation of selection pressure, the resistance ratio in the selected strain reached to 56.73 fold compared with the susceptible strain. The LC_{50} of resistant selected strain increased from 0.116 in F_1 generation to 3.404 µg/g artificial diet in F_{11} generation. The rate of resistance development ranged from 0.00024 to 0.0025 Table (1). This result shows the ability of *H. armigera* to evolve the resistance to Cry1Ac toxin when it exposes to selection pressure under laboratory condition; Moussa and Gujar (2005).

Table (1): Resistance development in *H. armigera* selected strain till the 11th generation.

Serial Number	Generation	LC_{50} [1]	95% fiducial limits	Slope ±Se	N [2]	RR [3]	R [4]
1.	F1 (selected)	0.116	0.018 - 0.294	0.562 ± 0.164	180	1.90	
2.	F2 (selected)	0.288	0.047 - 1.332	0.449 ± 0.162	180	4.8	0.0022
3.	F3 (selected)	0.792	1.224 - 2.600	1.912 ± 0.351	180	13.2	0.0025
4.	F4 (selected)	1.137	0.834 - 1.432	2.419 ± 0.451	180	18.95	0.0021
5.	F5 (selected)	1.255	0.876 - 1.635	2.117 ± 0.397	180	20.91	0.00024
6.	F6 (selected)	1.480	1.018 - 1.904	2.346 ± 0.436	180	24.67	0.00042
7.	F7 (selected)	1.712	1.186 - 2.215	2.209 ± 0.439	180	28.53	0.00040
8.	F8 (selected)	2.260	1.741 - 2.764	2.795 ± 0.583	180	37.67	0.00071
9.	F9 (selected)	2.526	4.968- 13.939	2.380 ± 0.514	180	42.10	0.00032
10.	F10 (selected)	2.928	2.189 - 3.563	2.990 ± 0.645	180	48.80	0.00041
11.	F11 (selected)	3.404	2.591 - 4.210	2.839 ± 0.678	180	56.73	0.00040

[1] Cry1Ac (µg/g)
[2] Number of larvae used in bioassay, including control
[3] Resistance ratio (LC_{50} of resistant strain/LC_{50} of susceptible strain)
[4] Rate of rising in resistance (log final RR/ Initial RR/ n)

As shown in Table (2), the data analysis of the biological parameters ranged from highly significant in larval mortality, pupation, emergency and sex ratio to significant in hatchability average. The average number of hatchability and larval mortality of susceptible strain was highly significant compared with resistant strain and F_1 population. Whereas the average

number of hatchability in resistant and F_1 strains was significantly similar but the degree of significance in larval mortality between resistant and F_1 strains was less (10.33 and 12.67). Contractedly, the average of pupation, emergency and sex ratio of resistant strain shows highly significant compared with susceptible and F_1 strain but the degree of significance between susceptible and F_1 population was slightly varied. On the other hand, the hatchability and larval mortality percentages in susceptible strain were higher than resistant and F_1 populations with values of 75.56-73.33%-48.44-57.57% respectively. But the pupation and emergency percentages were higher in resistant strain (51.56% and 93.93%) compared with the same in F_1 and susceptible strain (42.42-85.71%, 38.24- 88.46%) respectively. Also, the results show lesser pupation and emergency percentages in susceptible strain (38.23% and 88.46%). Overall, the data shows higher percentage number of males than the percentage number of females in all the strains, Table (2). The F_1 population had less number of females compared with susceptible and resistant strains. Despite that the hatchability percentage in susceptible strain was higher than other strains, it provide high larval mortality percentage than resistant and F_1 strains. In the life span experiment, the data analysis shows highly significant variation between all the strains in their developmental period but the variation of males longevity period was slightly significant and insignificant with females in all tested strains. The selected resistant strain had a longer larval, pupal and adult period compared with susceptible and F_1 strains.

Table (2): Biological parameters of resistant, susceptible and F1 population reread on normal artificial diet.

Factor / Strain	Hatchability		Larval mortality		Pupation		Emergency		Average number		Sex ratio %	
	Average	%	Average	%	Average	%	Average	%	male	Female	Male	Female
Suscep-tible	22.67 a	75.56	14.00 a	61.76	8.67 b	38.24	7.67 b	88.46	5.00 c	2.67 a	65.22	34.78
Resistant	21.33a b	71.11	10.33 c	48.44	11.00 a	51.56	10.33 a	93.93	7.33 a	3.00 a	70.97	29.03
F1	22.00 b	73.33	12.67 b	57.57	9.33 b	42.42	8.00 b	85.71	6.33 b	1.67 b	79.17	20.83
F. test	*		**		**		**		**	**		
L.S.D 5%	0.93		0.93		0.93		1.31		0.755	0.76		
1%	1.53		1.53		1.534		2.17		1.253	1.25		

Concerning to the larval and pupal body weight, among the three strains tested, the data analysis varied from highly significant and non significant. Also, the larval and pupal weight in susceptible strain was higher than F_1 and resistant strains. Overall, the data analysis shows that the life span of resistant strain was longer than susceptible and F_1 strain, Table (3). Also, Data observed that F_1 strain had intermediated developmental periods between susceptible and resistant strains. Additionally, the incubation period of eggs in all tested strains was not varied from strain to another. But the relative growth rate of F_1 population was slower than susceptible strain and

the growth rate of resistant strain was intermediate between the susceptible and F_1 population (Fig. 1). The results obtained here confirm the previous data published by Liu *et al.*, (1999), Tabashnik *et al.*, (2003). Bird and Akhurst (2005) found the fitness cost of resistance in *H. armigera* occurred on transgenic cotton, but was not evident on artificial diet. But Liang *et al.*, (2008) found that the fitness cost of resistance to Cry1Ac occurred not only on non-Bt cotton, but also on artificial diet. Similarly, in the current study, our finding indicates that there is a fitness cost observed in resistant population compared with susceptible and F_1 strains while feeding all populations on normal artificial diet. Liu *et al.*, (2001) found that the fecundity reduced, larval development delayed and pupal weight decreased in resistant strain of pink bollworm, *Pectinophora gossypiella*. Similarly, the differences between resistant, F_1 and susceptible strains of *H. armigera* while reared on artificial diets were measured using the following factors: fecundity, larval development, pupal weight and pupal duration period. Also, we found the relative growth rate associated with the resistance population reared on artificial diet reduced compared with the susceptible and F_1 strains.

Table (3): Life history of resistant, susceptible and F1 strains of *H. armigera* along with their larval development and pupal weight.

Factor / Strain	Eggs	Larval stage		Pupal stage			Adult longevity (d)	
					Weight (mg)			
	Inc. period (d)	Period (t)	Weight (mg)	Period (d)	Male	Female	Male	Female
Susceptible	3	14.04 b	0.23 a	10.64 b	0.28	0.32	9.90 ab	9.75
Resistant	3	15.19 a	0.16 b	13.13 a	0.27	0.31	11.47 a	10.15
F1	3	13.58 b	0.22 b	10.62 b	0.27	0.31	8.91 a	10.04
F. test		**	**	**	N.S.	N.S.	*	N. S.
L.S.D 5%		**0.598**	**0.077**	**0.668**	**0.015**	**0.025**	**1.581**	**1.949**
1%		**0.992**	**0.128**	**1.108**	**0.025**	**0.042**	**2.622**	**3.233**

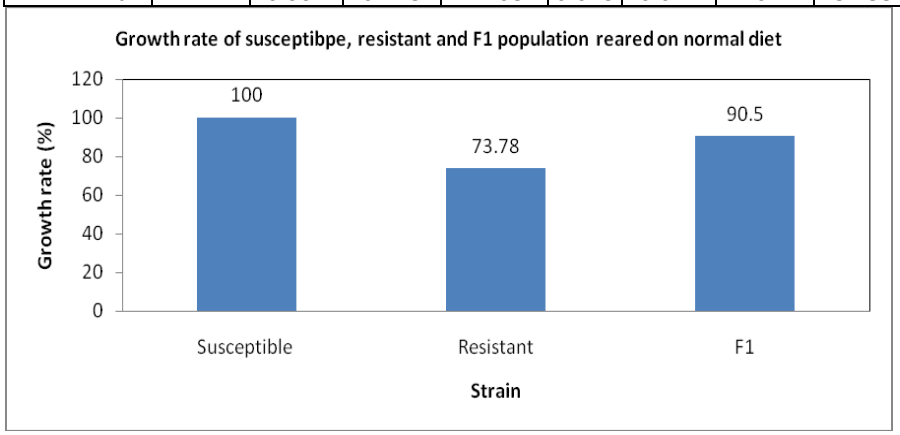

Fig. 1: Growth rate of susceptible, resistant, and F1 population reared on normal diet.

This result is in accordance with the data obtained by Liang *et al.*, (2008) who reported that the fitness costs associated with resistant on artificial diet and the relative fitness in resistant of *H. armigera* reduced as the resistance levels increased. It is very well know that the main goal for delaying insect resistance to transgenic Bt plants is to cultivate refuges of host plants that do not produce Bt toxins. This potentially delays the evolution of insect resistance to Bt crops by providing susceptible insects for mating with resistant insects. But our laboratory data with a cotton bollworm, *H. armigera*, disagree with an important assumption of the refuge strategy. We found that a resistant strain of larvae on normal diet takes longer to develop than susceptible and F_1 larvae, Table (3). Under field condition, the slower growth of resistant larvae on Bt cotton compared with susceptible larvae on non-Bt cotton could ease the possibility of mating between susceptible and resistant insects (Liu *et al.*, 2001). As shown in figure (2) the peak adult of resistant population does not meet the peak adult of susceptible population. Similarly, if the same situation occurred in the Bt field that has resistant individuals and non Bt field that has susceptible individuals, the possibility of mating among resistant individuals would increase and the probability of delaying the resistance would reduce.

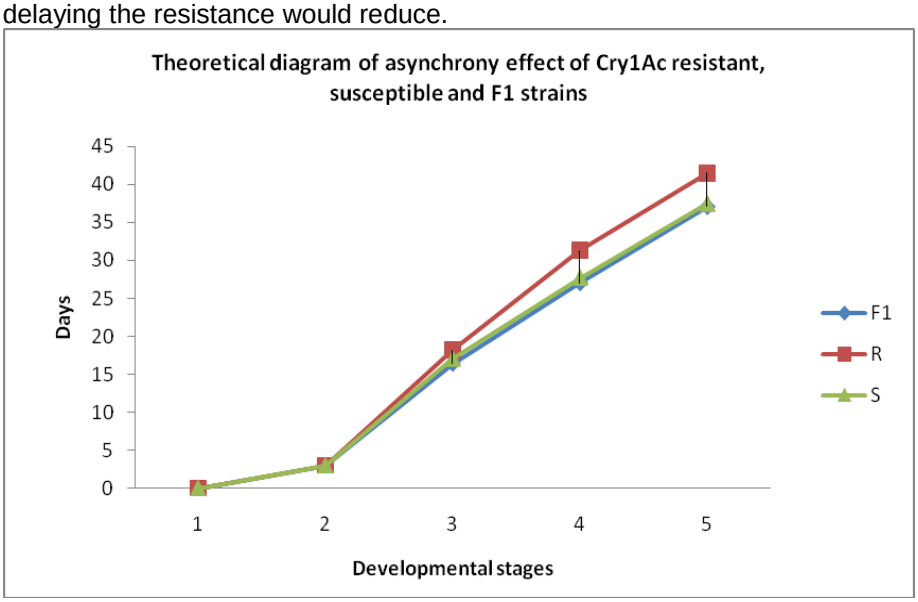

Fig. 2: Asynchrony effect of Cry1Ac resistant, susceptible and F1 strains reared on normal diet. 1: Represents egg laid,1-2: incubation time of eggs, 2-3: Represents larval period, 3-4: Represents pupal period, 4-5: represents adult stage period till death. 1-5: represents the life span period, F1: Represents F1 strain reared on normal diet, R: Represents resistant strain, S: represents susceptible strain.

If the overlapping in the distribution of emergence of susceptible and resistant *H. armigera* adults is decrease, this resulting nonrandom mating caused by developmental asynchrony. Thus, if the resistant adults are more likely to mate with each other instead of with susceptible adults, resistance could develop faster. This developmental asynchrony of resistant strain favors non-random mating that could reduce the expected benefits of the refuge strategy which is shown in Figure 2. Thus, the current study is therefore an important piece of research in order to reconsider the implementation of refuge strategy while adopting Bt crop under the field condition.

REFERENCES

Audtho M., Valaitis A. P., Alzate O. and Dean D. H. 1999. Production of chymotrypsin-resistant *Bacillus thuringiensis* Cry2Aa1 delta-endotoxin by protein engineering, Appl Environ Microbiol. 65(10):4601-4605.

Bird L. J. and Akhurst R. J. 2005. Fitness of Cry1A-resistnt and susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on transgenic cotton with reduced levels of Cry1Ac. J. Econ. Entomol. 98, 1311–1319.

Bradford M .1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding *Anal Biochem* 72: 248-254.

Carriére Y. and Tabashnik B. E. 2001. Reversing insect adaptation to transgenic insecticidal plants. Proc. R. Soc. Lond. Ser. B 268, 1475–1480.

Henneberry T. J., L. F. Jech, and T. de la Torre. 2001. Effects of transgenic cotton on mortality and development of pink bollworm (Lepidoptera: Gelechiidae) larvae. Southwest. Entomol 2001. 26:115–128.

Jenkins J. N., Parrott W. L., Mecarty J. C., Callahan F. E. , Berberich SA and Deaton W. R. 1993. Growth and survival of *Heliothis virescens* (Lepidoptera: Noctuidae) on transgenic cotton containing a truncated form of the delta endotoxin gene from *Bacillus thuringiensis*. J. of Econ. Entomol. 86: 182–185.

Liang G. M., Wu K. M., Yu H. K., Li K. K., Feng X., Guo Y.Y. , 2008. Changes of inheritance mode and fitness in *Helicoverpa armigera* (Hu¨bner) (Lepidoptera: Noctuidae) along with its resistance evolution to Cry1Ac toxin, J. Invert Pathol. 97:142-149.

Liu Y. B., Tabashnik B. E., Dennehy T. J., Patin A. L. and Bartlett A. C.1999. Development time and resistance to *B. thuringiensis* crops. *Nature* (Lond.) 400: 519.

Liu Y. B., Tabashnik B. E., Dennehy T. J., Patin A.L., Sims M. A., Meyer S. K. and Carrie`re Y., 2001. Effects of Bt cotton and Cry1Ac toxin on survival and development of pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 94, 1237–1242.

Moussa S. and G. T. Gujar. 2005. Inheritance of Cry1Ac resistance in the transgenic Bt cotton selected strain of the cotton bollworm, *Helicoverpa armigera* (Hubner), *Egypt. J. Agric Res*., 83 (3), 1061- 1078.

Rajagopal R., N. Arora, S. Sivakumar; G. V nagarjun, S. A. R. Nimbalkar and R. K. Bhatnagar. 2009. Resistance of *Helicoverpa armigera* to Cry1Ac toxin from *Bacillus thuringiensis* is due to improper processing of the protoxin, *Biochem. J.*; 419:309–316.

Shelton A. M., Zhao J. Z. and Roush R. T. 2002. Economic, ecological, food safety and social consequences of the deployment of Bt transgenic Plants. Annual Review of Entomology 47: 845–881.

Snedecor, G. W. and Cochran, W. G. 1967: Statistical Methods, Sixth edition, Iowa State College Press, Ames. Iowa, USA.

Tabashnik B. E, A. Patin, L., Dennehy T. J., Liu, Y. B., Carrière, Y., Sims M. A., and Antill L. 2001. Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. *Proc. Natl. Acad. Sci. U.S.A.* 97:12980-12984.

Tabashnik B. E., Carrie`re Y., Dennehy T. J., Morin, S., Sisterson M. S., Roush R. T., Shelton A. M. and Zhao, J. Z., 2003. Insect resistance to transgenic Bt crops: lessons from the laboratory and field. J. Econ. Entomol. 96, 1031–1038.

التأثير التزامنى لبكتريا الـــ *Bacillus thuringiensis* Cry1Ac على نمو وتطور سلالات دودة اللوز الأمريكية

سعد محمد موسى و عبد الجابر فتوح السيدعفصة

معهد بحوث وقاية النبات – مركز البحوث الزراعية - الدقى – جيزة - مصر

تم تعريض إحدى عشر جيلا متتالية من حشرة دودة اللوز الأمريكية للتوكسين الناتج من بكتيريا Bt توكسين ونتيجة لذلك تم تطور درجة المقاومة إلى ٥٦.٧٣ مرة في السلالة مقارنة بالسلالة الحساسة. و بدراسة تطور السلالات المقاومة والحساسة والجيل الأول الناتج من تزاوج كلا منهما معا وجد أن هناك اختلافات معنوية بين هذه السلالات في درجة نموها وتطورها. حيث كان معدل نمو السلالة المقاومة أقل من معدلة في حالة السلالة الحساسة والجيل الأول. أيضا أوضحت الدراسة أن هناك تأثير على تزامن فترة تطور السلالة المقاومة والسلالة الحساسة كنتيجة للتأثير الحيوي المركب البكتيري الـ Cry1Ac على درجة تطور ونمو السلالة المقاومة عندما تم تربيتها على بيئة صناعية جنبا إلى جنب مع السلالة الحساسة وأفراد الجيل الأول. حيث طالت فترة نمو السلالة المقاومة بالمقارنة بالسلالة الحساسة. لهذا فاحتمال تقابل وتزاوج الحشرات الكاملة من السلالتين في أن واحد لإنتاج أفراد الجيل الأول الأقل في درجة المقاومة تكون ضعيفة جدا. ونتيجة لذلك فرصة تزاوج الأفراد الكاملة من السلالة المقاومة بين بعضها البعض تكون أكثر من تزاوج أفراد السلالة المقاومة بأفراد السلالة الحساسة. وبناءا علية تكثر نسبة الأفراد المقاومة في الأجيال المتعاقبة ويصبح من الممكن تطور صفة المقاومة تحت الظروف الحقلية وعلى الجانب الأخر تقل نسبة الأفراد الحساسة ويصبح عامل تطور صفة المقاومة للحشرة في الحقل قائما بصورة أكبر. لذا تنصح الدراسة بإعادة النظر في نظرية الملاذ الحشري على أنة ليس هو العامل الهام فقط للتغلب على كسر صفة المقاومة.

قام بتحكيم البحث

أ. د/ محمد السيد رجب — **كلية الزراعة – جامعة المنصورة**

أ. د/ المتولى فراج المتولى — **مركز البحوث الزراعية**