Multifunctional properties of *Bacillus thuringiensis* bacteria strains and a new approach in struggle with a cotton bollworm

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Abstract

B. thuringiensis (Bt) strains showed 40.0 to 83.3% insecticidal activity against to cotton bollworm Helicoverpa armigera. High insecticidal activity occurred mainly on seventh day of experiment. When grown in medium containing different concentrations of NaCl, it was found that Bt1 and Bt31 strains were tolerant upto 600 mM, and 800 mM NaCl, respectively, while the rest were tolerant up to 1000 mM NaCl. All of B. thuringiensis strains synthesize gibberellic acid (GA). On the 1st day of the growth dynamics, the amount of GA synthesized was from 1.05 mg / l to 1.8 mg / l, on the third day of cultivation, the lowest (4.8 mg / l) and the highest (8 , 5 mg / l) GA synthesis was observed in the strains Bt31 and Bt26, respectively. In the first year of treatment with B. thuringiensis 94 strain against cotton bollworm, the biological efficiency was 20.8%, while the treatment before seeding in the second year led to 34.4% biological efficiency. Cotton yield increased by 2.4% compared to the control.

Keywords--- *B. thuringiensis, Helicoverpa armigera,* entomopathogenic bacteria, *insecticidal activity, salt tolerance, gibberellic acid (GA).*

Introduction

One of the most important areas in agriculture is the search for bacteria species that possess multifunctional features such as (entomocides, growth stimulators, phytopathogen resistancy, stress tolerance). Such research will help to solve the problem of obtaining environmentally friendly agricultural products, while reducing the costs of growing agricultural crops and increasing environmental sustainability. One of the promising directions is the comprehensive study of the bacteria *B. thuringiensis*.

B. thuringiensis is a gram-negative spore forming important entomocide agent. It is used as a bioinsecticide against pests in agriculture [1]. In addition, *B. thuringiensis* has been used to control various species of fungi and bacteria [2,3,4].

B. thuringiensis is known to grow in wide variety of habitats, it occurs in in milk as a probiotic, in highly salinized soil conditions, as well as in environments contaminated with phenolic compounds and various pesticides [5,6].

It has also been shown that these bacteria have endophytic properties [7] and have a positive influence on the growth and development of agricultural plants [8,9].

The main objective of our research is to identify *B.thuringiensis* strains that can be used not only to reduce the population of pests during the vegetation period but also to greatly reduce pest population by treating cotton seeds before planting thus increasing plant productivity and soil salinity.

1 Materials and Methods

1.1. Bacterial strains and growing conditions

Local strains of bacteria *B. thuringiensis Bt1, Bt18fo, Bt26, Bt31,Bt54, Bt81, Bt82, Bt84, Bt91, Bt93* and *Bt94* were used in experiments. Bacteria were grown in cultural media containing (w/v): pepton-1.0%; glucose 0.6%; NaCl-0.5; K2HP04-0.05; MgS04-0.02%, (pH-7.0). Cultures were grown on a shaker at 150 rpm, at 29-31 °C.

1.2. Insecticidal activity

Second and third generation larvae of *Helicoverpa armigera* Hbn. were used to test insecticidal activity of Bt strains.

Before infecting, the caterpillars were starved for 2-3 hours. Healthy and active caterpillars (10 caterpillars per glass jar) were used in the experiment and done in six replicates. Filter paper was

placed at the bottom of sterile Petri dishes, on which small "bouquets" of alfalfa previously moistened with bacterial suspension of *B. thuringiensis* strain under study were placed.

After the infected feed was completely eaten, subsequent feeding was carried out with the same plant but not treated with the pathogenic agent. In the control samples the feed was moistened with sterile water. The titer of bacteria used was $2x10^8$ U/ml. Dead caterpillars were microbiologically analyzed for the presence/absence of the bacteria under study. A method of preparing a biological product based on the Bt94 strain has been reported in previous works [10].

The calculation of biological effectiveness was carried out according to the Abbot formulation which provides adjustment for control.

1.3. Production of gibberellic acid

Bt strains were grown in peptone medium for 3 days. Cultures were precipitated by centrifugating at 6000 rpm. The supernatant until was adjusted to pH 2.5 by adding 3.75 N HCl. GA was extracted by adding ethyl acetate/$NaHCO_3$ fluid to the resulting liquid. GA amount was measured by UV spectrophotometer at 254 nm [11].

1.4. Salt tolerance ability

The Pepton-agar medium containing 100 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM, 800 mM, 900 mM, 1000 mM, 1500 mM, 2000 mM, or 2100 mM of NaCl was poured into the Petri dishes. Each Petri plate was inoculated bacteria in six 3cm lines. The average bacterial growth was calculated by measuring the length of the bacteria growing along the straight line.

2 Results and discussion

2.1. Insecticidal activity of *B.thuringiensis*

We conducted a 14-day laboratory test to determine whether eleven strains of *B. thuringiensis* possess any insecticide activity against 2- and 3-year-old cotton Bollworm.

Table 1.

The insecticidal activity of *B. thuringiensis* strains against to *Helicoverpa armigera* .

№	**Strains of *Bt***	**Number of caterpillars before experiment**	**Duration of the experiment (days)**				**Total number of dead insects**	**Biological efficiency,%**
			3 days	7 days	10 days	14 days		
			Number of dead insects					
1	control	60	0	0	0	0	0	0
2	*Bt*1	60	10	36	2	0	48	**80,0±0,46**
3	*Bt*18ϕo	60	11	27	7	0	45	**70,0±0,28**
4	*Bt*26	60	8	25	6	0	49	**81,6±0,39**
5	*Bt*31	60	10	30	8	0	48	**80,0±0,48**
6	*Bt*54	60	4	22	3	0	29	**48,3±0,22**
7	*Bt*81	60	6	19	1	0	26	**43,3±0,19**
8	*Bt*82	60	6	13	5	0	24	**40,0±0,26**
9	*Bt*84	60	13	22	10	0	45	**75,0±0,33**
10	*Bt*91	60	9	34	4		47	**78,3±0,58**
11	*Bt*93	60	7	30	8		45	**75,0±0,38**
12	*Bt*94	60	18	29	3		50	**83,3±0,19**

As can be seen from Table 1, 8 strains (Bt1, Bt18fo, Bt26, Bt31, Bt84, Bt91, Bt93 and Bt94) of the 11 strains used in this 14 day experiment showed greater than 50% insecticide activity.

Because strains Bt54, Bt81, and Bt82 exhibited less than 50% activity, we decided not to use these strains in our subsequent experiments against cotton bollworm larvae.

Among the strains used, Bt94 had the highest entomocide activity (83.3%). It should be noted that the main insecticidal activity of *B.thuringiensis* strains against cotton bollworm larvae was observed on the 7th day. The larvae that were still alive stopped feeding after 7 days and became inactive.

Bibi A. et al [12] reported that various concentrations of bioinsecticide formulas prepared using strains of *B. thuringiensis* led to 100% mortality of cotton bollworm larvae in 6 days. Similar to our study, Patel A. S. et al [13] found that 10^8 titer of *B.thuringiensis* ABt-10 strain showed 48.61% insecticidal activity in 5 days against cotton bollworm.

2.2. Salt tolerance ability

It is important that *B.thuringiensis* strains from treated seeds survive in the soil and will possess their insecticide activity in subsequent years.

In addition the survival of entomopathogenic bacteria in soils in our conditions requires that they are resistant to salinity, too. According to the International FAO (2015), 51% of Uzbekistan's irrigated fields is highly salinated [14].

Therefore, in our next study, we investigated tolerance of 8 strains with high insecticidal activity against cotton bollworm larvae using different NaCl concentrations (Figure 1). Bt1, Bt18fo, Bt26, Bt31, Bt84, Bt91, Bt93 and Bt94 strains of the B. thuringiensis bacterium were grown in nutrient medium containing NaCl ranging from 100 mM to 1500 mM. All the strains used in the experiment could optimally grow and develop in medium containing up to 500 mM NaCl. It was found that the width and length *B. thuringiensis* №1 strain reduced when NaCl concentration in nutrient medium was 600 mM (3.48%). It was observed that 700 mM of NaCl completely stopped growth and development of this strain. Bt31 strain could grow in peptone agar containing up to 800 mM NaCl.

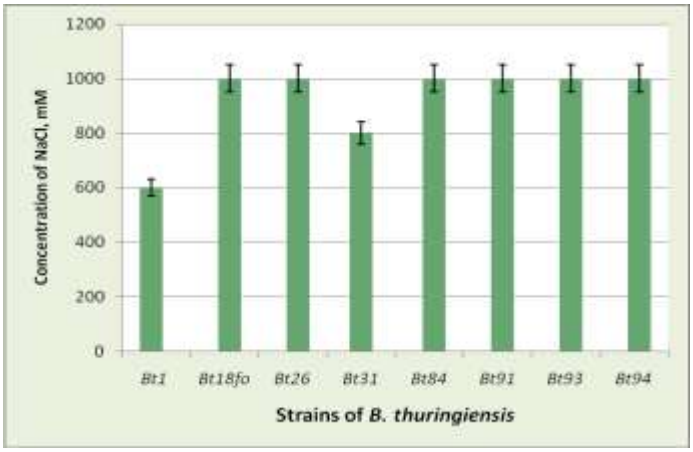

Figure 1. Survival of *B. thuringiensis* strains in agar medium containing different concentrations of NaCl.

The strains Bt18fo, Bt26, Bt84, Bt91, Bt93 and Bt94 showed tolerance upto 1000 mM (5.8%) NaCl. No bacterial growth or development was observed when NaCl concentration was 1100 mM (6.38%).

Sharma A. et al [15] reported on *Bacillus species* of *B. thuringiensis, B. megaterium, B. subtilis, B. licheniformis, B. firmus, B. horikoshii, B. pumilus, Bacillus sp.,* that were isolated from various environmental regions of India and that could grow at higher than 4% NaCl concentrations. They were found to be tolerant to high salinity, endospore forming, well adapted to suboptimal conditions, and promote plant growth under these conditions. These data confirm the high salinity tolerance ability of *B. thuringiensis* strains used in our experiments and indicate that they can be used for treating cotton seeds before sowing.

2.3. Gibberellic acid production

To determine the plant growth promoting properties of *B. thuringiensis* strains we studied gibberellic acid formation dynamics of the strains *Bt*1, *Bt*18fo, *Bt*26, *Bt*31, *Bt*84, *Bt*91, Bt93 and Bt94 for three days (Fig. 1). The first day of growing bacteria culture the highest GA secretion was observed in *Bt*1 (1.8 mg/L) and *Bt*93 (1.8 mg / L) strains, while for the remaining *Bt*18fo, *Bt*26, *Bt*31, *Bt*84, *Bt*91 and *Bt*94 strains it was 1.4 mg / L, 1.1 mg / L, 1.05 mg / L, 1.2 mg / L, 1.5 mg / L, and 1.7 mg / L, respectively.

In the log phase of growth which was on the 2nd day, the Bt1 strain produced more GA(3.4 mg / L) GA than the other strains. Bt18fo, Bt26, Bt31, Bt84, Bt91, Bt93 and Bt94 strains synthesized 1.8 to 2.9 mg / L GA. It was noted that on the 3rd day of growth (in stationary phase), GA produced by Bt26 strain reached 8.5 mg / L, showing increase in synthesis by 4 times compared to that on the first day. The strain Bt31 synthesized the lowest (4.8 mg / L) GA compared to other strains used in the experiment. Other strains that were used in the experiment namely Bt1, Bt18fo, Bt84, Bt91, Bt93 and Bt94 produced GA with concentrations from 6.9 to 8.4 mg / L on the third day of growth. It should be noted that as compared to the first day GA formation increased by 4-7 times on the third day of growth.

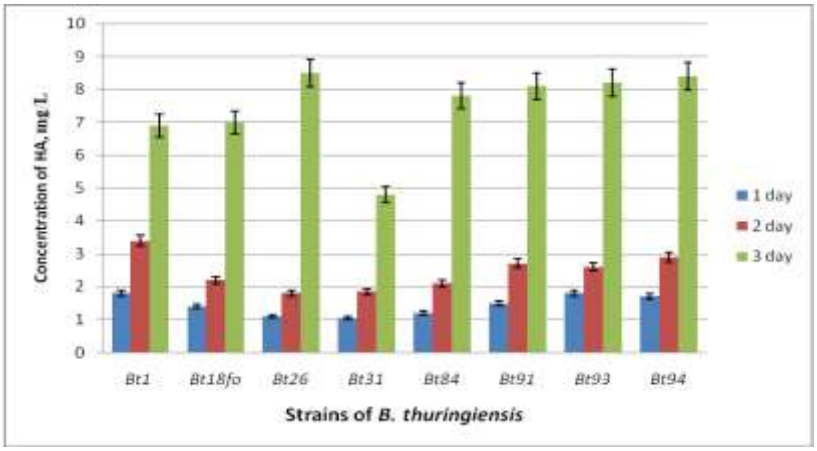

Figure 1. Dynamics of GA formation by *B. thuringiensis* strains in peptone nutrient medium.

A study by Pandya N.D. et al [16] showed that in Bacillus species GA synthesis begins in 12 h and synthesized 290 mg / L in 78h. In their research S. Sivasakthi et al. [17] found that a *Bacillus subtilis* strain could produce 2.89 mg/L GA. These results are consistent with the results we have obtained. GA concentrations produced by Bacillus species can vary depending on properties of a given strain.

2.4. Field test experiments

To investigate the influence on crop yields and effects on cotton bollworm as a result of planting Bt treated cotton seeds, we conducted field experiments for 2 years in the field where cotton bollworm larvae were found and quantified (Table 2).

Table 2.: The effectiveness of the use of the bacterial strain B.thuringiensis 94 against to cotton bollworm larvae of in the field (June 2018, 2 hectares of land)

№	Treatments	Used amount of formulation for processing 1000 kg	Average number of caterpillars per 100 plants			Biological effectiveness in 2 years after seed treatmen,	Cotton yield, t / ha
			Number of caterpilla	Number of larvae in 1 year after	Number of larvae in 2 years after		

		of seeds	rs average for two years (before treatment)	seed treatment	seed treatment	%	
1.	Control (not treated cotton seeds)	-	19.9±3.8	23.8±6,5	26.3±7,0	0	2.67±2,1
2	Cotton seeds treated with B.thuringiensis 94	2.0 L	20.3±5,0	16.8±3,5	13.3±3,0	34.4±2,6	2.91±5,3
3.	Cotton seeds treated with Beta Pro (commercial preparation)	160 gramm	21.2±4,0	17.3±2,0	14.1±2,0	33.4±4,5	2.88±3,9

Table 2 shows that first year of treatment of cotton seeds with *B.thuringiensis 94* resulted in 20.8% biological efficiency against cotton bollworm, while after treating seeds second year biological efficiency reached 34.4%. Commercial formulation Beta Pro also led to a decrease in cotton bollworm population, with a biological efficiency of 22.5% in the first year and 33.4% in the second year.

In the experimental fields with cotton seeds not treated with *B.thuringiensis*, the population of cotton bollworm increased by 19.5% first year and 32.1% second year. At the same time, the yield of cotton obtained in control fields was 2.67 t/ha, the yield was 2.4% higher in fields with Bt treated seeds than in control fields.

Cotton yield was 2.1% higher in fields sown with Beta Pro formulation treated seeds than the yield in control fields.

In fields sown with Bt treated seeds B.thuringiensis bacteria can survive in the soil and contribute to the reduction of cotton bollworm population. Bacteria of *Bt* have the ability to promote plant growth thus increasing cotton productivity.

Studies have shown that accumulation of *B.thuringiensis* bacteria as endophyte in the plant root was determined using GFP tagged Bt strains. In this processes, bacteria cells penetrate the roots of cotton and cabbage followed by spread to other parts of the plant by xelema. Leaves of these plants were found to have toxic effect on insects when fed to Lepidoptera Spodoptera frugiperda (cotton plant) and Plutella xylostella (cabbage) [18].

In a study by Ali M. M. et al [19], they detected the presence of *B.thuringiensis* strains in the plant Withania somnifera using phase-contrast microscopy. They noted that the synthesis of IUA by bacteria affects the growth of this medicinal plant. In addition, there have been studies on phytogormone synthesis, phosphorus mobilization, sidenophore forming, and against anti phytopathogens antimicrobial properties of *B.thuringiensis* strains. Treating corn seeds with Bt promoted fast root and shoot growth in plants [20].

The polyfunctional character of *B.thuringiensis* strains positively influences plant productivity and yield.

Conclusion:

It was determined that *B.thuringiensis* strains under current study grew in salinized (up to 1000 mM) soils, synthesized gibberellin, and had insecticidal activity. Treating cotton seeds with the bioinsecticide formulation based on these Bt strains protects cotton plants from insect pests during the vegetation period and increases the yield due their positive influence on plant growth.

References

1. Crickmore N., Zeigler D. R., Feitelson J., Schnepf E., Van Rie J., Lereclus D., Baum J., Dean D. H. Revision of the Nomenclature for the *Bacillus thuringiensis* Pesticidal Crystal Proteins//Microbiology and molecular biology reviews, 1998, 62(3): 807–813.
2. Stabb E.V., Jacobson L.M., Handelsman J. Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils// Appl. Environ. Microbiol, 1994, 60(12): 4404-4412.
3. Arora N., Ahmad T., Rajagopal R., Bhatnagar R.K. A constitutively expressed 36 kDa exochitinase from *Bacillus thuringiensis* HD-1// Biochem. Biophys. Res. Commun., 2003, 307: 620-625.
4. Magay E.B., Fedorova O. A., Mavzhudova A. M., Nurmukhamedova Kh., Egamberdiev Sh. Sh. Biocontrol activity of local strains of Bacillus thuringiensis against the causative agent of cotton Fusarium wilt. - Modern mycology in Russia,2017, 7: 28-30.
5. Kweon CK., Choi SY., Kwon HY, Kim EH, Kang HM., Moon JS., Jang GC., Lee HS., Kang SW., Kim JM., Pyo S., Rhee DK. Isolation, characterization, and evaluation of Bacillus thuringiensis isolated from cow milk// J. Korean of Veterinary Research, 2012, 52(3):169-176.
6. Das J., Das B., Dangar TK. Microbial populations and *Bacillus thuringiensis* diversity in saline rice field soils of coastal Orissa, India// African Journal of Microbiology Research, 2008, 2: 326-331.
7. Minu M. A., Dipak V. Bacillus Thuringiensisas Endophyte of Medicinal Plants: Auxin Producing Biopesticide// Int. Res. J. Environment Sci, 2014, 3(9):27-31.
8. Raddadi N., Cherif A., Boudabous A., Daffonchio D. Screening of plant growth promoting traits of *Bacillus thuringiensis//* Annals of Microbiology, 2008, 58 (1):47-52.
9. Kryzhko A. V., Kuznetsova L. N. The stimulating effect of entomopathogenic strains of Bacillus thuringiensis on the morphometric parameters of lettuce plants // Bulletin of the Voronezh State University, 2017, 4: 51-55.
10. Khalilov I.M., Kadirova G.Kh., Sultanov R.A., Shakirov Z.S. Development of regulations for obtaining bacterial insecticidal preparation based on the *B. thuringiensis* 94 against to gipsy moth// Reports of the Academy of Sciences of the Republic of Uzbekistan, 2019, 3: 75-79.
11. Berryos J, Illanes A., Aroca G. Spectrophotometric method for determining gibberellic acid in fermentation broths// Biotechnol. Lett., 2004, 26: 67–70.
12. Bibi A., Ahmed K., Ayub N., Alam S. Production of low cost Bacillus thuringiensis based-biopesticide for management of chickpea pod-borer Helicoverpa armigera (Huebn) in Pakistan//Natural Science, 2013, 5(11): 1139-1144.
13. Patel A. S., Shelat H.N., Patel H. K. Isolation and Insecticidal Potential of Native Bacillus thuringiensis against Helicoverpa armigera and Spodoptera litura// International Journal of Current Microbiology and Applied Sciences, 2018, 7 (2): 1330-1339.
14. European commision on agriculture. Thirty-ninth session. Combating land degradation for food security and provision of soil ecosystem services in Europe and Central Asia –International Year of Soils 2015/ Budapest, Hungary, 22 and 23 September 2015, http://www.fao.org/3/a-mo297e.pdf.
15. Sharma A., Singh P., Kumar S., Kashyap L. P., Srivastava A.K., Chakdar H. Deciphering Diversity of Salt-Tolerant Bacilli from Saline Soils of Eastern Indo-gangetic Plains of India//Geomicrobiology Journal, 2014, 32(2):170-180.
16. Pandya N.D., Desai P.V. Gibberellic Acid Production by *Bacillus cereus* Isolated from the Rhizosphere of Sugarcane// J. Of pure and applied microbiology, 2013, **7(**4):1-4.
17. Sivasakthi S., Kanchana D., Usharani G., Saranraj P. Production of Plant Growth Promoting Substance by *Pseudomonas fluorescens* and *Bacillus subtilis* Isolates from Paddy Rhizosphere Soil of Cuddalore District, Tamil Nadu, India// International Journal of Microbiological Research, 2013, 4(3):227-233.
18. Monnerat R. G., Soares C. M., Capdeville G., Jones G., Martins É. S., Praça L., Cordeiro B. A., Braz S. V., dos Santos R. C., Berry C. Translocation and insecticidal activity ofBacillus
a. thuringiensis living inside of plants// Microbial Biotechnology, 2009, 2(4): 512–520.
19. Ali M. M., Vora D. Bacillus Thuringiensisas Endophyte of Medicinal Plants: Auxin Producing
20. Biopesticide// International Research Journal of Environment Sciences, 2014, 3(9):27-31.

21. Kassogué ., Dicko A. H., Traoré D., Fané R., Valicente F. H., Babana A. H. Bacillus thuringiensis Strains Isolated from Agricultural Soils in Mali Tested for Their Potentiality on Plant Growth Promoting Traits//British Microbiology Research Journal, 2016, 14(3):1-7.
22. Sabirova SHîista Mirayupovna, Abdullaeva Djamilya Fazlitdinovna Increasing Ecological Culture In The Youth. Journal of biology and ecology, 2019, №2, pp. 80-82. http://dx.doi.org/10.26739/2181-0575-2020-1-13
23. S.R. Hajiyeva E.M.Gadirova U.N.Rustamova Application Of Moss Transplants In Biomonitoring Of The City Atmospheric Air. Journal of biology and ecology, 2019, №1, pp.14-17. http://dx.doi.org/10.26739/2181-0575-2019-2-3