ISOLATION AND IDENTIFICATION NATIVE *BACILLUS THURINGIENSIS* IN DIFFERENT HABITAT FROM WEST AZERBAIJAN AND EVALUATE EFFECTS ON INDIAN MOTH *PLODIA INTERPUNCTELLA* (HUBNER) (LEPIDOPTERA: PYRALIDAE)

Shahram Aramideh*, Mohammad Hassan Saferalizadeh*, Ali Asghar Pourmirza*, Mahmuod Rezazadeh Bari*, Mansureh Keshavarzi and Mahdi Mohseniazar***

*Department of Plant Protection, Institute Biotechnology, University of Urmia, Urmia, IRAN. E-mail: Shahramaramideh@gmail.com
**Seed and Plant Improvement Institute, Karaj, IRAN.

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ABSTRACT: The bacterium *Bacillus thuringiensis* is characterized by the production crystalline parasporal inclusion in stationary phase in sporulation growth stage with insecticide activity. This bacterium can be isolated and identified from different environment with navel toxin and can be evaluated toxicity against different host. In this report, 48 native strains were isolated from 740 samples by acetate selection method. All isolates were characterized by crystal morphology and toxicity against 2nd instars Indian moth (*Plodia interpunctella)* larvae. Majority of strains (58%) had bipyramidal crystals. 16 isolates had percentage mortality more than 75% and 11 Isolates namely wz-105, wz-111, wz-120, wz-122, wz-125, wz-149, wz-155, wz-157, wz-184, wz-187 and wz-189 had percentage mortality (>90%) equal or more than *B.thuringiensis kurstaki* as positive control.

KEY WORDS: *B.thuringiensis,* Isolates*, Plodia interpunctella,* toxicity.

Stored-product moths as Indian meal moth, *Plodia interpunctella*, one of the economically important lepidopteran pests, are mostly controlled chemically, especially due to the desirable cost/efficiency ratio of chemical control (Zettler & Arthur, 2000). However, the increasing resistance to some chemical insecticides, as well as the presence of insecticidal residues after chemical treatment, advocate for alternatives to chemical pesticides, including biological control (Arthur, 1996; Kramer et al., 2000, Flinn et al., 2006). Several entomopathogenic microorganisms are used for the biological control of insect pests and one of the most widely used is *B. thuringiensis* (*Bt*) (Samsonov et al., 1997). Stored-product moths are susceptible to *B. thuringiensis* (Johnson & McGaughey, 1996). *B. thuringiensis* is a Gram-positive, spore-forming bacterium, which during the sporulation phase produces a protein crystal. The crystal proteins (cry and cyt) are toxic against a large number of insects, mainly species of the Lepidoptera, Diptera and Coleoptera orders (Feitelson et al., 1992; Schnepf et al., 1998), some of which are important pests in agriculture or vectors of human diseases. *B. thuringiensis* has been isolated from different natural sources, as soil (Arango et al., 2002), dead or sick insects (Kaelin et al., 1994; Bernhard et al., 1997), stored plant products (Hongyu et al., 2000a), phylloplane (Maduell et al., 2002) and other natural sources (Iriarte et al., 1998).

In this study, the distribution, frequency and diversity of *B. thuringiensis* were assessed in different environments of WA province of Iran. *B. thunrngiensis* isolates were differentiated on the basis of crystal morphology and toxicity on *Plodia interpunctella*.

MATERIALS AND METHODS

Sample collection

Totally, 740 samples were collected from 11 locations in West Azerbaijan province. *B. thuringiensis* subspecies were isolated from uncultivated site that have no history of treatment with *B. thuringiensis* products include soil, beaches, forests, stored product, agricultural fields, insect cadavers and grasslands. Soil samples were collected by scraping off surface material with spatula and then obtaining a 10g sample from 5-15cm below the surface. All samples were stored in sterile plastic bags at ambient temperature.

B.thuringiensis isolation

The samples were processed by acetate selective method (Travers et al., 1987) in four concentrations of acetate sodium (0.2, 0.25, 0.3 and 0.35M.) (pH=6.8). Each concentration was applied for 186 samples. In this procedure, acetate inhibits germination of *B. thuringiensis* spores, so other spore germinates and non-spore forming bacteria eliminated by heat treatment (7min at 80°C). The surviving spores were plated and grown on nutrient agar and incubated at 30°C for 24 h to obtain colonies. Anywhere from 5 to 20 different colony types were usually obtained. The colonies were cultured onto T3 medium (3g tryptone, 2g tryptose, 1.5 g yeast extract, 0.05M sodium phosphate [pH=6.8], 0.005g of $MnCl_2$ per liter) for 4-5 days and studied for crystal morphology and bioassay tests.

Crystal morphology

Smears of bacterial strains were stained with coomassie brilliant blue solution (0.25% coomassie brilliant blue, 50% ethanol and 7% acetic acid) for 3min, washed with tap water, dried, and observed under a light microscope without cover and oil emersion (Fadel et al., 1988).

Bioassay

The activity of *B. thuringiensis* isolates against insects of order lepidoptera was tested using *Plodia interpunctella*. For toxicity testing, spore-crystal preparations were grown on T3 plates. The spores and crystals from the agar were floated on 10ml of sterile water and suspension was stored in sterile vials until it was tested. *Plodia interpunctella* (Lepidoptera: Pyralidae) was reared in clear plastic containers (50 cm length, 20 cm width, 10 cm height) on a 2:1:0.25:0.50:0.25:0.25 mixture of rough wheat bran, corn flour, dry yeast, honey, milk powder, glycerin containing approximately 250 g sterilized food (Ozkan, 2006).From each isolate five ml spore-crystal suspension sprayed on 1.5 gr artificial food on Petri dish and air dried at room temperature. Then ten 2nd instars larvae of *P. interpunctella* were released on this contaminated food. A standard strain *B. thuringiensis* subsp. kurstaki was used as positive and sterile distilled water as negative control. Each isolate was tested on 30 larvae in three replicates and mortality recorded after incubation at 20±2°C for 48h.

RESULTS

The inability of *B. thuringiensis* strains to germinate in the presence of acetate buffer allows screening of organism from samples. One hundred eighty six samples were tested for each four concentrations acetate-buffered medium. More *Bt* isolate obtain from Samples that incubated in 0.25 M. acetate at 4 h (56.25%).

Soil samples were the most abundant and diverse sources of *B. thuringiensis*. From 3010 different colonies of spore-forming bacteria, 48 *B. thuringiensis* isolates were obtained after microscopic observation. The average of *Bt* index was 6.4% (48 isolate from 740 sample). Most strains were isolated from Urmia soil but the highest *Bt* index was obtained in Khoy (Table1).

After screening of the isolates for the presence of parasporal bodies, all 48 isolates showed the *B. thuringiensis* parasporal bodies and were divided into eight classes based on crystal morphology: Spherical (S); Bipyramidal (BP); Cubical (Cu); Irregular (I); Cubical Plus Bipyramidal (Cu+BP); Spherical Plus Bipyramidal (S+BP); Cubical Plus Spherical (CU+S); and (Unknown)(UN). The results indicated that crystals produced by *B. thuringiensis* isolates from West Azerbaijan habitat were Bipyramidal crystals with 17 isolates (35.41%), Spherical and Bipyramidal+Spherical classes with 8 isolates (16.66%) and others classes with 3 isolates (6.25%) (Fig 1).

There was a wide range of toxicity to *Plodia interpunctella* in the isolates with mortality ranging from 10 to 100%. The distribution of mortality for the 48 isolates was separated into 4 groups. 12 (25%), 12 (25%), 8 (20%) and 16 (30%) isolates/percentage were distributed in 0-25%, 25-50%, 50-75% and 75-100% mortality groups; respectively. 16 isolates had percentage mortality more than 75%. Among them, 11 isolates namely wz-105, wz-111, wz-120, wz-122, wz-125, wz-149, wz-155, wz-157, wz-184, wz-187 and wz-189 were highly toxic on *P. interpunctella* (90-100% mortality) (Table 2). The results clearly show that isolates wz-149 were more effective (100% mortality) than the other isolates and standard strain.

DISCUSSION

Primarily identification of *Bt* is based on the presence of crystalline inclusions (Rampersad & Ammons, 2005). In the present study, from 3010 stained bacterial colonies in 48 isolate crystalline inclusions were observed under light microscopy, and were identified as *Bt* Based on the shape and size. The 48 new isolates of *Bt* were characterized into seven groups and no identified (unknown) (Fig. 1). Martin & Travers (1989) have isolated *B. thuringiensis* from several locations in Eastern Asia. They found that isolates with bipyramidal and spherical crystals were the most common. In this study majority of the isolates (58.32%) showed the presence of bipyramidal crystals. The diversity in the dominancy of parasporal shapes between West Azerbaijan Iran habitats and Eastern Asia might be related to the difference in sample location, habitat and genetic variation.

An average *Bt* index (the ratio of crystal producing isolates of *Bt* to all isolates) was found to be 0.064 for all samples (48 isolates from 740 samples) but the index changed according to sample types and origins. However, Martin and Travers (1989) found the highest *Bt* index (0.85) in the soil samples collected from Asia. This may be related to climate and geographic conditions. The abundance of *B. thuringiensis* was the highest in soil samples. Unlike this study, Hongyu et al., (2000b) and Bernhard et al., (1997) reported that *B. thuringiensis* is more abundant in stored product environments than soil. However, in our

study, among the stored product samples, only 6% of *B. thuringiensis* strains were isolated, but soil samples were the most abundant and diverse sources of *B. thuringiensis* (66%).

Plodia interpunctella, selected for resistance to Btk (Van Rie et al., 1990), but in this research some isolate were very toxic to *P. interpunctella*. 11 isolates were highly toxic on *P. interpunctella* (90-100%mortality). Bioassay results obtained in the present study show that some of the isolate (wz-149) was more toxic than the standard strain (*B. thuringiensis kurstaki*). It is important to discover new *B. thuringiensis* strains that can be effectively used in the biological control of insect pests.

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LITERATURE CITED

Arango, J. A., Romero, M. & Orduz, S. 2002. Diversity of *Bacillus thuringiensis* strains from Colombia with insecticidal activity against *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Journal of Applied Microbiology, 92: 466–474.

Arthur, F. H. 1996. Grain protectants: current status and prospects for the future. Journal of Stored Products Research, 32: 293–302.

Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D., Roberts, G., Pauli, S., & Rodgers, P. 1997. Natural isolates of *Bacillus thuringiensis*: world wide distribution, characterization, and activity against insect pests. Journal of Invertebrate Pathology, 70: 59-68.

Fadel A., Sharif N. & Alaeddinoglu, G. 1988. A rapid and simple method for staining of the crystal protein of *Bacillus thuringiensis*. Journal of Industrial Microbiology, 3: 227–229.

Feitelson, J. S., Payne, J. & Kim, L. 1992. *Bacillus thuringiensis*: insects and beyond. Biotechnology, 10: 271–275.

Flinn, P. W., Kramer, K. J., Throne, J. E. & Morgan, T. D. 2006. Protection of stored maize from insect pests using a two-component biological control method consisting of a hymenopteran parasitoid, *Thelocolax elegans*, and transgenic avidin maize powder, Journal of Stored Products Research, 42: 2218–2255.

Hongy,u Z., Ziniu, Y., & Wangxi, D. 2000a. Isolation, distribution and toxicity of *Bacillus thuringiensis* from warehouses in China, Crop Protection, 19: 449–454.

Hongyu, Z., Ziniu, Y. & Wangxi, D. 2000b. Composition and ecological distribution of Cry proteins and their genotypes of *Bacillus thuringiensis* isolates from warehouses in China. Journal of Invertebrate Pathology, 76: 191–197.

Iriarte, J., Bel, Y., Ferrandis, M., Andrew, R., Murillo, J., Ferre, J. & Caballero, P. 1998. Environmental distribution and diversity of *Bacillus thuringiensis* in Spain. Systematic and Applied Microbiology, 21: 97–106.

Johnson, D. E. & McGaughey, W. H. 1996. Contribution of *Bacillus thuringiensis* spores to toxicity of purified Cry proteins towards Indianmeal moth larvae, Current Microbiology, 33: 54–59.

Kaelin, P., Morel, P. & Gadani, F. 1994. Isolation of *Bacillus thuringiensis* from stored tobacco and *Lasioderma serricorne* (F.). Applied Environmental Microbiology, 60: 19–25.

Kramer, K. J., Morgan, T. D., Throne, J., Dowell, F. E., Bailley, M. & Howard, J. A. 2000. Transgenic avidin maize is resistant to storage insect pests, Nature Biotechnology, 18: 670–674.

Maduell, P., Callejas, R., Cabrera, K. R., Armengol, G. & Orduz, S. 2002. Distribution and characterization of *Bacillus thuringiensis* on the phylloplane of species of Piper (Piperaceae) in three altitudinal levels. Microbial Ecology, 44: 144–15.

Martin P. A. W. & Travers, R. S. 1989. Worldwide Abundance and Distribution of *Bacillus thuringiensis* Isolates. Applied Environmental Microbiology, 55: 2437-2442.

Ozkan, C. 2006. Laboratory rearing of the solitary egg-larval parasitoid, *Chelonus oculator* Panzer (Hymenoptera: Braconidae) on a newly recorded factitious host *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae). Journal of Pest Science, 79: 27–29.

Rampersad, J. & Ammons, D. 2005. A *Bacillus thuringiensis* isolation method utilizing a novel stain, low selection and high throughput produced atypical results. BMC Microbiology, 5: 52–63.

Samsonov, P., Padrón, R. I., Pardo, C., Cabrera, J. & De al Riva, G. A. 1997. *Bacillus thuringiensis* from biodiversity to biotechnology. Journal *of* Industrial Microbiology and Biotechnology, 19: 202-219.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. & Dean, D. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiology and Molecular Biology Reviews, 62: 775–806.

Travers, R. S., Martin, P. A. W. & Reichelderfer, C. F. 1987. Selective process for efficient isolation of soil *Bacillus* spp., Applied and Environmental Microbiology, 53: 1263–1266.

Van Rie, J., Jansens, S., Höfte, H., Degheele, D. & Van Mellaert. H. 1990. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. Applied and Environmental Microbiology, 56: 1378-1385.

Zettler, J. L. & Arthur, F. H. 2000. Chemical control of stored product insects with fumigants and residual treatments, crop protection, 19: 577–582.

Table 1- Distribution of *B. thuringiensis* isolates in samples collected from different localities of WA province.

Location	No of samples	No of colonies	No of isolates	*Bt* index (%)[a]
Orumie	337	2120	14	0.041
Mahabad	49	170	4	0.081
Khoy	34	80	5	0.147
Salmas	39	85	2	0.051
Shahindezh	39	77	4	0.102
Miyandoab	36	84	2	0.055
Bokan	40	70	2	0.05
Naghadeh	46	90	4	0.08
Oshnaviyeh	46	88	5	0.108
Mako	34	72	3	0.088
Sardasht	40	74	3	0.075
Total	740	3010	48	0.064

[a] *Bt* index is the ratio of *Bt* isolates producing crystal to all isolates in each sample group

Table 2-Classification of the *B. thuringiensis* isolates according to their toxicity levels against *Plodia interpunctella* after 48h.

Percent of morality	*Plodia interpunctella*		Isolate name
	No. of isolates	% of isolates	
Isolates causing a mortality of 0-25%	12	25	wz-77, wz-108, wz-117, wz-160, wz-166, wz-172, wz-179, wz-270, wz-500, wz-555, wz-666 and wz-734
Isolates causing a mortality of 25-50%	12	25	wz-116, wz-130, wz-141, wz-154, wz-183, wz-200, wz-216, wz-300, wz-352, wz-400 , wz-401 and wz-600
Isolates causing a mortality of 50-75%	8	20	wz-101, wz-172, wz-178, wz-182, wz-186, wz-188, wz-192 and wz-193
Isolates causing a mortality of >75%	16	30	wz-102, wz-105, wz-107, wz-111, wz-120, wz-122, wz-125, wz-149, wz-155, wz-157, wz-159,wz-181, wz-184, wz-187 wz-189 and wz-190

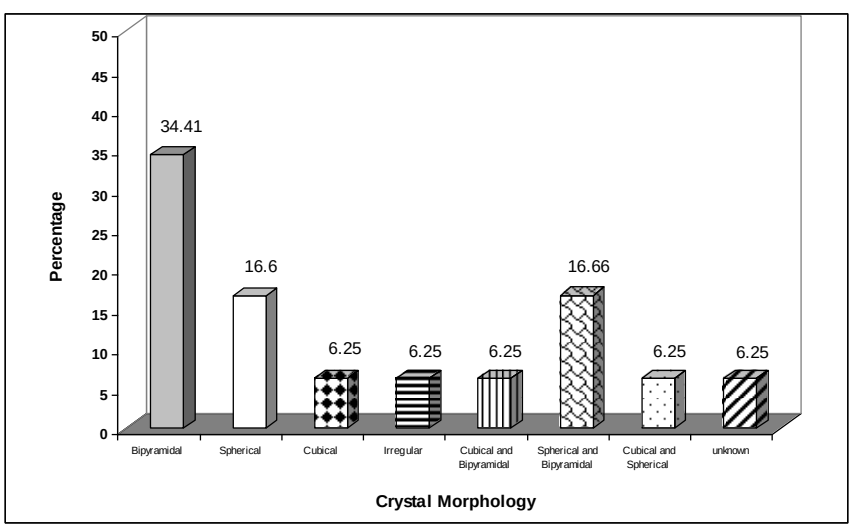

Figure 1. Percentage distribution of crystal morphologies of *B. thuringiens.*