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RESEARCH NOTES

Isolation, Identification and Differentiation of Local *B. thuringiensis* Strains

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ABSTRACT

In this research, 514 soil samples and dead larvae were collected from Khorassan, Lorestan, Tehran, Ghazvin, East Azarbaijan, West Azarbaijan, Mazandaran and Hamedan Provinces. *B. thuringiensis* was isolated from the samples using a heat-acetate method and the isolates were identified and classified using biochemical tests. The frequency of *B. thuringiensis* in soils with different plant communities was studied. According to results, 127 isolates were collected from the samples collected. Most isolates produced atypical and heterogenic and some bipyramidal crystals. Nearly all the isolates were able to hydrolyze starch and gelatin and ferment glucose and fructose, but could not produce indole and H_2S or ferment galactose and lactose. The isolates were divided into 8 biochemical types, among which *B. thuringiensis* subsp. *kurstaki* was the most frequent type. Total *Bt* frequency, corresponding to the whole sampling areas, was calculated as being 3.1%; the highest frequency was recorded for Khorassan Province (5.1%) and the lowest for Lorestan Province (0%). No realtionship was found between *B. thuringiensis* frequency and vegetation status of the soils examined.

Keywords: *B. thuringiensis*, Biochemical type, Isolation.

INTRODUCTION

B. thuringiensis has been used as a successful biological insecticide for more than 40 years and is a uniquely specific, safe and effective tool for the control of a wide variety of insect pests (Nester *et al.,* 2002). Intensive screening programs leading to the establishment of many *B. thuringiensis* collections have been conducted over the last few decades (Martin and Travers, 1989; Delucca *et al*., 1989; Smith and Couche, 1991; Meadows *et al*., 1992; Chilcot and Wigley, 1993; Chaufaux *et al*., 1997; Forsyth and Logan, 2000; Uribe *et al*., 2003; Ibarra *et al*., 2003). The International Entomopathogenic Bacillus Collection (IEBC collection) of the Pasteur Institute has more than 3600 *B. thuringiensis* strains.

B. thuringiensis strains, in any particular collection may be characterized in a number of ways such as biochemical typing, flagellar serotyping, profiling plasmid arrays or proteins, use of monoclonal antibodies and hybridization or PCR amplification, based on sequences of known *cry* genes (Porcar and Juarez-Perez, 2003; Schnepf *et al*., 2005). Originally *B. thuringiensis* strains were divided into subspecies or varieties on the basis of their spectra of activity against insects and complementary biochemical tests (Heimple, 1967). However, for high numbers of strains, this method was too cumbersome and a serological shorthand method was used instead (Bonnefoi and de Barjac, 1963). Over time, it was found that serology is also a time-consuming, expensive method for identifying thousands of isolates and a rapid method based on biochemical tests was developed (Martin *et*

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al., 1985). The initial purpose of this research was the collection of *B. thuringiensis* strains from Iran. Consequently, a shorthand biochemical schedule was used to differentiate isolates. Finally, the relationship between plant community and frequency of *B. thuringiensis* isolates was studied.

MATERIALS AND METHODS

Sample Collection

Soil samples were collected from Khorassan, Lorestan, Tehran, Ghazvin, East Azarbaijan, West Azarbaijan, Mazandaran and Hamedan Provinces, in Iran. It was attempted to collect soil from locations with diverse plantation statuses including those with little or no plant communities (high–altitude mountains, non-cultivated lands and beaches) and those that have plantations (agricultural lands, urban locations, forests). On some occasions, a number of dead larvae were found which were collected for *B. thuringiensis* isolation.

Isolation and Identification

The method of Traves *et al*. (1987) was used for isolating *B. thuringiensis* from both insect and soil samples. The isolates were cultured onto T3 sporulating plates (3 g tryptone, 0.05 M sodium phosphate pH 6.8, 0.005 g $MnCl_2$ per liter). After 48 hours incubation at 28ºC, colonies with typical *B. thuringiensis* morphology were picked and crystal-producing colonies were purified by restreaking onto T3 medium and, after 72 hours incubation at 28ºC, morphology of crystals was studied in parallel with *B. thuringiensis* subsp. *kurstaki* obtained from the commercial Dipel®. For further confirmation of crystal-forming isolates, two categories of biochemical tests were used (Lecadet *et al*., 1999). The first category consisted of generally positive charactersties for all *B. thuringiensis* strains including hydrolysis of starch and gelatin and fermentation of glucose and fructose. The second category, including production of indole and H_2S and fermentation of galactose and lactose, were negative for all strains. The biochemical tests were performed using standard methods as described by Schaad *et al*. (2001) and using Phenol-Red Broth Base (Merck) basal medium for carbohydrate fermentation.

Bt Frequency

The *B. thuringiensis* frequency was determined for each province corresponding to the percentage of soil samples with at least one *B. thuringiensis* isolate (positive samples) to the total number of soil samples of that province. Total *Bt* frequency was calculated as a perecentage of soil samples with at least one *B. thuringiensis* isolate to the total number of the examined soils.

Biochemical Typing

Four highly relevant biochemical tests including esculin utilization, acid formation from salicin and sucrose, and lecithinase production (Table 1) were used to subdivide the crystal-forming bactera into bibiochemical types (Martin and Travers, 1989).

RESULTS AND DISCUSSION

Isolation and Identification

The bacterium has been found to colonize many different habitats (Heimpel, 1967; Goldberg and Margalit, 1977; Martin and Travers, 1989; Smith and Couche, 1991; Meadows *et al*., 1992) but its normal habitat is the soil (Dulmage and Aizawa, 1982) and our sampling was mainly focused on soils. For *B. thuringiensis* isolation, heat-acetate method (Travers *et al*., 1987) was used. The major advantage of this method over traditional methods, which are based merely on heat treatment, is acetate usage. Acetate

Table 1. Biochemical types of *B. thuringiensis* strains[a].

Biochemical type (described subsp)	Biochemical and physiological test result[b]			
	Hydrolysis of		Utilization of	
	Esculin	Lecithin	Sucrose	Salicin
thuringiensis	+	+	+	+
kurstaki	+	+	-	+
indiana	+	-	+	+
galleriae	+	-	-	+
aotto	+	+	+	-
dendrolimus	+	+	-	-
morrisori	+	-	+	-
darmstadiensis	+	-	-	-
ostriniae	-	-	-	+
israelensis	-	+	-	-

[a] Martin and Travers, 1989
[b](+) Positive reaction; (-) Negative reaction

is known to inhibit germination of *B. thuringiensis* spores, so other spores germinate and then the growing cells and other non-spore-forming bacteria are killed by heat treatment. Using this method, 127 *B. thuringiensis* strains were isolated from 514 soil samples (Table 2). The total number of dead larvae examined was 14 (Table 2) among which 12 larvae, including 10 *Heliothis sp.* from Khorassan Province and two unknown larvae from Tehran Province, contained *B. thuringiensis.* On LB and T3 media, the putative *B. thuringiensis* isolates produced flat, dry, white colonies with uneven borders.

The main criterion for *B. thuringiensis* differentiation from other soil spore-forming bacteria was crystal production by sporulating cultures (Lecadet *et al*., 1999). However, for further confirmation, a number of biochemical tests was also used. The results of biochemical tests indicated that all the isolates except two (one starch minus and one lactose plus) followed the described pattern of reactions to the negative and positive tests. By re-screening crystal in these two strains, they were positively identified as *B. thuringiensis*. Minor variations in the biochemical reactions of bacteria to biochemical tests could occasionally be observed and are negligable (Lecadet *et al*., 1999).

Crystal Morphology

Most strains produced atypical crystals, often heterogenous in size and shape. Only a low percentage of th strains (17%) formed typical, bipyramidal crystals. Abundance of heterogenous crystals in *B. thuringiensis* strains has already been reported by Lecadet *et al*. (1999) who found more than 50% of *B. thuringiensis* strains produce irregular or heterogenous crystals. The protein profiles of heterogenic crystals consist of many poorly defined components which could be a source of novel insecticidal properties (Juarez-Perez *et al*., 1994; Burtseva *et al*., 1995; Chaufaux *et al.*, 1997). Few strains, including two strains isolated from dead *Heliotis* sp. larvae, produced bipyramidal crystal similar to those of *B. thuringiensis* subsp. *kurstaki*, isolated from the commercial anti-lepidopteran product, Dipel®. It has already been demonstrated that some bipyramidal or cuboid crystals are active against lepidopteran species and, thus, crystal morphology may reflect a kind of specificity towards special pest (Lecadet *et al*., 1999). In a worldwide *B. thuringiensis* isolation program, 47% of the bipyramidal crystal-forming isolates were found to be toxic to lepidopteran, 1% to dipteran, 0.5%

Table 2. Distribution of *B. thuringiensis* in soil and larvae samples by province.

Province	Place collected	No. soil samples	No. dead larvae [c]	No. positive soil samples	Total no. isolates	*Bt* index (%) [a]
Khorassan	Agricultural	84	12	3	43	5.1
	Non-cultivated	14	0	2	5	
Lorestan	Mountains	24	0	0	0	0
	Agricultural	10	0	0	0	
Tehran	Agricultural	41	0	1	3	
	Urban [b]	33	2	1	19	4.5
	Non-cultivated	14	0	2	4	
Ghazvin	Agricultuiral	46	0	1	7	4.5
	Non-cultivated	21	0	2	12	
West Azarbaijan	Agricultural	54	0	2	17	3.6
	Non-cultivated	29	0	1	9	
East Azarbaijan	Agricultural	27	0	1	3	2.8
	Non-cultivated	9	0	0	0	
Hamedan	Agricultural	31	0	1	3	3.2
Mazandaran	Forest	47	0	1	2	1.8
	Beach	10	0	0	0	
Total	All locations	514	14	16	127	3.1

[a] Correspods to percentage of positive soil samples of each province to total number of soil samples of that province.
[b] An introduced rather than a native plant community.
[c] Dead larvae collected from Khorassan Province were *Heliothis* sp. and those from Tehran Province were unknown.

to both diptera and lepidoptera and 34% non-toxic; thus a definite relationship between crystal morphology and toxicity could not always be concluded (Martin and Travers,1989). Thus crystal morphology, although important, may not always predict toxicity.

Bt Frequency

Total *Bt* frequency was calculated as 3.1%. The highest numbers of positive soil samples were collected from Khorassan Province which gave rise to a *Bt* frequency of 5.1%, and the lowest *Bt* frequency was recorded 0% from Lorestan Province (Table 2). In order to clarify the relationship between plant community and *Bt* frequency two opposite soils, including those having plant community (agricultural, urban and forest) and those with little to no plantation (non-cultivated, beach, mountains), were studied. The results indicated that *Bt* frequency of the planted locations was 3% while that of plant-poor areas was 5.3% indicating that a high level of plantation is not a prerequestic for high *B. thuringiensis* occurrence. This result is in contrary to the general opinion that, due to the existence of a close relationship between insects and plants, *B. thuringiensis* could more frequently be found in planted places. Martin and Travers (1989) in a worldwide program on *B. thuringiensis* isolation, found more *B. thuringiensis* isolates in those environments with no detectable insects and plantations than those with high plant communities. It is known that the normal environment of *B. thuringiensis* is soil (Dulmage and Aizawa, 1982) but the bacterium is not normally toxic to insect larvae that live in the soil such as black cutworms, corn root worms, Japanese beetles or wireworms. Also, insects such as fireants, lice and ticks, that are frequent

residents of planted land, are not affected by the bacterium. However, the bacterium is mostly toxic to insects that have aerial or water-born larval stages, such as cabbage loopers, gypsy moths and mosquitoes (Martin and Travers, 1989). Whether the bacterium makes a crystal toxin for insects so that it very rarely contacts them or it makes the crystal for some other purpose than to kill these insects, remains to be clarified. Demonstration of the purpose of the crystal production may lead to clarification of the fundamentals of crystal toxicity .

Biochemical Typing

Using the biochemical typing method, all the *B. thuringiensis* strains isolated were divided into eight biochemical types (Table 3). In some cases, an undescribed combination of biochemical tests was yielded which were referred to by numbers. The usual methods for identifying *B. thuringiensis* by the serotyping of flagellar antigens (Bonnefoi and de Barjac, 1963) is expensive and requires a complete set of specific antibodies. However, a set of biochemical tests was developed for the rapid identification of different biochemical types of *B. thuringiensis* isolates (Martin *et al.,* 1985). This system is based on the biochemical tests that have been published for known varieties for which the serotypes have been identified (de Barjac, 1981), and have been used for *B. thuringiensis* classification in many investigations (Dow and Lonc, 1999; de Barjac and Frachon, 1990; Elubieta *et al*., 2001).

Based on biochemical typing, *B. thuringiensis* subsp. *kurstaki* (Es^+ Sa^+ Le^+ Su^-, lepidopteran-specific), was the most common biochemical type in Iran and it constituted 38% of the whole isolates. Based on biochemical typing, the abundance of *B. thuringiensis* subsp. *kurstaki* in Asia and New Zealand has already been demonstrated, whereas *B. thuringiensis* subsp. *israelensis* (Es^- Sa^- Le^+ Su^-, dipteran-specific) was the most common biochemical type occurring in the United States, Europe, Africa and Central America (Martin and Travers, 1989).

There are many *B. thuringiensis* characrterization methods, among which biochemical typing was used in this study. Although this method in general does not make any distinction at a fine taxonomic level and does not exactly imply specific larvacidal activity, it may provide complenmentary information for more reliable identification and compaprative studies (Swiecicka and De Vos, 2003). In general, due to the presence of multiple genes per strain, variable gene families in a given serotype, differing expression levels of the genes and different activities after solubilization in/of the larval gut, an exact correlation between the insecticidal

Table 3. Frequency of *B. thuringiensis* iochemical types in Iran.

Biochemical type[a] (described subsp) thuringiensis	Number (%) of isolates 10 (8)	Biochemical type (described subsp) morrisori	Number (%) of isolates 3 (2)
kurstaki	48 (38)	darmstadiensis	0 (0)
indiana	9 (7)	ostriniae	0 (0)
galleriae	0 (0)	israeliensis	18 (14)
aotto	0 (0)	1	5 (4)
dendrolimus	21 (17)	2	12 (10)

[a] Numbers correspond to undescribed combinations of biochemical types; *i.e*., number 1 is Es^- Sa^- Le^- Su^+ and number 2 is Es^- Sa^- Le^+ Su^+.

activity and most characterization methods (such as biochemical typing, flagellar serotyping, plasmid and protein profiling, monoclonal antibodies and hybridization or PCR of known *cry* genes) has not been always determined (Martin and Travers, 1989; Porcar and Juarez-Perez, 2003; Swiecicka and De Vos, 2003; Schnepf *et al*., 2005).

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جمع‌آوری و بررسی سوش‌های بومی *Bacillus thuringiensis*

م. کشاورزی

چکیده

باکتری باسیلوس ترونژین سیس مهمترین عامل کنترل میکروبی آفات در سراسر جهان می‌باشد که ۹۵٪ کل تولیدات تجاری آفت‌کش‌ها را به خود اختصاص داده است. به لحاظ تنوع خصوصیات آفت‌کشی جدایه‌های مختلف بی‌تی، تلاشهای زیادی به منظور یافتن جدایه‌های جدید با خواص نوین سمی در سراسر جهان صورت گرفته است. در این راستا در تحقیق حاضر از خاک و لارو مرده حشرات از استانهای تهران، قزوین، آذربایجان شرقی، آذربایجان غربی، مازندران، همدان و لرستان ۵۱۴ نمونه‌برداری صورت گرفت. جداسازی اولیه بی‌تی از سایر باکتری‌های اسپورزا بر اساس متد Acetate-heat selection صورت گرفت و باکتریها به‌وسیله روشهای بیوشیمیایی شناسایی و تأیید شدند. شاخص بی‌تی که عبارت از درصد تعداد جدایه‌ها بر تعداد نمونه‌ها در کل مناطق یا در هر استان بود، محاسبه شد. براساس نتایج، در مجموع ۱۲۷ جدایه از سراسر کشور جداسازی شد که درصد زیادی (بیش از ۵۰٪) از جدایه‌ها دارای کریستالهای غیر تیپیک و هتروژن در اندازه و شکل بودند. پاسخ کلیه جدایه‌ها به تستهای هیدرولیز نشاسته، ژلاتین، تخمیر گلوکز و فروکتوز مثبت و به تست‌های تولید اندول، تولید H2S و تخمیر گالاکتوز و لاکتوز منفی بود. جدایه‌ها به ۸ تیپ بیوشیمیایی تقسیم شدند. شاخص کل بی‌تی معادل ۳/۱٪ براورد شد. بالاترین شاخص بی‌تی متعلق به استان خراسان (معادل ۵/۱٪) و کمترین آن استان لرستان (۰٪) بود، اما رابطه مستقیمی بین فراوانی پوشش گیاهی و شاخص بی‌تی به‌دست نیامد.