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https://doi.org/10.5109/1854015

出版情報：九州大学大学院農学研究院紀要. 62 (2), pp.419-428, 2017-09-08. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **62** (2), 419–428 (2017)

Characteristics of New *Bacillus thuringiensis* Strains Isolated from Soils in the Vicinity of the Baekdu Mountain and Biological Activity against *Spodoptera exigua*

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(*Received April 28, 2017 and accepted May 10, 2017*)

From among 16 *B. thuringiensis* strains collected and isolated from soils in the vicinity of the Baekdusan mountain, four strains (YN1–1, YN2–6, YN4–2, and YN6–2) that showed high LC95 values for *Spodoptera exigua* 3rd larvae of 2.43×10^6, 7.00×10^6, 3.26×10^6, and 2.62×10^6 cfu/ml, respectively, were finally selected. SDS–PAGE experiments were conducted to identify the toxoprotein patterns of the new four strains and, in the results, all the strains showed major bands of ca. 130 kDa, which were changed into ca. 65 kDa through digestion by *Spodoptera exigua* midgut fluid. To identify the Cry gene compositions of the strains, plasmid DNAs were extracted and PCR was conducted using specific primers (cry1Aa–cry1L). Six genes (cry1Aa, cry1Ab, cry1C, cry1D, cry1I and cry1L) were identified in three strains: YN1–1, 2–6, and 4–2 and the amplification of five genes; cry1Aa, cry1Ac, cry1C, cry1D and cry1L in the YN6–2 strain. All four *B. thuringiensis* strains were identified as *B. thuringiensis* subsp. *aizawai* strains by comparing the H serotypes of 16S rRNA and flagellin (*fliC*) gene.

The possibility of commercialization of YN1–1 strain was examined by insecticidal activity test for the *Spodoptera exigua* 3rd larvae of six *B. thuringiensis* pesticide products sold in Korea.

Key words: *Bacillus thuringiensis*, Bioassay, Biological control, Isolation, *Spodoptera exigua*

INTRODUCTION

Spodoptera exigua has been currently reported to be a polyphagous pest that id not only widely distributed in the tropical, subtropical, and temperate regions (Mochida and Okada, 1974), but also lives in a wide host range, harming almost all plants such as vegetables, flowers, fruit trees, field crops, and special purpose crops. Its host plants are known to reach 52 species in South Korea alone (Goh *et al*., 1991) and ca. 140 species globally (Minamikawa, 1937; Mochida and Okada, 1974). *S. exigua* usually occurs 4–5 times per year. In the case of the southern area, it occurs from early June to late November in the southern area, many of its imagoes appear between later July and late October, and its peak is known to be around mid–August (Goh *et al*., 1991; Kim *et al*., 1995a, b; Park *et al*., 1991). In South Korea, damage due to *S. exigua* was first identified in 1986 in Jin–do, Jeonnam (Goh *et al*., 1993; Park *et al*., 1991) and it has occurred massively throughout the country from 1998, causing serious damage (Ahn *et al*., 1989; Han *et al*., 2013). In addition, the number of generations occurred has been increasing due to temperature rises and the expansion of protected cultivation areas; furthermore, the number of cases of occurrence has been increasing due to the possibility of overwintering increased caused by cold resistance mechanisms (Kim and Kim, 1997).

To control *S. exigua*, chemical control using pesticides, biological control using natural enemies, attracticide using sex pheromones, and mating disruption are used (Jung *et al*., 2003; Kim *et al*., 2004; Yoo *et al*., 1995). Although chemical control is mainly conducted using chemical pesticides, such as organophosphorus, carbamate, and pyrethroid pesticides (Eveleens *et al*., 1973), the 3rd or later generation larvae cannot be easily controlled by chemically due to the emergence of high resistance to organosynthetic pesticides (Meinke and Ware, 1978). Therefore, biological control has been introduced to control such resistant and hardly controllable pests.

Control agents using *Bacillus thuringiensis* are the most frequently agents used for biological control. *B. thuringiensis* that forms spores and parasporal crystals in rod cells was first isolated in 1901 form the body of ill silk worm larvae in Japan. *B. thuringiensis*'s insecticidal crystal proteins come in diverse forms, such as bipyramidal forms, spherical froms, and indeterminate forms. In general, bipyramidal form crystal proteins show high toxicity to Lepidoptera larvae (Jin *et al*., 2009). Based on their insecticidal ranges and similarity in gene sequences, those insecticidal crystal proteins are divided into two types. Cry proteins show toxicity in the bodies of organisms in the case of Lepidoptera, Diptera, and Coleoptera larvae, while Cyt proteins show toxicity in the bodies of organisms in the case of only Diptera larvae, but have a wide range of insecticidal activities outside the bodies of organisms. Raymond *et al*. (2010) newly isolated and disclosed 149 cry proteins (Cry1–Cry51) and 9 Cyt proteins (Cyt1–Cyt2). Among them, the Cry1

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protein shows insecticidal activities against Lepidoptera larvae (Bravo *et al*., 2005).

Globally, products released using *B. thuringiensis* include Gomelin, Bactospeine (*B. thuringiensis*), Dendrobacillin (*B. thuringiensis* subsp. *dendroliomus*), Thuricide, Dipel, Bathurin82'S' (*B. thuringiensis kurstaki*), and Thuricide90TS (*B. thuringiensis galleriae*) (Travis and M. O'Callaghan, 2008). These products have been used since the 1920s to control diverse Lepidoptera larvae (Fangneng *et al*., 2001; McWhorter *et al*., 1972). South Korean products include Solvitchae, Tobagi, Geumulmang, and Shuricide, which were registered for Lepidoptera pests such as *S. exigua*, *Plutella xylostella*, *Pyrausta panopealis*, *Dendrolimus spectabilis*, *Acrolepiidae*, *Phyllonorycter ringoniella*, and *Palpita indica Saunders* (Kil *et al*., 2007).

In the present study, the characteristics of *B. thuringiensis* strains isolated from soils in the vicinity of the Baekdusan mountain are reviewed. Since old larvae of *S. exigua* cannot be controlled because individuals in different stages of development,t ranging from eggs to imagoes, are mixed up due to the generation characteristics of *S. exigua*, more active new *B. thuringiensis* strains were selected. The protein characteristics of the newly isolated strains are investigated and their genes are analyzed. In addition, in order to find out the possibility to commercialize the newly isolated *B. thuringiensis*, we conducted experiments to compare and review the insecticidal activities of the newly isolated B. thuringiensis strains with the *B. thuringiensis* products sold in South Korea.

MATERIALS AND METHODS

Isolation of *B. thuringiensis* strains

Sixteen *B. thuringiensis* strain isolated from soils collected in the vicinity of the Baekdusan mountain were bought from the Department of Forest Science, Agricultural School, Yanbian University, China. To observe the shapes of the spores and endotoxin protein crystals of the *B. thuringiensis* strains, the *B. thuringiensis* strains were smeared on the nutrient agar medium (Difco, USA) and cultured for 4–5 days at 27°C. The **B. thuringiensis** strains proliferated and autolyzed on the medium were observed through a phase microscope (Olympus BX51) (Kim *et al*., 1995a).

After testing the biological activity, four *B. thuringiensis* strains with high activity against *S. exigua* were sent to the Micro–Ecological Resources Research Institute of Mokwon University for identification to obtain the results of 16S rRNA gene base sequence, 16S rRNA gene base sequence analysis, and the use of flagellin genes.

Experimental insect

The individuals of *S. exigua* and *S. litura* used in the present experiment were bought from the National Institute of Agricultural Sciences of the Rural Development Administration and successively cultured using artificial feed (Goh *et al*., 1990). The individuals of *P. xylostella* were collected from outdoor individual groups and successively cultured by supplying Chinese cabbage and feed; 10% sugar water was supplied to the spawning box (plastic with diameter: 20 cm, height: 15 cm) as feed for imagoes. All the insects were bred under the conditions of temperature 25 ± 1°C, light condition 16L:8D, and relative humidity of 50–60%. The individuals of *Helicoverpa armigera* used in the experiment were directly collected from a chili field in Yesan, Chungnam.

Biological activity test

The *B. thuringiensis* strains were inoculated on a nutrient agar medium, cultured for 5 days at 27°C, and bacteria were collected after identifying the occurrence of autolysis through a phase microscope. After centrifugation, the supernatant was removed and 2 ml of sterilized water were added to the remaining pellet to use the bacterial solution corresponding to a concentration of ca. 1.0×10^8 in the biological assay.

To test the biological activity of the *B. thuringiensis* strains against *S. exigua* and *S. litura*, 200 μl of the diluted solution corresponding to the concentration of ca. 1.0×10^8 (cfu/ml) were sprayed on every 1 g of the artificial feed; the artificial feed was inoculated with five 3rd larvae per 1 g. In the case of *H. armigera*, 2 × 2 cm sized pieces of chili were immersed in a diluted solution corresponding to the concentration of ca. 1.0×10^8 (cfu/ml), dried in the shade, and inoculated with five 3rd–5th larvae per piece to observe the larvae. These processes were repeated three times in total and the mortality of the larvae was investigated for a total of 120 hours in the units of 24 hours. To test the biological activity of the *B. thuringiensis* strains against P. *xylostella*, the method presented by Tabashnik *et al*. (1990) was used with some modification of the leaf immersion method. Pieces of Chinese cabbage leaves sized 3 × 3 cm were immersed in a diluted solution corresponding to the concentration of ca. 1.0×10^8 (cfu/ml), dried in the shade, and inoculated with 10 3rd larvae per piece. These processes were repeated three times in total and the mortality of the larvae was investigated for a total of 72 hours in the units of 24 hours.

The 50% lethal concentration (LC50) was calculated using a PC program (Raymond, 1985) based on the probit calculation method presented by Finney (1971) using the mortality of the *S. exigua* larvae investigated for 5–7 sections of concentrations ranging from the concentration for complete death to the concentration for complete survival.

To compare insecticidal activities, the biological activity levels of six different products being sold in domestic markets against *S. exigua* were tested through pot experiments. In the pot experiments, evenly diluted solutions of the six products and the *B. thuringiensis* strains were smeared on lettuce in a 72 × 72 × 200 cm container, and the lettuce was inoculated with the *S. exigua* 3rd larvae for larvae observation.

SDS–PAGE analysis

To prepare subject samples, a Nutrient agar medium

Table 1. Crystal protein gene–specific primers for the PCR analysis (Cry1Aa–Cry1L)

Cry genes	Forward primer	Reverse primer	Size of product (bp)
Cry1Aa	GAGCCAAGCGACTGGAGCAGTTTACACC	ATCACTGAGTCGCTTCGCATGTTTGACTTTCTC	724
Cry1Ab	GGTCGTGGCTATATCCTTCGTGTCACAGC	GAATTGCTTTCATAGGCTCCGTC	238
Cry1Ac	TCACTTCCCATCGACATCTACC	ATCACTGAGTCGCTTCGCATGTTTGACTTTCTC	487
Cry1B	CAGAAACAACAGAACGACC	CACTTCCCCACCATCCAT	921
Cry1C	TAATCCACAGTTACAGTC	TATTATCCTCAGGCGGTAA	432
Cry1D	AAGGGAAGGAAATACAGAGC	CGAACGAACGAGATGTTAG	641
Cry1E	CAGCTATTCCTCTTTTTTCAGT	ATGAGAAGTTACACGATGCC	540
Cry1F	TACTGGCAGATTACCGTTAG	AAATGTTCGGGTGTGGTTCG	1080
Cry1G	AATCTTCATTCAGGTGCCAC	GAAAAGGTAAATGGAGTAGTAA	300
Cry1H	GGGGAGTTATTGGTCCTGAT	GTTATTGGTGTGAAAAGAGTTG	1500
Cry1I	TGAATATGTGGGGAGGACA	CTAATGGTATTTGTGTAATGCT	468
Cry1J	TTTTGGATGGGGAGAGGATA	AGCCGTCATTTCAAGTCCTG	911
Cry1K	CTCGGACTTATCCCATTCCA	TGGCTGTTCTGTCGTTTCAG	542
Cry1L	AATAGGCAACCAGAACGTGG	ACGCTGTGAAAATACGTCCC	830

was inoculated with the *B. thuringiensis* strains to culture the *B. thuringiensis* strains for 5 days at 27°C. After identifying the occurrence of autolysis through a phase microscope, the cultured *B. thuringiensis* strains were subjected to centrifugation for 10 min at 15,000 rpm and 4°C in a centrifugal tube using a PBS buffer. The supernatant was removed and the remaining pellet was washed three times with washing buffer I (500 mM NaCl, 2% Triton X–100) and two times with washing buffer Ⅱ (500 mM NaCl). The washed parasporal inclusions were added with sterilized water and preserved at −20°C (Lee *et al*., 2015).

The midgut fluid used in digestion reactions was obtained by separating the midguts of 30 5th *S. exigua* larvae kept −4°C for 10 sec using sterilized dissecting knives, putting the separated midguts into 2 ml tubes to centrifuge them for 15 min at 13,000 rpm (Jin *et al*., 2015). Only the light brown supernatant was put into Eppendorf Tubes® and kept at −20°C (Zhong *et al*., 2000).

SDS–PAGE experiments were conducted using the method presented by Laemmli (1970) with some modifications. To analyze the protein patterns of the *B. thuringiensis* strains selected in the present experiments, Eppendorf Tubes® were inoculated with parasporal inclusions 5 μl per tube, added with 50 mM NaOH aqueous solution 700 μl per tube, and left unattended at room temperature for 5 min for reactions. The reactant was centrifuged for 5 min at 13,000 rpm, 4°C; then, the supernatant was removed, 20 μl of the sample buffer was added to each tube, and the tubes were subjected to vortexes until the bacteria dissolved. After the vortexing, the Eppendorf Tubes® were immediately heat–treated for 10 min at 100°C and immersed in ice for 1 min to prevent protein denaturation. The Eppendorf Tubes® were taken out from the ice, centrifuged for 1 min at 13,000 rpm and room temperature, and the bacteria were inoculated into 12% separating gel and 5% stacking gel (Garcia–Carreno *et al*., 1993). The gels were electrophoresed, stained with 30% ethanol, 10% acetic acid, and 0.5% Coomassie Brilliant Blue G–250 and then decolorized with 30% ethanol and 10% acetic acid. To experiment *S. exigua* digestion by midgut fluids, parasporal inclusions 5 μl and midgut fluid 1 μl were mixed, stirred, and left unattended at 37°C for 15 min for reactions (Jin *et al*., 2009).

PCR analysis

To extract plamid DNA the selected *B. thuringiensis* strains were inoculated into 10 ml of Luria–Bertani medium (Difco, USA) and cultured for 16 hours under the conditions of 30°C and 220 rpm. The culture solution (1 ml) was inoculated into 100 ml of the SPY medium (0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2 yeast extract, 0.1% glucose) to culture the bacteria for ca. 4 hours until the OD_{600} measured values became 0.6–0.8. The cultured bacteria were extracted using plasmid mini prep kit ver2.0 (Biofact, Korea).

Gene–specific primer sets larvae (Porcar and Juárez–Pérez, 2003; Thammasittirong and Attathom, 2008) were used to identify the Cry genes among *B. thuringiensis* endotoxin genes (Table 1). PCR was amplified using Thermal Cycle C1000TM (BIO–RAD, USA). The sample was made by mixing the premix (Bioneer, Korea) containing buffer solution components and dNTP with template DNA 1.0 μl, primer set 1.0 μl, and distilled water 17 μl to make the final volume into 20 μl. The PCR conditions used were 95°C, 5 min, 30 cycles; 94°C, 1 min; 57°C, 1 min; 72°C, 1 min, and 72°C, 5 min; PCR was identified through electrophoresis in 1.2% agarose gel under the conditions of 100V and 30 min (Abdullah *et al*., 2009, Yang *et al*., 2011, Ye *et al*., 2009).

RESULTS AND DISCUSSION

Isolation of *Bacillus thuringiensis* strains

Due to its physiological and ecological characteristics, *S. exigua* that causes a great damage to crops cannot be easily controlled using agricultural chemicals; the lack of an appropriate control method causes troubles to the recent production of environment–friendly agricultural products. Therefore, studies aiming to isolate new *B. thuringiensis* strains, i.e. microbiological control agents, and to figure out their characteristics are important. The isolation of the *B. thuringiensis* strains from soils have been tried from long ago and has been actively implemented in various countries in the world. For example, in Turkey, ca. 63.5% of the *B. thuringiensis* strains were isolated from storage warehouses and dead insects (Apaydin *et al*., 2005); in Mexico, ca. 63.5% of the *B. thuringiensis* strains were isolated from soil samples from cultivated lands (Bravo *et al*., 1998). In Colombia, ca. 60% of the *B. thuringiensis* strains were isolated from soil samples from cultivated lands and mountains (Armengol *et al*., 2007) and, in Spain, ca. 23% of the *B. thuringiensis* strains were isolated from soil samples (Quesada–Moraga *et al*., 2004). In Japan, ca. 1% of the *B. thuringiensis* strains were isolated from soils throughout the country and, in the USA, similar ratios of the *B. thuringiensis* strains were isolated and reported from soils (Ohba and Aizwa, 1978; Martin and Travers, 1989). In South Korea, it has been reported that ca. 10% of the *B. thuringiensis* strains were isolated (Kim *et al*., 1995b, 2006).

The *B. thuringiensis* strains isolated from soils in four areas (Helong Xichengzhen, Helong Longchengzhen, Helong Xichengzhen, Antu xian, and Longjing Tongfosizhen) in the vicinity of the Baekdusan mountain were bought from the Department of Forest Science, Agricultural School, Yanbian University, China (Table 2). Strains YN1–1, 2–6, 4–2, and 6–2 that show a high biological activity against S. exigua were sent to the Micro–Ecological Resources Research Institute of Mokwon University for identification. 16S rRNA genes have a large diversity according to differentiation between species and between genera and contain information that can be used to classify bacteria at the level of species. Changes in 16S rRNA gene base sequences are used as the most useful information in understanding taxonomic relationships between bacteria. Flagellin (*fliC*) gene is a specific gene in the proteins of the *B. thuringiensis* strains having H serotype, which is used for phylogenetic classification of the *B. thuringiensis* strains. According to the results of checking of changes in the 16S rRNA gene base sequences of strains YN1–1, 2–6, 4–2, and 6–2 and the H serotype of Flagellin (*fliC*) genes, these strains belong to the phylogenetic group that includes species in genus Bacillus and show phylogenetic relationships of 99.4% with the strain *B. thuringiensis* subsp. aizawai (DQ400633). Therefore, strains YN1–1, 2–6, 4–2, and 6–2 were identified as *B. thuringiensis* subsp. aizawai.

Table 2. Isolation of *B. thuringiensis* from soils at collecting areas

Number of soil sample	Locality
YN 1–1, 1–2, 1–3	Helong Xichengzhen
YN 2–1, 2–2, 2–3, 2–4, 2–5, 2–6	Helong Longchengzhen
YN 4–1, 4–2, 4–3, 4–4	Helong Xichengzhen
YN 6–1, 6–2, 6–3	Longjing Tongfosizhen

Biological activity test

To test the host ranges and insecticidal activities of each of the 16 *B. thuringiensis* strains isolated from soils in the vicinity of the Baekdusan mountain, the insecticidal activities of the strains at the stock solution concentration of 1.0×10^8 (cfu/ml) against four different pests; *P. xylostella*, *S. litura*, *S. exigua*, and *H. armigera* (Table 3). All 16 strains showed a high toxicity not below 90% against *P. xylostella*. In the case of *S. litura*, five strains (YN1–1, 2–1, 4–1, 4–4, and 6–1) showed high insecticidal activities not below 90%; eight strains (YN1–2, 1–3, 2–2, 2–3, 2–4, 2–6, 4–2, and 6–2) showed insecticidal activities of 70–89%, and the remaining three strains showed low insecticidal activities of 50–69%. In the case of S. exigua, four strains (YN1–1, 2–6, 4–2, and 6–2) showed high insecticidal activities not below 90%, while 11 strains (YN 1–2, 1–3, 2–1, 2–2, 2–3, 2–4, 2–5, 3–1, 4–1, 4–4, and 6–1) showed insecticidal activities of 70–89%; the remaining two strains showed low insecticidal activities of 50–69%. In the case of H. *armigera*, strain YN1–1 showed high insecticidal activities not below 90% and strains YN2–6 and 6–2 showed insecticidal activities of 70–89%. Therefore, strains YN1–1, 2–6, 4–2, and 6–2 with the insecticidal activities against *S. exigua* that causes the greatest damage to South Korean crops were selected.

Based on the results of the tests of insecticidal activities of the selected *B. thuringiensis* subsp. *aizawai* strains YN1–1, 2–6, 4–2, and 6–2 against *S. exigua* 3rd larvae, LC_{50} and LC_{95} values were calculated (Table 4). The LC50 values of strains YN1–1, 2–6, and 4–2 were shown to be similar as 1.57×10^5, 5.23×10^5, and 3.07×10^5 (cfu/ml), respectively. The LC_{50} value of strain YN6–2 was 1.34×10^6 (cfu/ml), which is ca, 10 times lower as compared to the three strains mentioned above. Similarly to the LC_{50} values, the LC_{95} values of strains YN1–1, 2–6, and 4–2 were shown to be 2.43×10^6, 3.26×10^6, and 2.62×10^6 (cfu/ml), respectively, and the LC_{95} value of strain YN6–2 strain was shown to be 0.7×10^7 (cfu/ml), which is ca. 10 times lower as compared to the three strains mentioned above. Based on the results of tests of insecticidal activities against *S. exigua* 4th larvae (Table 5), in the case of *S. exigua*, its chemical treatment when it exists as young larvae is considered to be the most effective control method. To identify the effective insecticidal concentrations of the four selected strains, the insecticidal activities of these strains against *S. exigua* 4th larvae at concentrations of 10^3–10^7 (cfu/ml) were tested and compared (Fig. 1). At the concentration of 10^3 (cfu/ml), strain YN1–1 showed insecticidal effects ca. 10% higher than other strains among the four strains.

Table 3. Insecticidal activities of *B. thuringiensis* isolates against lepidopteran larvae

Strains	Tested insects			
	Lepidopteran			
	P. xylostella	*S. litura*	*S. exigua*	*H. armigera*
YN 1–1	+++	+++	+++	+++
YN 1–2	+++	++	++	NT
YN 1–3	+++	++	++	NT
YN 2–1	+++	+++	++	NT
YN 2–2	+++	++	++	NT
YN 2–3	+++	++	++	NT
YN 2–4	+++	++	++	NT
YN 2–5	+++	+	++	NT
YN 2–6	+++	++	+++	++
YN 4–1	+++	+++	++	NT
YN 4–2	+++	++	+++	+
YN 4–3	+++	+	+	NT
YN 4–4	+++	+++	++	NT
YN 6–1	+++	+++	++	NT
YN 6–2	+++	++	+++	++
YN 6–3	+++	+	+	NT

+++: Highly effective, 90% lethality; ++: Effective, 70~89% lethality; +: Low effective, 50~69% lethality; −: not effective, 0~49% lethality; NT: No Test

Table 4. Toxicity of the *B. thuringiensis* YN1–1, YN2–6, YN4–2, YN6–2 isolates against *S. exigua* 3rd larvae

Strains	n	LC*$_{50}$ (cfu/ml)	LC$_{95}$ (cfu/ml)
YN1–1	200	1.57×10^5	2.43×10^6
YN2–6		1.34×10^6	7.00×10^6
YN4–2		5.23×10^5	3.26×10^6
YN6–2		3.07×10^5	2.62×10^6

* LC : Lethal concentration

Table 5. Toxicity of the *B. thuringiensis* YN1–1, YN2–6, YN4–2, YN6–2 isolates against *S. exigua* 4th larvae

Strains	n	LC$_{50}$ (cfu/ml)	LC$_{95}$ (cfu/ml)
YN1–1	200	1.33×10^6	1.98×10^7
YN2–6		1.20×10^8	4.32×10^8
YN4–2		7.04×10^6	2.96×10^7
YN6–2		1.37×10^7	5.99×10^7

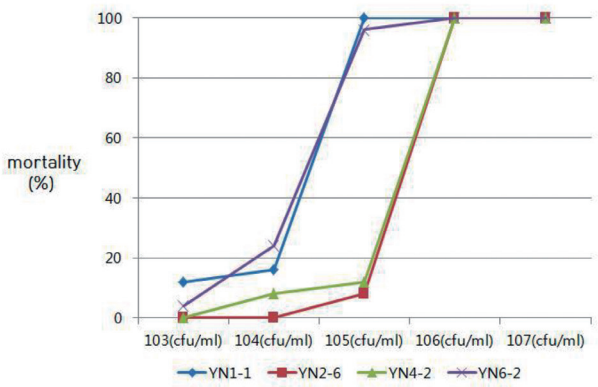

Fig. 1. Toxicity of the *B. thuringiensis* YN1–1, YN2–6, YN4–2, YN6–2 concentrations against *S. exigua* 3rd larvae.

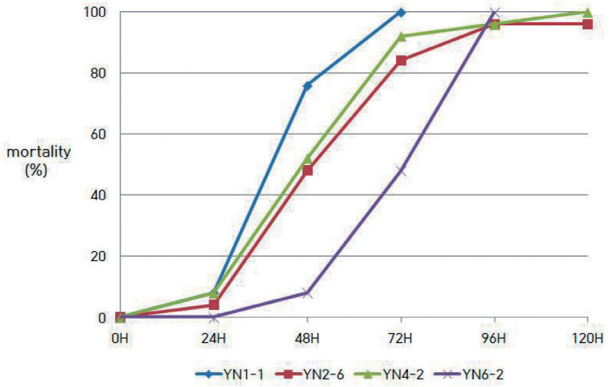

Fig. 2. Toxicity of *B. thuringiensis* YN1–1, YN2–6, YN4–2, YN6–2 treated at the same concentrations against *S. exigua* 3rd larvae.

Likewise, at the concentration of 10^5 (cfu/ml), strain YN1–1 showed the highest insecticidal effects. In addition, when *S. exigua* 4th larvae were treated with the four strains at the same concentrations to check fast–acting properties, strain YN1–1 showed 100% insecticidal rate in the shortest time (Fig. 2). Based on these results, the *B. thuringiensis* subsp. aizawai strain YN1–1 could be assumed to be the best strain for use in the biological control of *S. exigua*.

Morphological characteristics of the *B. thuringiensis* strains

Since the morphology of the *B. thuringiensis*' endotoxin proteins is closely related to the insect species to which the endotoxin proteins show insecticidal properties (Maeda *et al*., 2000), the morphology of the endotoxin proteins under phase microscopes is important information. The morphology of the crystalline endotoxin proteins of the newly isolated *B. thuringiensis* subsp. *aizawai* strains YN1–1, 2–6, 4–2, and 6–2 with high insecticidal activities against *S. exigua* was observed through a phase microscope. Based on the results, the four strains were typical bipyramidal forms as with the reference strain *B. thuringiensis* subsp. *aizawai* that has activity against *Lepidoptera* (Fig. 3). Similarly, in the case of the *B. thuringiensis* strains isolated by Kim *et al*. (2006), when the crystalline proteins were observed through a phase microscope, bipyramidal forms showed high insecticidal activities against moth pests and bipyramidal form crystals were reported to have cry1 toxin protein and have activity against *Lepidoptera* (Armengol *et al*., 2007; Donovan *et al*., 1988).

SDS–PAGE analysis

The molecular weight of the Cry1 protein that constitutes bi–pyramidal form crystals with insecticidal activities against *Lepidoptera* corresponds to ca. 130–140 kDa and the Cry1 protein is decomposed into toxoproteins with molecular weights of ca. 60–65 kDa to show insecticidal effects (Aronson, 1986). Major protein patterns of 130 kDa were identified in the *B. thuringiensis* subsp. *aizawai* strains YN1–1, 2–6, 4–2, and 6–2 that showed high insecticidal activities against *S. exigua* (Fig. 4, lane1, 3, 5, 7).

To identify the active toxin of the parasporal inclusions produced by the *B. thuringiensis* strains, the four strains were induced to react with *S. exigua* midgut fluids and subjected to electrophoresis. According to the results, all four strains were decomposed into proteins of approximately 65 kDa that show insecticidal activities (Fig. 4, lane2, 4, 6, 8). Based on these results, how the Cry1 protein shows insecticidal activities in the midguts of the *S. exigua* larvae could be estimated.

***B. thuringiensis* strain Cry gene analysis**

The PCR analyses were conducted to identify the cry genes in the *B. thuringiensis* strains. The *B. thuringiensis* subsp. aizawai line was reported to have four genes: cry1Aa, cry1Ab, cry1C, and cry1D (Aronson *et al*., 1991; Höfte and Whitely, 1989; Visser *et al*., 1988, 1990). The cry1 genes with insecticidal activities against *S. exigua* were expected to be cry1Aa, cry1Ab, cry1C (de Maagd *et al*., 2003), cry1D (Bravo *et al*., 1998; Lee *et al*., 2001; Porcar *et al*., 2000), and cry1F (Hernández and Ferré, 2005; Luo *et al*., 1999). In addition, it has been reported that the cry1 gene shows activity against

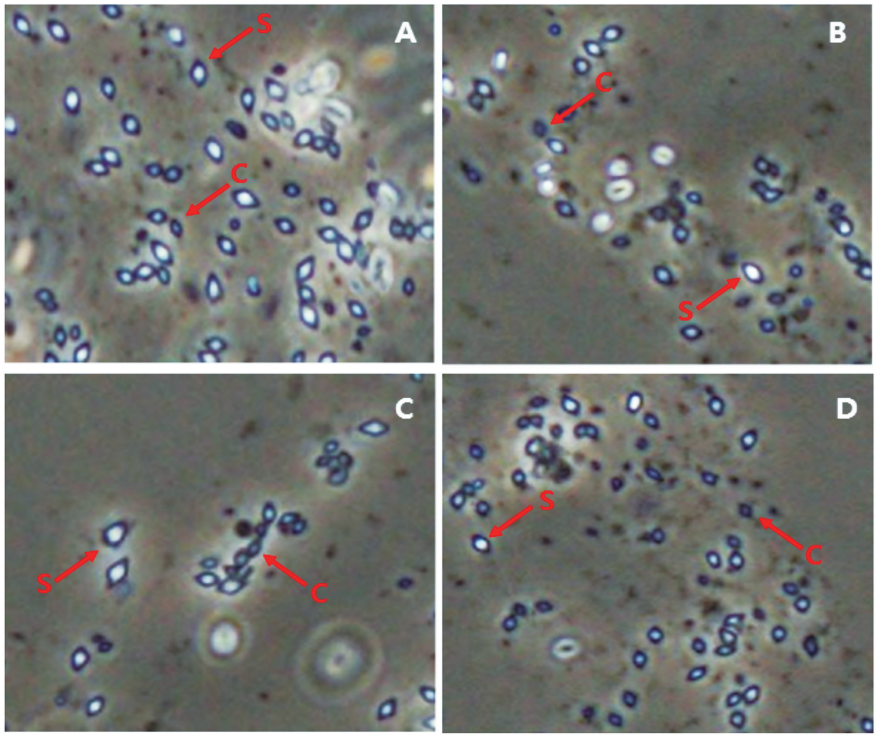

Fig. 3. Phase–contrast microscope photographs (×1,000) of crystal shapes of *B. thuringiensis*. (A): YN1–1; (B): YN2–6; (C): YN4–2; (D) YN6–2. C: crystal; S: spore

Lepidoptera (Tamez–Guerra *et al*., 2004; Zhong *et al*., 2000).

The cry genes of *B. thuringiensis* subsp. *aizawai* strains YN1–1, 2–6, 4–2, and 6–2 that were selected in the present study were identified (Fig. 5). Strains YN1–1, 2–6, and 4–2 were identified as having Cry1Aa, Cry1Ac, Cry1C, Cry1D, Cry1I, and Cry1L genes and strain YN6–2 showed amplification of Cry1Aa, Cry1Ac, Cry1C, Cry1D, and Cry1L genes. It could be assumed that all the four strains have high insecticidal activities against *S. exigua* due to Cry1 genes. The effects of Cry1I gene could not be identified in the present study.

Tests and comparison of biological activities of *B. thuringiensis* pesticides

To review the possibility to commercialize the *B. thuringiensis* subsp. *aizawai* strain YN1–1 that was newly isolated from soils in the vicinity of the Baekdusan mountain, the biological activities of six *B. thuringiensis* pesticide products sold in South Korea against *S. exigua* 3rd larvae were tested (Table 6). Artificial feed was treated with the chemical fluids according to the indicated doses of the products; according to the results, whereas products A showed insecticidal activities exceeding 70%, the remaining four products showed large differences in insecticidal activities, ranging from 54.4% to 4.4%. Although the *B. thuringiensis* strains directly act as intestinal toxins to kill insects, they also have the insecticidal mechanism of inhibiting feeding to induce death from starvation (Broderick *et al*., 2006). Lettuce was treated with the *B. thuringiensis* products to identify the foregoing mechanism and the differences in the quantities of feeding between the product group with high insecticidal activities; the product group with low insecticidal activities could be visually identified based on previous experimental results (Fig. 6).

Recently, with the increase of the comfort of human living, consumers' demands for safety of agricultural products have increased and environment–friendly agriculture has developed accordingly. Consequently, the number of environment–friendly agricultural materials has rapidly grown and these materials are diversely

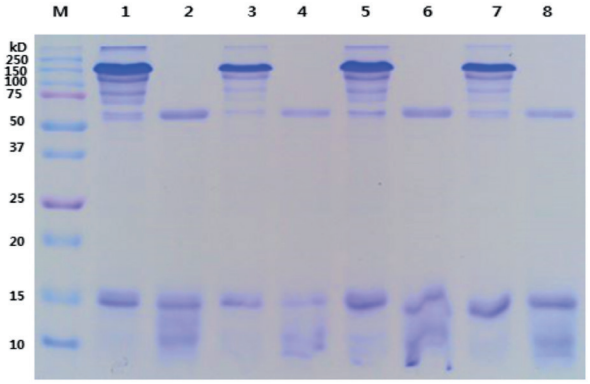

Fig. 4. SDS–PAGE analysis of parasporal inclusions of the *B. thuringiensis* YN1–1, YN2–6, YN4–2, YN6–2. M: Standard marker; lane 1: *B. thuringiensis* YN1–1; lane 2: *B. thuringiensis* YN1–1 digested with gut juice; lane 3: *B. thuringiensis* YN2–6; lane 4: *B. thuringiensis* YN2–6 digested with gut juice; lane 5: *B. thuringiensis* YN4–2; lane 6: *B. thuringiensis* YN4–2 digested with gut juice; lane 7: *B. thuringiensis* YN6–2; lane 8: *B. thuringiensis* YN6–2 digested with gut juice

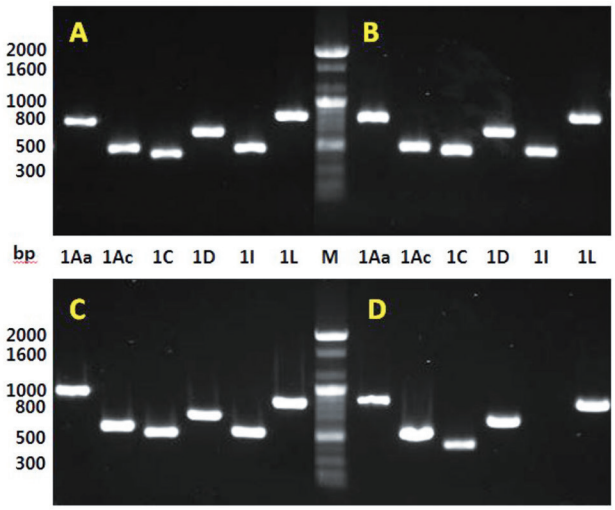

Fig. 5. Agarose gel (1.2%) electrophoresis of the PCR products obtained with specific primers for the cry genes. A: YN1–1; B: YN2–6; C: YN4–2; D: YN6–2

Table 6. Toxicity of *B. thuringiensis* products at recommended use concentraions against *S. exigua* 3rd larvae

Products	*B. thuringiensis* strains	Usage dose per 20 L of water	Mortality (%)
A	*B. thuringiensis* subsp. *aizawai*	40 ml	72.2 ± 5.1
B–1	*B. thuringiensis* subsp. *aizawai*	50 ml	41.1 ± 5.1
B–2	*B. thuringiensis* subsp. *aizawai*	100 ml	51.1 ± 3.8
C	*B. thuringiensis* subsp. *aizawai* + *B. thuringiensis* subsp. *kurstaki*	10 g	32.2 ± 3.8
D	*B. thuringiensis*	8 g	4.4 ± 1.9
E	*B. thuringiensis*	20 g	54.4 ± 3.8
F	*B. thuringiensis* subsp. *aizawai*	10 g	42.2 ± 1.9
Control	*B. thuringiensis* subsp. *aizawai*		2.2 ± 1.9

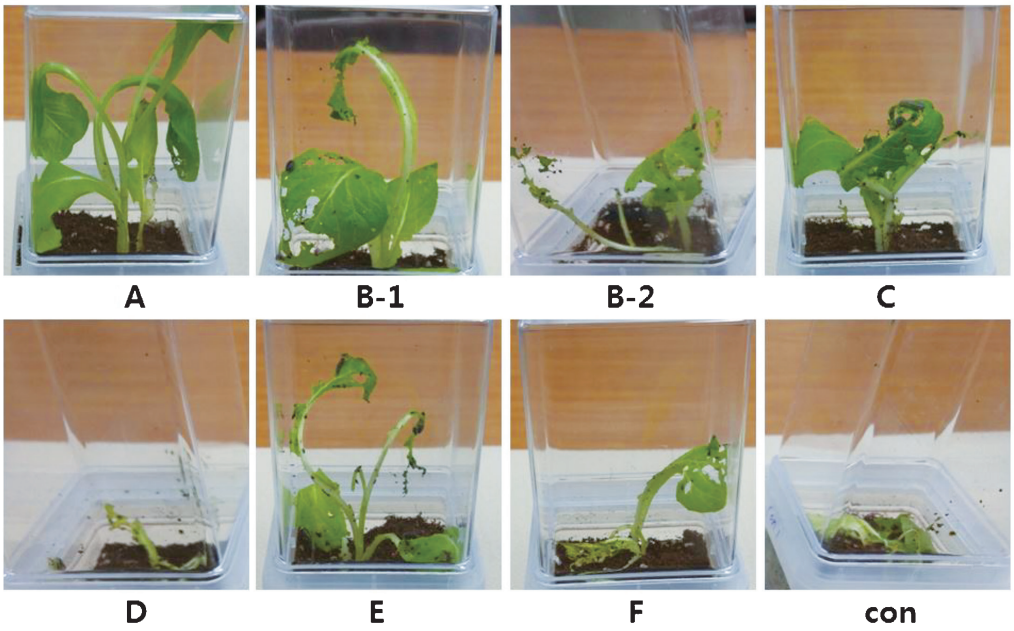

Fig. 6. Comparative toxicity of the *B. thuringiensis* products and the *B. thuringiensis* YN1–1 isolates against *S. exigua* 3rd larvae.

used; however, the results remain insufficient in terms of the effects of these materials (Kil *et al*., 2007). In the case of the *B. thuringiensis* products, the number of active spores included in the products is very important, because it determines biological effects in proportion to the number of cry proteins. Based on the observed increase of insecticidal activities as a function of dosage, priority in producing (developing) products should be assigned to biological activity, rather than to commercial (economic) aspects.

In the present study, the insecticidal effects and insecticidal agents of the newly isolated *B. thuringiensis* strains were identified through biological tests on *Lepidoptera* pests and molecular biological experiments. Since the shapes of crystals and protein patterns of the 12 strains with a little lower insecticidal activity against *S. exigua* are similar to the four identified strains, they are assumed to be *B. thuringiensis* with the same serotype. The diverse distributions of the *B. thuringiensis* strains reflecting the regional specificity of the Baekdusan mountain could not be identified. Some differences in cry proteins were identified among *B. thuringiensis* strains with the same serotype and even the strains consisting of the same cry proteins showed differences in biological activity. Finally, further research is needed to find strains with a high biological activity.

AUTHOR CONTRIBUTIONS

Yu Seop Kim designed the study, performed the comprehensive experiments, analyzed the data and wrote the paper. Na Young Jin performed biological activity test experiments. You Kyoung Lee and Hee Ji Kim bred pests. Young A Hur conducted the control value analyses. Young Nam Youn edited the paper. Chisa Yasunaga–Aoki participated in the design of the study and discussed on the experiments and the results. Dae Young Kim performed the isolation of *B. thuringiensis* strains. Yong Man Yu supervised the work and wrote the paper. All authors assisted in editing of the manuscript and approved the final version.

ACKNOWLEDGEMENTS

This work was supported by research fund of Chungnam National University.

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