



***Bacillus thuringiensis* Isolation from the Environments of Boron Mines and Effects of Boric Acid on Bioactivity**

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Abstract

Bacillus thuringiensis (Bt) isolation from the environments of boron mines, the characterization based on *cry* gene content, boron tolerance, insecticidal crystal protein production and bioactivity of Bt isolates were examined in this study. PCR analysis indicated the presence of Bt strains with *cry1* (100%), *cry2* (41%) and *cry1* plus *cry2* (41%) genes. Boron tolerance of Bt isolates grown in different boric acid concentrations changed from 25 to 75 mM. Moreover, boric acid prolonged the lag phase of the growth curve. Furthermore, Bt-KE63-64 isolate at 50 ppm caused 76% mortality against *Cadra cautella* larvae. Two protein bands at 130 kDa and 65 kDa were detected with SDS-PAGE analysis. Increasing concentration of boric acid resulted in a decrease at the level of Cry protein expression. Finally, addition of 1% boric acid to spore-crystal mixtures of Bt isolate didn't cause any additive effect on the bioactivity. In conclusion, it is the first time that Bt with high bioactivity was isolated from the environments of boron mines and boric acid tolerance of some of the Bt isolates was up to 75 mM.

1. INTRODUCTION

Gram-positive and spore-forming bacterium *Bacillus thuringiensis* has entomopathogenic effect due to its insecticidal crystal (Cry) proteins (ICP) formed during the stationary phase of the growth [1]. *cry* genes encoding the Cry proteins are generally known to be carried on plasmids [2]. ICPs were classified according to their insecticidal activities. *cry* genes encode lepidoptera specific-Cry1, lepidoptera and diptera specific-Cry2, coleoptera specific-Cry3 and diptera specific-Cry4 proteins [3].

Nowadays, ICP and spore mixtures of Bt have been widely used to control pests in agriculture as an alternative to chemical pesticides. However, commercial application of these pesticides requires economic and adequate manufacturing processes. Therefore, there have been numerous research in the literature to reach the optimum level of Bt growth, sporulation and ICP production. For instance, İçgen et al. [4] investigated the effects of different mineral elements and pH on Bt growth and crystal protein synthesis. They found that Mg and Cu were the most important metals for biosynthesis of Cry1 and Cry2 proteins. In addition, Özcan et al. [5] indicated that the pretreated poultry litter with 2 N HCl yielded 95% more Cry1 and Cry2 protein than the poultry litter pretreated with 2 N NaOH in *Bt kurstaki*. In another study, addition of tannic acid into a diet increased the efficacy of *Bt kurstaki* against *Heliothis virescens* larvae by 68-84% [6].

Boron is an intermediate element between metals and non-metals and has semiconductor property [7]. It is essential for plant growth [8], embryonic development of zebrafish [9] and frogs [10]. In addition, boron stimulates growth in yeast [11]. Furthermore, increasing evidence suggests the function of boron in animal metabolism as well [12]. However, there have been no studies on the occurrence of Bt in boron containing environments and effects of boric acid on Bt isolates. Therefore, the aim of this work was to isolate Bt from boron-related environments and to investigate *cry* gene profile, the effect of boric acid on the Cry protein production and bioactivity.

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2. MATERIALS AND METHODS

2.1. *Bacillus thuringiensis* Reference Strains

Reference strains *Bt* subsp. *kurstaki* (BGSC 4D1), *Bt* subsp. *aizawai* (BGSC 4J3), *Bt* biovar. *israelensis* (BGSC 4Q2) were obtained from Bacillus Genetic Stock Center, Columbus, Ohio, USA.

2.2. Sample Collection

Thirty-five soil samples were collected from 5 different locations in Balıkesir (Bandırma-Yıldızlar) and Kütahya (Simav-Emet-Hisarcık) boron mineral deposits and their environments. About 200 g of soil samples were collected with a sterile spatula at 10 cm depth. Samples were placed in sterile plastic bags and stored at 4°C until processed.

2.3. *Bacillus thuringiensis* Isolation

Bt strains were isolated according to the method of Santana et al. [13]. Briefly, soil samples were exposed to heat treatment for 5 hr at 80°C. After suspending the samples in saline solution, they were exposed to heat treatment again for 12 min at 80°C, plated on LB agar and allowed to grow overnight at 37°C. *Bt*-like colonies described as cream-coloured and fried egg appearance on the plates [14] were labeled and subcultured 3 times. Each pure subculture was grown on nutrient agar (NA), dispersed in sterile water and examined with phase contrast microscope for the crystal formation. Duplicate stock samples in 25% glycerol were kept at -80°C.

2.4. DNA Template and PCR Analysis

PCR analysis was carried out to determine *cry* gene content of *Bt* isolates. Five pairs of universal primers for *cry1*, *cry2*, *cry3*, *cry4* and *cry9* genes as described by Ben-Dov et al. [15,16] were used. Genomic DNA was isolated according to the method of Hansen and Hendriksen [17] and reference strains served as positive control for PCR reactions. Each amplification process was carried out in a 50 µl reaction mixture containing 200 µM dNTP, 0.5 µM of each universal primer; 1.5 mM MgCl₂, and 2 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania) in an Advanced Primus 96 Thermal Cycler (PiqLab, Erlangen, Germany). PCR conditions were as described in Alper et al. [18]. After amplification, 10 µl of each PCR product were electrophoresed on 1% ethidium bromide agarose gel and DNA bands were visualized in a gel documentation system (Vilber Lourmat, Marne-la-Vallée, France).

2.5. Boron Tolerance and Growth Curve

One single colony of each *Bt* isolate grown on NA was transferred to 5 ml PBS. After adjusting to Mc Farland 3 density (OD₆₀₀ of 0.05), the bacterial suspension was diluted to 1, 0.2, 0.06, and 0.03 densities and 4 µl of each bacterial suspension was spotted on NA containing 0,20,30,40,50,75 mM boric acid and incubated for 48 hours at 37 °C in order to determine the boric acid tolerance.

For the growth curve, each *Bt* isolate was inoculated to NA and incubated overnight at 37 °C. A single colony was transferred into 5 ml of PBS and adjusted to Mc Farland 3 density. One ml of the bacterial culture was inoculated into 50 ml LB broth containing different concentrations of boric acid and incubated at 150 rpm at 37 °C in a shaking incubator. Bacterial growth was monitored by OD₆₀₀ measurements with a spectrophotometer (Shimadzu UVmini-1240) and growth curves were drawn using Microsoft Excel program.

2.6. Extraction of Spores plus Crystals and SDS-PAGE Analysis

A procedure described in Alper et al. [18] was used for protein extraction. Spore plus crystal mixture was washed twice in 1.4 mL of 0.5 M NaCl, and resuspended in ice-cold 1.4 mL of Tris EDTA buffer (10 mM Tris, 1 mM EDTA pH 8 and 0.5 mM PMSF) and centrifuged for 10 min at 10,000 rpm at 4 °C. After that, spore plus crystal mixture was dissolved in sterile H₂O with 1mM PMSF. The protein concentration of each sample was measured by Bradford assay [19]. SDS-PAGE of each sample (5µg /lane) was performed as described by Laemmli [20].

2.7. Bioactivity

Second instar larvae of *Cadra cautella* were grown as described by Magana et al. [21] and used to test the toxic potency of boric acid and/or Bt isolates. A spore-crystal mixture was prepared according to the technique of Bravo et al. [22]. Briefly, Bt culture was grown in 100 ml of nutrient broth at 28 °C by shaking at 150 rpm for 3 days. The culture was centrifuged at 4 °C at 5000 rpm for 20 min. The pellets were washed twice with ice-cold 1M NaCl and 3 times with sterile H₂O. The pellets were dried overnight at 37 °C and stored as powder at – 20 °C until used.

Spore-crystal powders were suspended in distilled water containing 0.1% Tween80. Suspensions were mixed with a diet that included wheat bran/corn powder (3:1) at the concentration of 500 ppm (i.e., 2500 µg of spore-crystal mixture in 5 gr compost) and dried. Assays were carried out using 15 larvae per dose with three replicates. Diet without toxin served as negative control. Toxicity tests were carried out at 25°C, 70% RH with 16:8h, L:D schedule; and larval mortality was recorded after 7 days. The mortality data were corrected by Abbott's formula [23].

2.8. Statistic Analysis

The difference among the treatments in bioassay was tested by using one way analysis of variance (ANOVA). Data normality and homogeneity of variance were tested using the Shapiro-Wilk test and Bartlett's test. Post-hoc comparisons were conducted with Tukey's honestly significant difference test at $\alpha = 0.01$.

3. RESULTS

3.1. *Bacillus thuringiensis* Isolation and *cry* gene Profile

In total, 17 Bt isolates were obtained from 35 soil samples collected from the environments of Balıkesir and Kütahya boron mines. PCR analysis showed that all isolates were positive for the *cry1* genes; however, isolates carrying *cry2* genes constituted 41% of all isolates. The percentage of isolates carrying both *cry1* plus *cry2* genes was 41.2. On the other hand, *cry3*, *cry4* and *cry9* genes were not detected in any of the samples examined (Table 1).

Table 1. *Bt* isolates and *cry* gene profile

Location	Bt isolates	<i>cry</i> gene content
Kütahya-Emet	Bt-KE63-64	<i>cry1, cry2</i>
Balıkesir-Bandırma	Bt-BB99	<i>cry1</i>
Kütahya-Hisarcık	Bt-KH58	<i>cry1, cry2</i>
Balıkesir-Bandırma	Bt-Ba11	<i>cry1</i>
Kütahya-Hisarcık	Bt-KH3	<i>cry1, cry2</i>
Kütahya-Emet	Bt-KE20	<i>cry1</i>
Kütahya-Emet	Bt-KE67-68	<i>cry1</i>
Balıkesir-Bandırma	Bt-Ba14	<i>cry1</i>
Kütahya-Emet	Bt-KE30	<i>cry1</i>
Balıkesir-Yıldızlar	Bt-BY7	<i>cry1, cry2</i>
Kütahya-Hisarcık	Bt-KH51	<i>cry1, cry2</i>
Kütahya-Emet	Bt-KE50-51	<i>cry1</i>
Kütahya-Hisarcık	Bt-KH63	<i>cry1, cry2</i>
Balıkesir-Bandırma	Bt-BB48	<i>cry1</i>

Balıkesir-Simav	Bt-Bsi6	<i>cry1</i>
Kütahya-Hisarçık	Bt-KH1	<i>cry1</i>
Kütahya-Emet	Bt-KE12	<i>cry1, cry2</i>

3.2 Boron Tolerance of Bt Isolates

Boron tolerance test was performed in order to determine the boron tolerance of Bt isolates. According to the results, the isolates Bt-KE20 and Bt-KE63-64 tolerated 20 mM and 30 mM boric acid, respectively. The majority of the isolates could grow in medium containing 50 mM boric acid (Table 2). Only 3 isolates, Bt-KH1, Bt-KE12 and Bt-KE67-68 had ability to grow in 75 mM boric acid concentration.

Table 2. Boron tolerance of Bt isolates

Isolate name	0 mM	20 mM	30 mM	40 mM	50 mM	75 mM
Bt-KE63-64	++++	++++	+ + - -	- - - -	- - - -	- - - -
Bt-BB99	++++	++++	++++	++++	++++	- - - -
Bt-KH58	++++	++++	++++	++++	++++	- - - -
Bt-Ba11	++++	++++	++++	++++	++ * *	- - - -
Bt-KH3	++++	++++	++++	++++	++++	- - - -
Bt-KE20	++++	++++	- - - -	- - - -	- - - -	- - - -
Bt-KE67-68	++++	++++	++++	++++	++++	* * * *
Bt-Ba14	++++	++++	++++	++++	++++	* * - -
Bt-BY7	++++	++++	++++	++++	+++ *	- - - -
Bt-KH51	++++	++++	++++	++++	+++ *	- - - -
Bt-KE50-51	++++	++++	++++	++++	+++ *	- - - -
Bt-KH63	++++	++++	++++	++++	+++ *	- - - -
Bt-BB48	++++	++++	++++	++++	+++ *	- - - -
Bt-Bsi6	++++	++++	++++	++++	+++ *	- - - -
Bt-KH1	++++	++++	++++	++++	+++ *	* * * *
Bt-KE12	++++	++++	++++	++++	+++ *	* * * *
<i>B.t. kurstaki</i>	++++	++++	++++	++++	* * * *	- - - -
<i>E. coli</i>	++++	++++	++++	++++	++++	++++
<i>P. tolasii</i>	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -

+ : Strong growth; * : Weak growth; - : No growth

Based on the growth curve, Bt-KE63-64 isolate could grow up to 25 mM boric acid concentration and lag phase of the growth cycle was found to be 2 times longer than that of the isolate grown in boric acid-free medium. However, in both medium, the Bt isolate entered stationary phase at the same time (Figure 1A). Similarly, *Bt kurstaki* (4D1) entered into log phase immediately in boron-free medium, but lag phase of *Bt kurstaki* took 3 hr and 9 hr at 25 mM and 50 mM boric acid, respectively (Figure 1B). Other isolates also showed the similar pattern of growth curve at 25 mM boric acid whereas initiation of log phase changed from isolate to isolate at 50 mM boric acid (Data not shown).

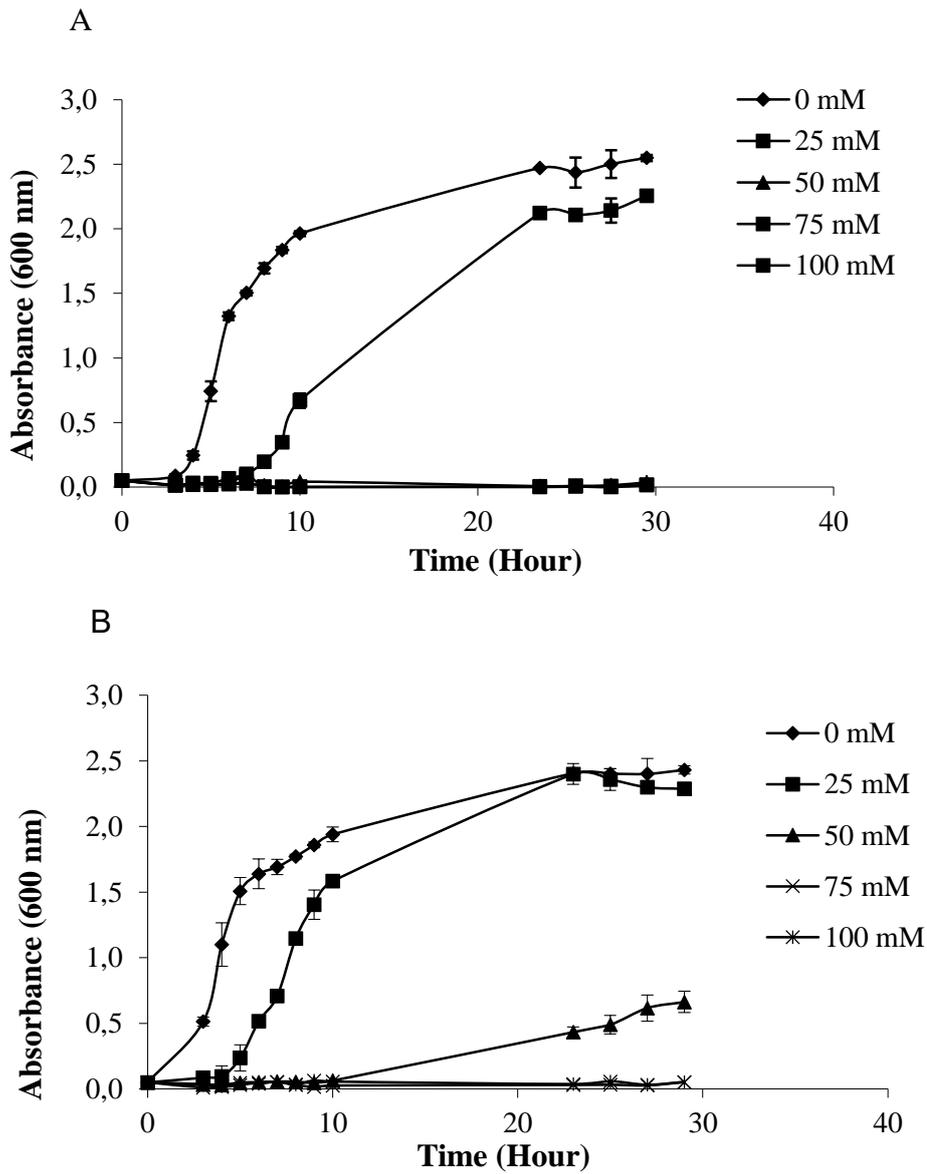


Figure 1. Growth curve of *Bt-KE63-64* isolate (A) and *Bt kurstaki* (B) grown in different boric acid concentrations.

3.3.The Effect of Boric Acid on Cry Protein Production

Bt-KE63-64 isolate and *Bt kurstaki* (4D1) were grown in medium containing 0-5-10-20-40 mM boric acid and the amount of protein production was determined by Bradford analysis. The highest protein level was observed in the boric acid-free medium. However, the level of protein production decreased gradually by increasing amount of boric acid in the culture medium (Figure 2A, 2B). For example, the concentration of protein was reduced approximately by twofold in the presence of 5 mM boric acid. The boric acid concentration at 40 mM gave rise to ninefold and fourfold decrease in the amount of protein produced by *Bt kurstaki* (4D1) and *Bt-KE63-64* isolate, respectively.

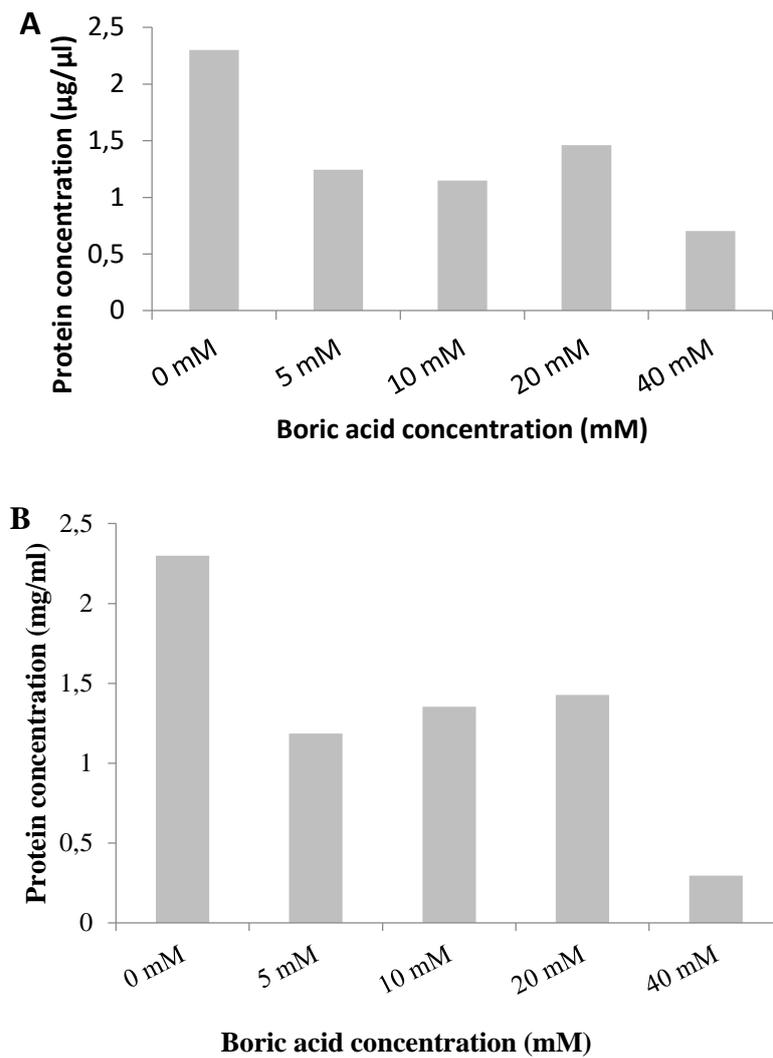


Figure 2. Determination of Cry protein level of *Bt-KE63-64* isolate (A) and *Bt kurstaki* (B) grown in different boric acid concentrations.

SDS-PAGE analysis exhibited 2 main protein bands, 130 kDa for Cry1 and 65 kDa for Cry2 proteins (Figure 3A, 3B). Increased concentration of boric acid caused a decrease in the amount of the Cry proteins produced. The most significant decrease was observed in the medium containing 40 mM boric acid. This is most likely to be due to a decrease in the number of bacterial cells that were able to survive in the high boric acid concentration as well as due to the retardation of log phase.

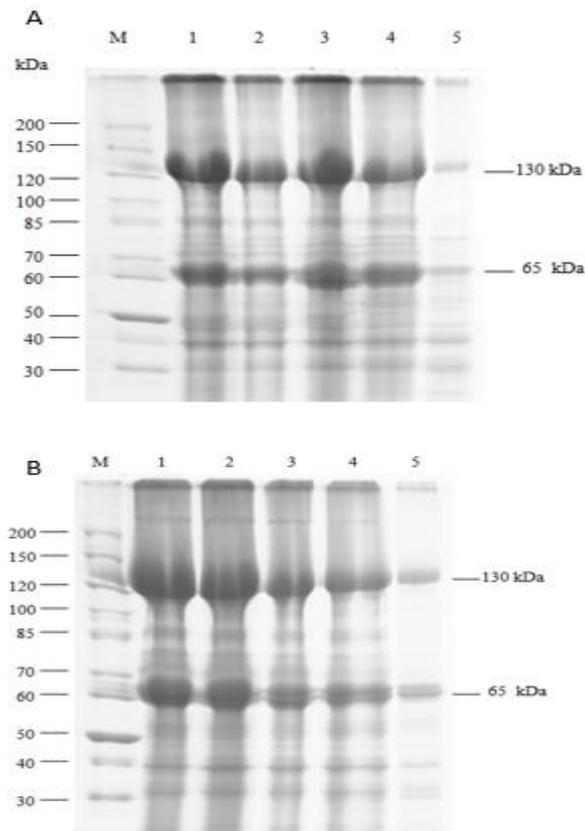


Figure 3. SDS-PAGE analysis of Cry proteins from *Bt-KE63-64* and *Bt kurstaki* grown in different boric acid concentrations. *Bt-KE63-64* (A) or *Bt kurstaki* (B). M: marker; lane 1: 0 mM; lane 2: 5 mM; lane 3: 10 mM; lane 4: 20 mM; lane 5: 40 mM.

3.4. The Effect of Boric Acid on Bioactivity

Bioactivity of spore crystal mixture of *Bt-KE63-64* isolate or in combination with boric acid were tested on *C. cautella* larvae. In our previous study, we showed that 9MY and 44 MY isolates exhibited low bioactivity (10% and 30% mortality), whereas 42MY isolate indicated high bioactivity (97% mortality) on *C. cautella* larvae at 500 ppm [18] and they were used as control in this present study. *Bt-KE63-64* isolate was chosen for bioactivity assay, because it had the highest incidence of crystal inclusions among the other isolates in comparison with reference strain 4D1 by phase contrast microscopy. Figure 4 indicated that none of the boric acid treatments alone was statistically ($p < 0.01$) different from the control. Addition of 1% boric acid to the spore-crystal mixtures of 9MY (50 ppm) and 44MY (50 ppm) isolates, did not cause any increase in the bioactivity compared to spore-crystal mixture alone (Figure 4). Spore-crystal mixture of 42MY (50 ppm) showed toxic effect over 80%. However, no statistically significant difference was observed when this toxin was applied with 1% boric acid ($p < 0.01$) (Figure 5). In addition, *Bt-KE63-64* isolate (50 ppm) alone showed 76% toxic effect on *C. cautella* larvae. Similarly, there was no statistical difference between bioactivity of 50 ppm spore crystal mixture of *Bt-KE63-64* and that of combination with 1% boric acid treatment (Figure 5). The results showed that, 1% boric acid, applied to *C. cautella* larvae with Bt toxin had no additive effect on the mortality.

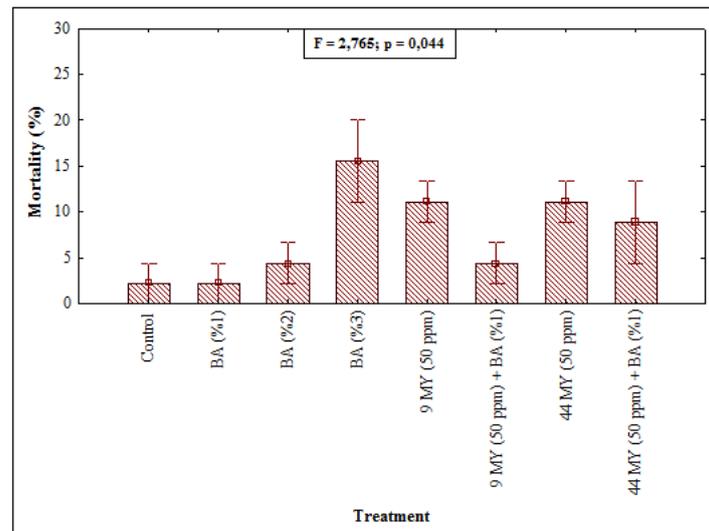


Figure 4. Mortality of Bt spore-crystal mixture in combination with different boric acid concentrations on *C. cautella* larvae.

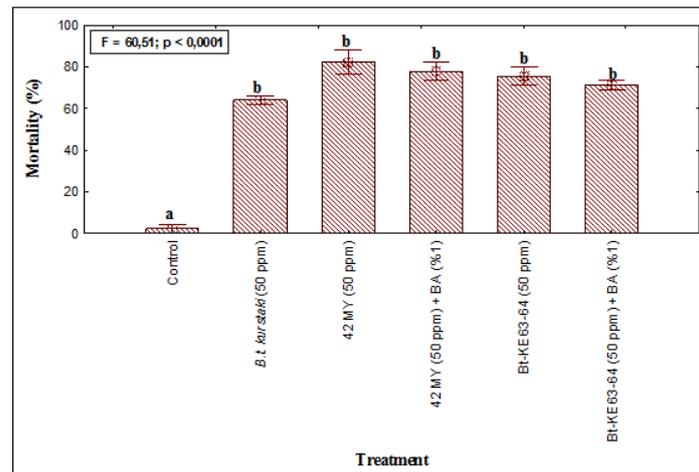


Figure 5. Mortality of Bt-KE63-64 or Bt kurstaki spore-crystal mixture in combination with boric acid on *C. cautella* larvae. Values sharing the same letter are not significantly different at $p < 0.01$.

4. DISCUSSION

In recent years, Bt based biopesticides have been widely used in agricultural applications. However, in order to overcome the development of insect resistance against Bt preparations, isolation of new Bt strains from different geographical environments is important. In addition, identification of the optimum growth conditions and improving the bioactivity of Bt strains are important in terms of the pesticide production at the economic level. Boron element has been known to be required for many organisms. Although there have been studies on the metabolic function of boron on plants and animals, there are very few data on Bt in the literature. Therefore, Bt isolation from boron containing environment and the effects of boric acid on these Bt isolates were carried out in this study.

Isolation of 17 Bt strains from 35 soil samples implies that Bt may have ability to tolerate the boron element available at different concentration. Range of the boron level among soil samples from Eskisehir-Kırka boron mines and environments was found to be between 1 ppm and 75000 ppm [24]. Thus, these isolates may serve as an important source for investigation of boron metabolism in bacteria. Substantial work in the literature examined the distribution of *cry* genes and showed that occurrence of *cry1* and *cry2* genes are higher than that of the other *cry* genes [22, 25, 26, 27]. Similarly, when the *cry* gene distributions of the 17 Bt isolates from boron containing environments was analyzed, the presence of *cry1* and *cry2* genes was

detected. In other words, soil from boron containing environment didn't play a negative role on the presence of plasmids carrying these genes among Bt strains. In addition to boron, pH of soil, nutritional and other environmental factors may play an important role in the presence or absence of the plasmids carrying the certain *cry* genes in Bt isolates. Future studies on detailed examination of each parameter will clarify this issue.

According to the boron tolerance test, Bt isolates except Bt-KE63-64 and Bt-KE20 generally showed boron tolerance up to 50 mM boric acid. Ahmed and Fujiwara [28] determined that different genus of bacteria such as *Arthrobacter*, *Rhodococcus*, *Lysinibacillus*, *Algoriphagus*, *Gracilibacillus* and *Bacillus* isolated from soil samples in Kütahya and Tokyo showed boron tolerance at 80, 100, 150, 300 and 450 mM. Likewise, boron tolerance of different species of bacteria isolated from Bursa Kestelek boron mines and their environment was shown to vary between 50 mM and 300 mM [29]. In the same study, boric acid tolerance of 37 standart microorganisms ranged between 25 mM - 75 mM. These studies suggest that different molecular structures might be developed for boron tolerance in different genera and species of bacteria.

When the growth rates of Bt isolates at various boric acid concentration were examined, boric acid at 25 mM and 50 mM delayed the initiation of logarithmic phase compared to the control group (Figure 1 A, B). Extension of the lag phase in the presence of increased boric acid concentration may result from the biosynthesis of some proteins and receptors in order to overcome the toxic effects of boric acid. In addition, no contribution of boric acid on the Bt growth may imply that boron may not be necessary for Bt or probably it is sufficient for the growth at very low level such as less than 5mM. Indeed, boron is one of the micro-nutrients in plant growth [8] and there is a small difference between the boron levels that causes deficiency or toxicity in plants [30].

Bt kurstaki and Bt-KE-63-64 were grown in different boric acid concentration in order to see how boric acid affect the Cry protein level. Increasing boric acid concentration from 5 mM to 20 mM caused a gradual decrease in the level of Cry protein. However there was a sharp decrease in the Cry protein level at 40 mM boric acid concentration. This could be due to the inhibition of bacterial growth rather than a direct effect of boric acid on the transcriptional or translational expression of Cry protein genes. Indeed, growth curves demonstrate that increasing boric acid concentrations delayed the lag and log phases of the growth. Therefore, the late initiation of the stationary phase caused a delay in the crystal protein synthesis because it is known that sporulation and crystal protein synthesis occurs in the stationary phase of the growth [1].

In the literature, there are studies about how combination of spore-crystal mixture of Bt with different compounds affect the mortality of agricultural pests. For example, Doane and Wallis [31] demonstrated that application of boric acid and Bt endotoxins together increased mortality of *Porthetria dispar* larvae. In addition, Govindarajan et al. [32] searched mortality by applying Bt toxin with boric acid to *Spodoptera litura*. In that study, Bt caused 100 % larval mortality when it was applied in combination with 0.1, 0.5, 1.0, 1.5 and 2 % boric acid. However, application of boric acid or Bt toxin alone was less effective than the combination of them. In addition, when boric acid was applied alone, it did not cause mortality in the concentrations of 0.1% and 0.5%. In contrast, concentrations of 1% and 2% boric acid resulted in nearly 100% mortality at the end of day 5. Finally, Khan [33] aimed to increase the pathogenicity against various termite species by using *Bacillus thuringiensis* with 1% boric acid together. It was shown that 1% boric acid application increased Bt virulence by 1.8 folds.

In contrast to the above data, 1% boric acid application in this present study did not show any toxic effects on the mortality of *C. cautella* larvae. Additionally, the boric acid did not increase the toxicity of various Bt strains against *C. cautella*. The reason could be due to the type of the Bt strain and various species of larvae used. In general, the toxic effect of Bt depends on the type, age, and the physiology of larvae. These results may imply that boric acid, known as a stomach poison, doesn't cause a damage in the protective membrane of the digestive system of *C. cautella* larvae at low concentrations (1%). In fact, spore-crystal mixture of Bt, activated by proteases, form large pores on the cell surface by binding to receptors on the epithelial surface [34]. In this present study, boric acid does not appear to provide a further contribution to this mechanism.

As a result, Bt occurrence was detected in boron containing soil samples in this present study and spore-crystal mixture of Bt-KE63-64 from boron containing environment, had higher toxicity (76%) than that

(65%) of *B. kurstaki* at 50 ppm against *C. cautella* larvae. However, addition of 1% boric acid did not cause any additive effect on the toxicity of the spore-crystal mixture. Therefore, it seems that the use of boric acid at more than 1% concentrations may not provide an economical contribution on the toxic effects of Bt-KE63-64 isolate. On the other hand, detection of Bt-KE63-64 isolate with 76% toxic effect at 50 ppm against *C. cautella* larvae will serve as an important alternative to available Bt formulations in the case of development of insect resistance.

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors

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