Research Article
Insecticidal Activity of *Bacillus thuringiensis* Strains Isolated from Soil and Water

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We attempted to search novel *Bacillus thuringiensis* strains that produce crystals with potential utility in plant protection and with higher activity than strains already used in biopesticide production. Seven *B. thuringiensis* soil and water isolates were used in the research. We predicted the toxicity of their crystals by cry gene identification employing PCR method. The isolate MPU B63 with interesting, according to us, genes content was used in evaluating its crystal toxicity against *Cydia pomonella* caterpillars. The strain MPU B63 was cultured from water sample and had cry1Ab, cry1B, and cry15 genes. The LC50 crystals of MPU B63 were compared to LC50 of commercial bioinsecticide Foray determined against *C. pomonella* (codling moth). The activity of MPU B63 inclusions against codling moth larvae was approximately 24-fold higher than that of Foray. The results are a promising introduction for further study evaluating the potential usefulness of isolate MPU B63 crystals in plant protection.

1. Introduction
Biopreparations based on spore-crystals mixtures of *Bacillus thuringiensis* seem to be a good alternative for chemical pesticides. They are environment friendly, do not have a negative influence on nontarget animals, including vertebrates, and are effective in reducing the number of insect pests [1]. However, novel *B. thuringiensis* isolates with higher and broader spectrum of activity are searching in their natural habitats. New strains are cultured from samples collected from soil [2], leaves [3], dead insects [4], and other sources [5–7].

Bacteria *B. thuringiensis* produce crystals comprised of Cry and Cyt proteins active against insect pest [8]. Sixty-eight groups of Cry and 3 groups of Cyt toxins have been known [9]. The toxicity of the most Cry and Cyt proteins are determined [10]. The knowledge on crystal composition leads to prediction of its potential activity [5]. An effective tool in estimating the utility of crystals against pests is identification of genes coding for insecticidal toxins [11]. For example, genes cry1, cry2, cry7, cry8, cry9, cry15, cry22, cry51, and cry1 code for proteins active against *Lepidoptera* pests [10]. Similarly, cry54 codes for protein that is harmful to moths [12]. Other cry genes determine the synthesis proteins toxic for insects of *Diptera* [13, 14], *Coleoptera*, *Hemiptera*, *Hymenoptera*, *Hemiptera*, *Siphonoptera* [10], *Homoptera*, *Orthoptera*, and *Phthiraptera* orders [15]. Furthermore, detection of cry genes by PCR method enables discovering genes of novel crystalline toxins [11].

The protection of plants against some insects can be difficult. An example is protection of fruit trees against codling moth (*Cydia pomonella*) from *Lepidoptera* order. The pest forms tunnels inside the fruit and is hardly available for insecticides dispersed on the fruit surface. Moreover, *C. pomonella* is resistant to most chemical pesticides [16].

We cultured *B. thuringiensis* strains from samples of soil and water in searching for novel isolates synthesizing crystals with high and wide insecticidal activity. We determined the potential toxicity of their crystalline inclusions by detection of cry gene profiles with PCR technique. The isolate with interesting, according to us, gene content was used in evaluating its crystal activity against *C. pomonella* caterpillars.

2. Materials and Methods

2.1. Bacteria. Seven *Bacillus thuringiensis* strains were used in the study (Table 1). Six bacterial isolates were cultured
Identification of cry15, cry16, cry18, cry20, cry22, cry25, were accomplished as proposed by Ben-Dov et al. [26]. and steps of PCR for cry3, cry40, genes was described by Ejiofor and Ibarra et al. [13]. Identification of cry39 genes was conducted as depicted by Ibarra et al. [13]. Identification of cry19 and cry39 genes was done according to instruction of Salehi Jouzani et al. [27].

The gene amplifications were carried out in MyCycler Termal Cycler (Bio-Rad, USA). The PCR products were electrophoresed in 1.5% agarose gel NOVA Mini (Novazym, Poland), stained with ethidium bromide and documented with Bio-Print V.99 System (Vilber Lourmat, France). The sizes of amplicons were estimated by GelCompar II 3.5 software (Applied Maths, Belgium).

2.3. Activity of B. thuringiensis Crystals against C. pomonella Caterpillars. The activity of B. thuringiensis crystals against C. pomonella was determined using the strain MPU B63 with cry1Ab, cry1B, and cry15 genes. The B. thuringiensis strain was cultured on M.B.Th medium for 5 days during sporulation. The mixture of spores and crystals was collected, washed with 1 M NaCl and then in distilled sterile water [28]. The spore-crystal mixture was suspended in 50 mM Tris HCl, 10 mM KCl, and pH 7.5 and placed on sucrose density gradient (67%, 72%, 79%, and 87%). After centrifugation, the layer of crystals was gathered and washed in sterile distilled water [29].

The number of crystals in the suspension was evaluated in a Bürker cell. Five dilutions of toxins \(10^2 \text{–} 10^6\) were prepared and applied to two-day-old Cydia pomonella caterpillars. The spore-crystal mixture of commercial pesticide Foray was prepared in the same manner, at the same time, and using the same conditions as for MPU B63. The larvae were cultured on medium according to Guennelon et al. [30]. The suspension of MPU B63 crystals or Foray spore-crystal preparation with known concentrations was spread on the medium surface. The larvae are cannibalistic; therefore, they were reared individually at a 16 : 8 (day : night) period, 26°C and 40–60% humidity. The number of dead insects was estimated after 7 days.

The 50% lethal concentration (LC\(_{50}\)) of MPU B63 crystals against C. pomonella was calculated by using probit analysis with the consideration of dead caterpillars in control sample [31]. The obtained value was compared to LC\(_{50}\) commercial bioinsecticide Foray determined against C. pomonella. The insecticidal activity of Foray preparation was 21200 IU/mg. The potency (IU/mg) of isolate MPU B63 was counted using the following formula [32]: potency of isolate crystals (IU/mg) = [LC\(_{50}\) of Foray × potency of Foray (IU/mg)]/LC\(_{50}\) of isolate crystals.

3. Results

3.1. Distribution of Crystalline Toxin Genes. The B. thuringiensis strains had from three to eight crystalline toxin genes. We found that the isolates had cry1Aa, cry1Ab,
cry1Ac, cry1B, cry1C, cry1D, cry1I, cry2Aa, cry2Ab, cry9B, cry9E, and cry15. The obtained results are given in Table 1.

*B. thuringiensis* soil isolates harbored cry1, cry2 and cry9 genes. Strain MPU B63 cultured from water possessed cry1 and cry15 genes. The cry1A gene was present in all isolates. All *B. thuringiensis* strains obtained from soil samples carried cry2A and cry1I. Strains with cry1C had also cry1D, cry9B, and cry9E. The soil isolate MPU B30 had large number and diversity of cry genes; it possessed cry1Aa, cry1Ba, cry1Ca, cry1D, cry1H, cry2Ab, cry9B, and cry9E genes. The amplicons of some cry genes are shown in Figure 1. None of the isolates had cry1J, cry1K, cry5, cry6, cry7, cry8, cry11, cry12, cry13, cry14, cry16, cry17, cry18, cry19, cry20, cry21, cry22, cry24, cry26, cry27, cry28, cry29, cry30, cry32, cry39, cry40, cry1T, and cryT2 genes.

### 3.2. Toxicity of *B. thuringiensis* MPU B63 Crystals for *Cydia pomonella* Larvae

The strain MPU B63 was chosen to determine its crystal activity due to unique cry gene profile. The isolate had cry15 gene. The LC$_{50}$ value of MPU B63 toxins against *C. pomonella* was $1.55 \times 10^5$ crystals per larva. The obtained value was compared to the LC$_{50}$ of commercial biopesticide Foray containing spores and crystals of *B. thuringiensis* subsp. *kurstaki* that is recommended to protect plants against lepidopteran insects. LC$_{50}$ of Foray for *C. pomonella* was $3.69 \times 10^6$ spores and crystals per larva (Table 2). The LC$_{50}$ of MPU B63 crystals was approximately 24-fold lower than LC$_{50}$ of bioinsecticide against *C. pomonella* caterpillars. The potency of MPU B63 toxins was approximately 890 IU/mg, and it was higher than the potency of Foray.

### 4. Discussion

*Bacillus thuringiensis* bacteria are ubiquitous in soil [2, 13, 33, 34], dead larvae [4], sand [5], leaves [3], water [7], or dust from stored grains [6]. Wild strains isolated form environmental samples can synthesize crystals that display higher activity against insect pests in comparison to *B. thuringiensis* strains already used in pesticide production. We attempted to culture *B. thuringiensis* isolates from soil and water samples and estimate their potential usefulness in plant protection.

The knowledge on coding for genes toxins in crystalline inclusion is useful in predicting potential pathogenicity of *B. thuringiensis* isolates against insects [5, 7, 11]. Cry1 toxins display activity against lepidopteran, dipteran, and coleopteran pests. Cry2 genes code for crystalline proteins toxic for *Diptera* and *Hemiptera*. Cry9 proteins indicate activity against insects of *Coleoptera* and *Lepidoptera* order. Cry15 is toxic for lepidopteran pests [10]. Two of soil-isolated strains (MPU B30 and MPU B55) had genes of Cry1, Cry2, and Cry9 toxins. Other *B. thuringiensis* isolates cultured from soil possessed cry1 and cry2 genes. Their crystals showed potential activity against pests of *Coleoptera*, *Diptera*, *Hemiptera*, and *Lepidoptera*. Water-isolated strain harbored genes coding Cry1 and Cry15 toxins that indicate the crystals activity against coleopteran, dipteran, and lepidopteran insects.

All isolates had cry1 gene, and seven of eight strains harbored cry2 gene. These genes were also noted as the most frequent in *B. thuringiensis* strains [2, 3, 5, 6, 33, 34]. All analyzed *B. thuringiensis* harbored cry1I genes that have been reported as the most abundant in *B. thuringiensis* isolates [11]. Soil-isolated strains with cry1A possessed also cry2A gene, which is with agreement in notice done by Saadaoui et al. [3] in strains from soil samples collected in Tunisia. We observed that strains with cry1C had also cry1D, cry9B, and cry9E.

Strain *B. thuringiensis* subsp. *kurstaki* HD-1 applied in production of insecticide Foray harbored cry1Aa, 1Ab, 1Ac, 1I, 2Aa, 2Ab, and 2Ac genes [35]. Soil isolate MPU B30 had the largest number of cry genes among the isolates analyzed (Table 1). In comparison to Foray, it additionally carries cry1B, cry1C, cry1D, cry9B, and cry9E genes, which can indicate wider spectrum of toxicity and higher insecticidal
activity of their crystals than the commercial insecticide. Our attention was directed to MPU B63 with cry15 gene isolated from water sample. The gene is rarely detected on mouse erythrocytes [37].

Our searching for a novel isolate producing crystals with higher activity than commercial biopesticide revealed the MPU B63 strain. The toxicity of Foray insecticide was approximately 24-fold lower compared to that of MPU B63 crystals. The results are a starting point for future research determining potential usefulness of MPU B63 isolate in plant protection.

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