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Efficiency and Midgut Histopathological Effect of the Newly Isolated *Bacillus thuringiensis* KS δ-Endotoxins on the Emergent Pest *Tuta absoluta*

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Tuta absoluta (Povolny, 1994) is a devastating moth to the Solanaceae plants. It is a challenging pest to control, especially on tomatoes. In this work, we studied the entomopathogenic activity of the Cry-forming δ-endotoxins produced by *Bacillus thuringiensis* strain KS and *B. thuringiensis kurstaki* reference strain HD1 against *T. absoluta*. These strains carried the *cry2*, *cry1Ab*, *cry1Aa* / *cry1Ac*, and *cry1I* genes, and KS also carried a *cry1C* gene. The δ-endotoxins of KS were approximately twofold more toxic against the third instar larvae than those of HD1, as they showed lower 50% and 90% lethal concentrations (0.80 and 2.70 μg/cm² (δ-endotoxins/tomato leaf)) compared with those of HD1 (1.70 and 4.50 μg/cm²) ($p < 0.05$). Additionally, the larvae protease extract showed at least six caseinolytic activities, which activated the KS and HD1 δ-endotoxins, yielding the active toxins of about 65 kDa and the protease-resistant core of about 58 kDa. Moreover, the histopathological effects of KS and HD1 δ-endotoxins on the larvae midgut consisted of an apical columnar cell vacuolization, microvillus damage, and epithelial cell disruption. These results showed that the KS strain could be a candidate for *T. absoluta* control.

Keywords: Tomato borer, epithelium, Cry proteins

Introduction

The tomato leaf miner *Tuta absoluta* is a small lepidopteron moth, belonging to the family Gelechiidae, which includes the serious insect pests angoumois grain moth *Sitotroga cerealella*, the pink bollworm *Pectinophora gossypiella*, and the potato tuber moth *Phthorimaea operculella*. The main host for *T. absoluta* was thought to be tomato; however, it has been reported that it also feeds on eggplant, pepper, and potato leaves as well as various other plants [12]. This pest has a high reproductive potential and its damage occurrs throughout the entire growing cycle of tomatoes, reaching up to 100% in cultivations [31]. After devastating the tomato production in South America, *T. absoluta* was introduced accidently to Spain in 2006. Its spread across boarders has been phenomenal, reaching the entire shores of the Mediterranean [9]. It was in the EPPO A2 list of pests recommended for regulation as quarantine pest [32].

The problem associated with *T. absoluta* is the difficulty to achieve an effective control through application of chemical insecticides. In fact, the larvae has the ability to rapidly evolve strains with reduced susceptibility to chemical insecticides, like the reported cases of resistance in Brazil, Argentina, and Chile [29, 41–43]. However, potential biocontrol agents are under development, including predatory bugs such as *Macrolophus* and egg parasitoids like *Trichogramma*. The pheromone trap was used for the early detection and control of *T. absoluta*. Moreover, the entomopathogenic bacterium *Bacillus thuringiensis* was considered a successful biopesticide against several pests. It produced crystalline inclusion bodies during sporulation, constituted of insecticidal proteins named Cry and classified

into 70 groups according to their similarity of amino acid sequences [8, 40]. The Cry1 protoxins were activated by proteolysis into toxins upon ingestion by susceptible larvae, and then bound to specific receptors on the brush border membrane of the midgut epithelial cells, causing pore formation and cell lyses [20–22, 33–35]. The toxicity of *B. thuringiensis* sprayable formulations, largely due to Cry toxins, are considered highly effective, selective, and safe [10]. Transgenic crops producing *B. thuringiensis* toxins kill some major insect pests. Hence, genetically modified cotton-producing Cry1Ac resisted for several years to *Pectinophora gossypiella* [45, 46], and expression of the Cry9Aa2 N-terminal region gene in tobacco leaves conferred resistance to *Phthorimaea operculella* [5].

Owing to the hazards of *T. absoluta* on the future of tomato production, and given the scarcity of information about the relationship between the Cry proteins and the larvae midgut of this insect, we firstly evaluated its susceptibility to the δ-endotoxins produced by our *B. thuringiensis* strain KS by comparing with those of *B. thuringiensis kurstaki* strain HD1. We also examined the capability of *T. absoluta* to activate the *B. thuringiensis* protoxins and its midgut histopathological aspect after being exposed to the δ-endotoxins. The possibility of using the KS strain will be discussed to formulate a biological control agent of *T. absoluta*.

Materials and Methods

Bacterial Strains and Growth Conditions

B. thuringiensis strain KS was newly isolated from soil samples, and *B. thuringiensis* ssp. *kurstaki* strain HD1 was used as the reference strain [4]. Luria-Bertani (LB) medium was used for culturing the *B. thuringiensis* strains [39] and solid T3 medium [51] was used to produce the crystal inclusion bodies during the sporulation growth phase. Additionally, the liquid medium described by Ghribi *et al*. [15] was used in order to produce the parasporal crystals during growth. Flasks containing 50 ml of culture medium were incubated 4 days at 30°C and 200 rpm in a rotary shaker.

DNA Extraction and PCR Analysis

B. thuringiensis plasmid DNA was extracted by the alkaline lyses procedure including lysozyme treatment [39], and chromosomal DNA was extracted as described by Geiser *et al*. [14]. PCR assays were realized in a reaction mixture including 50 mM KCl, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer, 0.5 μg of DNA, and 1.25 U *Taq* DNA polymerase. Thermal cycler conditions consisted of a denaturing step at 94°C for 2 min, followed by 28 cycles constituted of denaturing at 94°C for 50 sec, annealing at 48°C for 45 sec, and extension at 72°C for 90 sec. Specific primer pairs were used for the detection of the *cry1Aa/cry1Ac* and *cry1Ab* genes [14], as well as *cry1B*, *cry1C*, *cry1D*, *cry1E*, *cry1F*, *cry1G*, *cry1H*, *cry1I*, *cry9* [48], and *cry2* [49]. An approximately 1.50 kb fragment sequence of the 16S rRNA gene was amplified by the primers Fd1-court (AGAGTTTGATCCTGGCTCAG) and Rd1-court (AAGGAGGTGATCCAGCC) and then sequenced by the primers Fd1-court, Rd1-court, BK1F (TCACCAAGGCAACGATGCG), and Fd2 (CAGGATTAGATACCCTGGTAG). An approximately 0.30 kb fragment sequence of the *gyrB* gene was amplified and sequenced using the primers BCFW1 (GTTTCTGGTGGTTTACATGG) and BCRW1 (CAACGTATGATTTAATTCCACC) [23, 30]. The partial sequences of the 16S rRNA and *gyrB* genes of the KS strain were deposited in the GenBank database under the accession numbers KC737848 and KC737849, respectively.

Preparation of the Larvae Protease Extract and Zymogram Analysis

T. absoluta larvae (5–6 mm in length) were chilled on ice for 30 min. After that, they were collected in MET buffer (Mannitol, 300 mM; EGTA, 5 mM; Tris, 20 mM; pH 7.2) at 4°C. They were disrupted in a blender and centrifuged for 10 min at 13,000 ×*g*. The supernatant was recovered (larvae protease extract) and the protein content was determined by using the Bio-Rad Protein Assay (Germany) according to the method of Bradford [1].

For zymogram analysis, an aliquot of the larvae protease extract (10 μg) was mixed with Laemmli sample buffer and separated by sodium dodecyl sulfate 13% Tris-glycine polyacrylamide gel electrophoresis. The gel was washed in 50 mM sodium carbonate buffer (pH 9.6) for 30 min and then incubated in sodium carbonate buffer (pH 9.6) containing 2% casein, at 37°C for 3 h. After Coomassie blue staining, clear bands of protease activities were visible against a dark background [13].

δ-Endotoxins Preparation and Proteolysis Assay

The crystal inclusion bodies were prepared from *B. thuringiensis* as described by Lee *et al*. [26]. Briefly, fresh cultures of the *B. thuringiensis* strains were plated on solid T3 medium [51] and incubated at 30°C for 3 days. The bacteria were collected from the plates and then washed twice with 1 M NaCl and twice with distilled water. The pellets were solubilized with 0.05 M NaOH, at 30°C for 2 h, centrifuged for 10 min at 13,000 ×*g*, and then the protein concentrations of the soluble fractions were determined according to the method of Bradford [1]. The soluble Cry proteins were mixed with the *T. absoluta* larvae protease extract at a ratio of 20/1 (v/v) or by using commercial trypsin at a Cry proteins/protease ratio of 20/1 (μg/μg). The mixtures were incubated at 37°C with constant agitation for diverse incubation times. Protein extracts suspended in Laemmli sample buffer (3×) were boiled for 5 min, analyzed by SDS-PAGE (9%), and stained using Coomassie blue [25].

Insecticidal Bioassays

The development of the *T. absoluta* larva needs four instars to reach the chrysalides and then the moth. Hence, the toxicity assays

against *T. absoluta* third instar larvae were done by displaying the spore-crystal mixtures on tomato leaf surface at various concentrations (δ-endotoxins/leaf surface (μg/cm²): 0.2, 0.5, 1, 2, 4, 6, and 8) in order to establish the 50% and 90% lethal concentrations (LC_{50} and LC_{90}) after 2 days. One tomato leaf and 10 larvae were placed in each Petri plate. They were kept in the insect culture room at 26–28°C and under a photoperiod of approximately 14 h light and 10 h dark. The experiment was repeated three times and the LC_{50} and LC_{90} of the δ-endotoxins were calculated from pooled raw data by probit analysis using programs written in the *R*. language [52].

Preparation and Sectioning of the Insect Tissues

T. absoluta larvae were alimented with tomato leaves containing the KS or HD1 δ-endotoxins. After 24 to 36 h, the larva guts were excised and collected in 10% formol. They were dehydrated *via* ethanol solutions with increasing concentrations, washed in 100% toluene, and fixed in paraffin wax. Four micrometer sections were obtained and placed in carriers loaded with a mix of 1.5% egg albumin and 3% glycerol in distilled water. For histopathological location of the δ-endotoxin effects, the sections already de-paraffinated in 100% toluene were stained with hematoxylin-eosin (HE) as reported by Ruiz *et al*. [36].

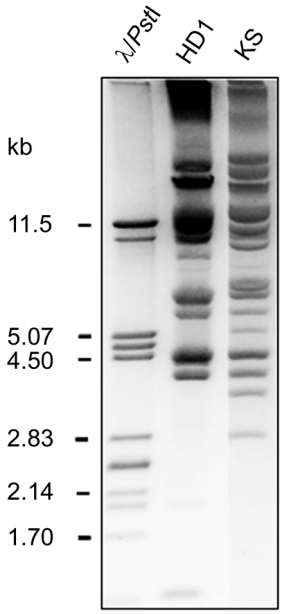

Fig. 1. Comparison of the native plasmid patterns of *B. thuringiensis* strains KS and HD1.

Results

Identification, *cry* Genes Content, and δ-Endotoxins Production of *B. thuringiensis* Strain KS

The sequencing of the PCR-amplified DNA corresponding to the partial 16S rRNA and *gyrB* genes showed 100% sequence similarity of the KS strain with the *B. thuringiensis* species, which are characterized by the presence of several plasmids in their genomes. Concordantly, we showed that KS harbored numerous plasmids and presented distinguished plasmids pattern when compared with that of *B. thuringiensis kurstaki* reference strain HD1 (Fig. 1). The PCR analysis revealed that besides the *cry2*, *cry1I*, *cry1Aa* / *cry1Ac*, and *cry1Ab* genes contained in HD1, KS harbored a *cry1C*-type gene. Additionally, the examination of the crystal forms showed that the KS and HD1 strains produced bipyramidal and cubic crystals. These strains, grown in liquid medium [15] in order to produce the crystal inclusions during growth, showed δ-endotoxin amounts for KS and HD1 of about 1,196 ± 78 and 1,577 ± 72 μg δ-endotoxins/ml, respectively.

Oral Toxicity of the *Bacillus thuringiensis* δ-Endotoxins Against *T. absoluta*

Several concentrations of the *B. thuringiensis* strains KS and HD1 δ-endotoxins were tested against *T. absoluta* third instar larvae. After 2 days, control larvae fed diet without δ-endotoxins developed fully, but several intoxicated larvae fed diet with δ-endotoxins died during the test. The determined LC_{50} was about 0.80 and 1.70 μg/cm² (toxin / tomato leaf) and the LC_{90} was about 2.70 and 4.50 μg/cm² for KS and HD1, respectively. Additionally, the confidence limits indicated that the lethal concentrations of the KS and HD1 δ-endotoxins were significantly different. Hence, the KS δ-endotoxins were approximately 2-fold more toxic than those of HD1 (Table 1). Concordantly, the bioassays conducted on *T. absoluta* larvae at the beginning of the fourth instar

Table 1. Toxicity of the δ-endotoxins of *B. thuringiensis* strains KS and HD1 against *T. absoluta* third-instar larvae after 2 days.

Strain	*Tuta absoluta* (L3) δ-endotoxin/tomato leaf surface (μg/cm²)			
	LC_{50}	95% Confidence limit	LC_{90}	95% Confidence limit
HD1	1.70	1.35-2.05	4.50	3.95-5.05
KS	0.80	0.60-1.00	2.70	2.20-3.20

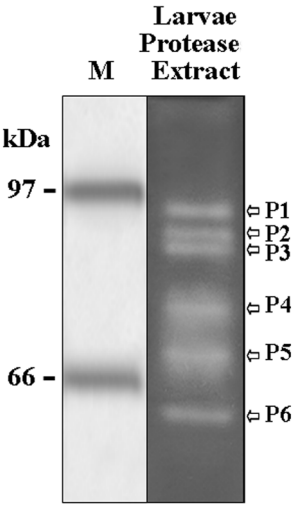

Fig. 2. Zymogram analysis of *T. absoluta* larvae proteases.
The larvae protease extract (10 μg) was separated in SDS-PAGE, and the proteolysis activities were revealed using casein as the substrate.

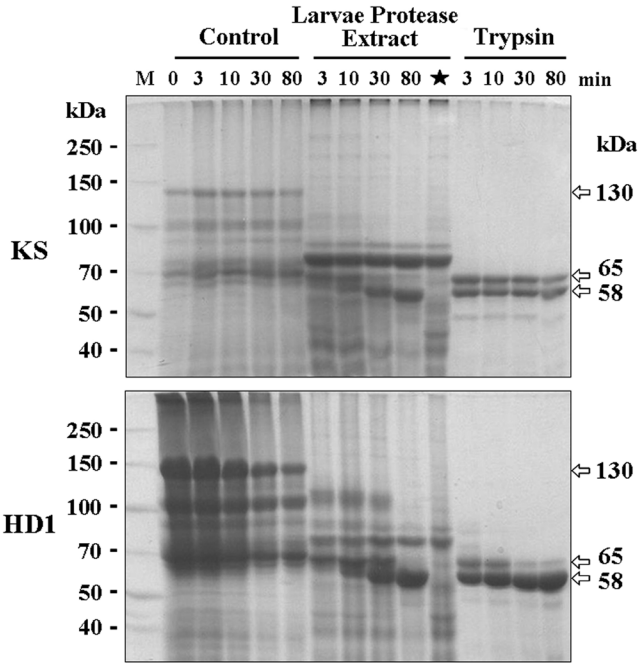

Fig. 3. Proteolysis of the KS and HD1 δ-endotoxins.
SDS-PAGE analysis of the protoxins digestion with *T. absoluta* larvae protease extract or the commercial trypsin for 3, 10, 30, and 80 min. Lanes: M, molecular mass markers. The control corresponded to the δ-endotoxins incubated without proteases. *, Larvae protease extract without δ-endotoxins.

confirmed that KS δ-endotoxins were significantly more toxic than those of HD1 (data not shown).

Zymogram Analysis of the Larvae Proteases

The number and molecular masses of the proteases recovered in the larvae protease extract were examined using zymogram analysis with casein as a universal substrate. Thus, the zymogram analysis indicated at least six discernable caseinolytic activities (P1-P6) comprised between 97 and 50 kDa. The bands corresponding to P1, P2, P3, and P6 had relatively delimited forms and high intensities, suggesting that each band corresponded to one or more proteases having similar molecular masses. Furthermore, the P4 and P5 bands were clearly large, vague and diffused, suggesting that each one resulted from various proteases running in close proximity (Fig. 2).

δ-Endotoxin Activation by Proteases

In order to determine if each step of the action mode was involved in the higher activity of KS against *T. absoluta*, the protoxin activation process was studied [24]. For such purpose, the KS and HD1 crystals were solubilized, and the obtained protoxins were activated by means of *T. absoluta* larvae protease extract or commercial trypsin at different times of incubation. Subsequent to SDS-PAGE analysis, we found that there was no major difference between the proteolysis patterns of KS and HD1 in the two cases. The control samples incubated without any added proteases showed two major bands. The first band (130 kDa) corresponded to Cry1 protoxins, which remained relatively stable during the incubation. The second band (approximately 65 kDa) corresponded to the Cry2 and/or Cry1 toxins after protoxins activation. Proteolysis activation of both the KS and HD1 protoxins (130 kDa) to an approximately 65 kDa toxin occurred rapidly *via* the larvae protease extract or trypsin, as we did not find detectable protoxins after 3 min. Moreover, the 65 kDa toxins were themselves resisted progressive proteolysis during the 80 min reaction time, producing the protease-resistant core of 58 kDa (Fig. 3). Furthermore, the trypsin proteolysis profile showed that the stable toxin band was observed very early (3 min), suggesting that the trypsin was very active on both toxin mixtures. The pattern similarity observed with the larvae protease extract or the trypsin indicated the presence of high trypsin-like activity in the midgut of *T. absoluta* larvae, as was described in several insects [3, 7]. Consequently, the involvement of the protoxin activation process in the enhancement of KS toxicity compared with that of HD1 was discarded.

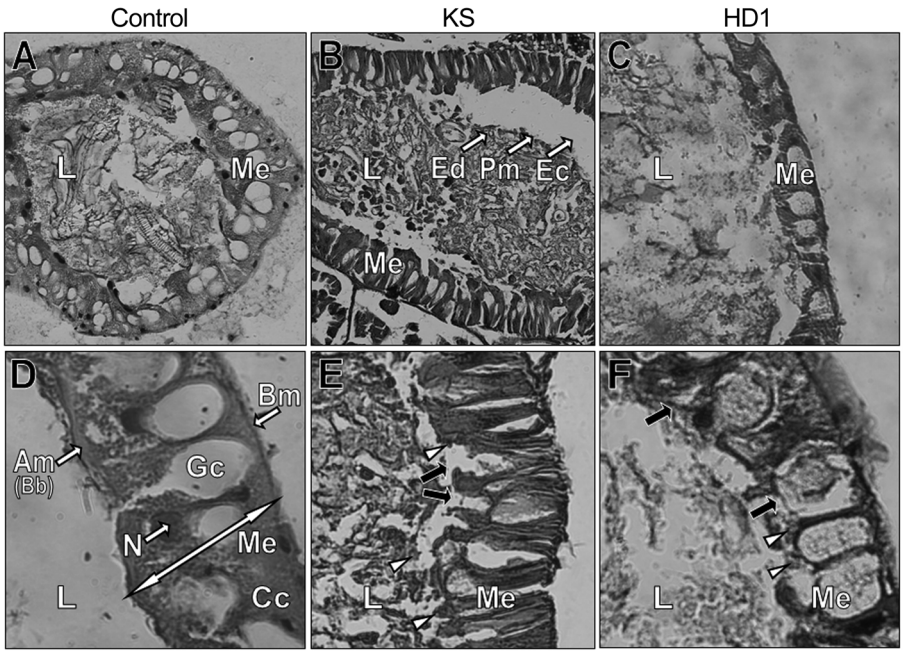

Fig. 4. Effects of the KS and HD1 δ-endotoxins on *T. absoluta* midgut.
(**A**), (**B**), (**C**) General aspects; and (**D**), (**E**), (**F**) histopathological effects of sections through the midgut epithelium. (**A**), (**D**) Larvae not exposed to toxins; (**B**), (**E**) larvae fed diet containing KS δ-endotoxins; (**C**), (**F**) larvae fed diet containing HD1 δ-endotoxins. In (**E**), (**F**), black arrows indicate lyses of columnar cells and head arrows show vacuolization of columnar cells and vesicle formation in the apical region of cells. Me, midgut epithelium; Cc, columnar cell; N, nucleus; Gc, goblet cell; Am, apical membrane = (Bb) brush border membrane; Bm, basement membrane; L, lumen; Pm, peritrophic membrane; Ec, ectoperitrophic space; Ed, endoperitrophic space. (**A**), (**B**), (**C**) Magnification 40×; (**D**), (**E**), (**F**) magnification 100x.

Histopathological Effects of KS δ-Endotoxins on *T. absoluta* Larvae

Histopathological observations of the effects of the KS and HD1 δ-endotoxins on *T. absoluta* were conducted on the early stage of the fourth instar larvae. As *T. absoluta* is a lepidopteron, the gut cross-sections of the untreated larvae showed a midgut wall composed of a peritrophic membrane and a midgut epithelium. The peritrophic membrane was composed of chitin and proteins that delimited the midgut lumen in the ectoperitrophic and endoperitrophic spaces [28, 37, 47]. The midgut epithelium was lined with columnar cells, which contained numerous microvilli forming the apical membrane (brush border membrane), and goblet cells that were intercalated with the columnar cells. The midgut epithelial cells of the control larvae were closely associated with one another, presenting a typical morphology and showing no evidence of damage (Figs. 4A and 4D). Furthermore, extensive damages to the midgut epithelium were induced by the KS and HD1 δ-endotoxins. Mostly, histopathological changes included hypertrophy of the epithelial cells and their nucleus, dilation of the intercellular spaces, and degeneration of the epithelium columnar cells, so the lumen was filled with debris of disrupted cells. Brush border membrane alteration allowed for cell cytoplasmic vacuolization and vesicle formation in the apical region toward the midgut lumen (Figs. 4B, 4C, 4E, and 4F).

Discussion

T. absoluta attacks the Solanaceae major crops and principally the tomato crops in South America and Mediterranean countries. Here, we reported a particular emphasis on the δ-endotoxins effect of our isolated *B. thuringiensis* strain KS and the *B. thuringiensis* ssp. *kurstaki* reference strain HD1 on *T. absoluta* larvae. The symptoms developed upon δ-endotoxins ingestion were typical, consisting of the cessation of feeding, loss of gut peristalsis, body retraction, overall paralysis, and then death of the insect. Quantitatively, insecticidal bioassays against third instar larvae revealed that the KS δ-endotoxins were approximately 2-fold toxic than those of HD1 because of their lower LC_{50} and LC_{90}. KS and HD1 bipyramidal crystals should contain Cry1Aa / Cry1Ac and Cry1Ab, and a supplementary Cry1C protoxin for KS. They also produced the Cry2 toxin (65–70 kDa), forming the cubic crystal, and the secreted Cry1I toxin, which did not integrate the δ-endotoxin crystals.

Numerous studies on the action mode of the δ-endotoxins described the solubilization of the crystal inclusion bodies and the proteolysis processing of the Cry proteins in the insect alkaline midgut environment. For *T. absoluta*, the zymogram analysis revealed at least six protease activities. Additionally, proteolysis pattern similarity was revealed when the KS and HD1 protoxins (130 kDa) were activated by larvae protease extract or trypsin, producing the toxins (65 kDa) and the proteolysis-resistant core (58 kDa). In fact, to be active, a Cry1 protoxin must first be deleted from its C-terminal moiety, necessary for its crystallization inside *B. thuringiensis* with midgut trypsin and/or chymotrypsin-like proteases, followed by the deletion of several N-terminal amino acids depending on the Cry sequences [24, 27]. After that, active Cry toxins interact specifically and sequentially with multiple receptors on the surface of the midgut brush border membrane of the target insects. These interactions trigger oligomerization of the toxin and its insertion into the membrane to form ion channels, leading to colloid osmotic lyses [2, 16, 44]. For illustration, by using brush border membrane vesicles (BBMV) from the midgut tissues of *Phthorimaea operculella* (Lepidoptera: Gelechiidae), *Mamestra brassicae*, and *Spodoptera exigua* (Lepidoptera: Noctidae), binding of the three structurally related insecticidal crystal proteins CryIAa, CryIAb, and CryIAc showed a common receptor [11]. Here, we had clearly observed the damage caused by the KS and HD1 δ-endotoxins on the *T. absoluta* midgut, consisting essentially of the disintegration of the epithelial cells and the vacuolization of the apical membrane, conducting to larvae death. Hence, it will be interesting to identify the binding specificities of the Cry proteins to their receptor (s) in the midgut epithelium cells of *T. absoluta*.

Several explanations for the KS enhanced toxicity were possible, like the presence of the *cry1C* gene. In such case, it is possible that the Cry1C effect was due to its higher toxicity against *T. absoluta* by comparing with other KS Cry types or due to a cumulative or a synergistic effect between the Cry proteins. Such synergistic effect was previously demonstrated for the *B. thuringiensis* Cry1Aa and Cry1Ia [50]. Other possible reasons were the sequences of the KS Cry1 and Cry2 proteins, which could specify their susceptibilities to midgut proteases and their interactions with their specific receptors in the larva midgut brush border membranes [11, 19]. For example, the binding sites for *B. thuringiensis* Cry2Ae toxin on Heliothine brush border membrane vesicles are not shared with Cry1A, Cry1F, or Vip3A toxin [17]. Additionally, a high copy number or expression level of one/several *cry* genes could modify the Cry protein proportion in the crystal, and consequently improve or decrease its toxicity. In fact, Chang *et al*. [6] described variability in the transcription and translation of the *cry1Ab3*, *cry1Ca1*, and *cry1Da1* genes by *B. thuringiensis aizawai,* leading to a proportion modification of the corresponding Cry proteins forming the crystal. Additionally, Saadaoui *et al*. [38] demonstrated that the *cry1A* copy number of the *B. thuringiensis* BLB1 was significantly higher than that of the *B. thuringiensis* HD1 strain, which could be one of the factors responsible of its hypertoxicity. Furthermore, the genetic background may be influential, like the presence of the proteases or any other insecticidal factor. For example, the three selected *B. thuringiensis* isolates conferring better or equal protection to the tubers than the reference strain *B. thuringiensis* HD1 against *Phthorimaea operculella* belonged to the subspaces *kurstaki* and *morrisoni* and carried combinations of *cry1* (*cry1Ab*, *cry1Ac*, *cry1D*, *cry1E*, *cry1F*, *cry1Ia*), *cry2,* and *cry4* [18].

The fermentation process could be optimized in order to increase the KS toxicity and to make it a candidate for an integrated pest management of *T. absoluta*. Additionally, we propose to use this strain against the *Tuta absoluta* closely related pests *Phthorimaea operculella* and *Keiferia lycopersicella* (Lepidoptera: Gelechiidae), which also damage several Solanaceae, where *P. operculella* is particularly dangerous to potato production worldwide in both field crops and storage (leaves, stems, exposed tubers), whereas the main host of *K. lycopersicella* is tomato.

In conclusion, the *B. thuringiensis* KS is a promising strain that produced more efficient δ-endotoxins against *T. absoluta* than the reference strain HD1 already largely used in biological control. Several reasons for its efficiency are possible, like the presence of the *cry1C* gene. Its Cry protoxins are activated by the proteases of the larvae protease extract into 65 kDa and then 58 kDa toxins, which cause the midgut epithelium damage and larvae death.

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