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Abstract

The lethal and sub-lethal effects of sporulated cultures of a novel *Bacillus cereus sensu lato* strain lacking detectable *cry* genes and identified through morphological and genetic analyses, have been studied on the Mediterranean fruit fly *Ceratitis capitata*. The lethal effects on young larvae were concentration dependent, with a median lethal concentration (LC_{50}) of 4.48 x 10^8 spores/g of diet. Sporulated cultures of this strain significantly extended development time and reduced immature survival, and the size of emerging fly adults. Besides spores, the toxicity has been associated to the insoluble extra-spore fraction characterized through a proteomic approach. The profile of the extra-spore protein fraction (ES) showed major protein bands within the 35-65 kDa range. The results of mass spectrometry analysis highlighted the presence of putative virulence factors, including members of protein families previously associated to the insecticidal action of other microbial entomopathogens. These proteins include metalloproteases, peptidases and other enzymes.

Keywords: Fruit fly; Tephritidae*;* bioinsecticide, entomopathogenic bacteria; *Bacillus thuringiensis*; toxicity.

1. Introduction

The Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) is a polyphagous species affecting more than 250 species of fruits and vegetables worldwide (White and Elson-Harris, 1994). In the Mediterranean basin, its host range includes several economically important crops, such as sour and sweet orange, grapefruit, loquat, apricot, peach, fig, pear, persimmon, prickly, pear and clementine (Liquido et al., 1991).

Due to its high reproductive potential, the management of this multivoltine pest is always necessary to protect fruits from the destructive action of larvae (Papadopoulos, 2008).

Most conventional management methods involve the application of various synthetic insecticidal formulations as foliage baiting or cover spraying. The negative impact of chemicals on non-target organisms (Damalas and Eleftherohorinos, 2011) has over time encouraged the research and development of safer methods of control including the use of low impact insecticides, mass trapping and the release of natural enemies. Efforts have been made to explore the opportunity to find and employ entomopathogenic microorganisms like fungi and bacteria against *C. capitata*. In this prospect, the potential of specific strains of fungal species *Beauveria bassiana* and *Metarhizium anisopliae* (Quesada-Moraga et al., 2006; Ortiz-Urquiza et al., 2010; Beris et al., 2013; Falchi et al., 2015), and of the entomopathogenic bacterium *Bacillus thuringiensis* (*Bt*) (Gingrich, 1987; Karamanlindou et al., 1991; Martinez et al,, 1997) on diverse fly stages has been investigated. Similarly, the effects of *B. thuringiensis* isolates have been studied on other Tephritid species such as the olive fly *Bactrocera oleae* Gmelin (Alberola et al., 1999) and the Mexican fruit fly *Anastrepha ludens* (Loew) (Robacker et al., 1996). In this context, the insecticidal action of bacterial protein toxins produced by *B. thuringiensis* strains have been demonstrated, thus envisioning their possible employment in bait sprays against adults or as a resource to develop transgenic plants resistant to endophytic larval stages (Vidal-Quist et al., 2009 and 2010). A main concern with

different *B. thuringiensis* strains showing toxicity against flies is the production of unwanted secondary metabolites like the β-exotoxin, whose use in agriculture is prohibited (Liu et al., 2014). However, Vidal-Quist et al. (2010) have demonstrated the activity of Cyt1Aa protein from *B. thuringiensis* serovar *israelensis* against *C. capitata*.

The infectivity of other bacterial species to the Mediterranean fruit fly has been in a few cases reported (Sarakatsanou et al., 2011; Molina et al., 2010), even if the molecular implications and the role of specific bacterial metabolites in the interaction with the host have not been explained.

The *Bacillus cereus* phylogenetic cluster, which includes *B. thuringiensis*, is represented by close related bacterial species whose pathogenic properties are highly divergent. While their genetic relationships are still under debate, the insecticidal potential of new isolates in this group is of actual interest. Due to the production of typical parasporal crystals, *B. thuringiensis* can generally be phenotypically distinguished from other *B. cereus* group members (Vega and Kaya, 2012).

The present study investigates the effects of a recently isolated acrystalliferous *Bacillus cereus sensu lato* strain on immature *C. capitata* survival and development in relation to specific bacterial fractions characterized through a proteomic approach.

2. Materials and methods

2.1. Bacterial strain identification and preliminary characterization

The present study was conducted with a bacterial isolate selected for its toxicity against *C. capitata* larvae in a screening program including several *B. thuringiensis*-like isolates. Among more than 300 isolates assayed, this strain was the most effective against *C. capitata* larvae, and no toxicity was observed against adults. In addition, no effects were detected on other fly

84 species like the olive fly *Bactrocera oleae* Gmelin (Diptera: Tephritidae) and the house fly
85 *Musca domestica* L. (Diptera: Muscidae). Pathogenic effects were associated to whole spore
86 suspensions and not to bacterial vegetative cells (Floris et al., 2007).
87 Spores of this strain were found in soil samples from Zimbabwe (Southern Africa) and were
88 permanently stored in glycerol at -80°C in the *B. thuringiensis*-like collection of the
89 Dipartimento di Agraria (University of Sassari, Italy). For taxonomic identification,
90 preliminary morphological examinations under light and transmission electron microscopy
91 (TEM) were conducted. Subsequently, different genes were amplified and sequenced. These
92 include the *16S rRNA* gene encoding for the Small subunit ribosomal RNA, the two house-
93 keeping genes *gyrB* and *aroE* encoding for the subunit B protein of DNA gyrase
94 (topoisomerase type I) and for the shikimate dehydrogenase, respectively. Specific flagellin
95 (H antigen) genes (*hag*) were also used to complement phylogenetic analyses.
96 Genomic DNA of the bacterium was routinely isolated from overnight cultured cells
97 employing the DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) in
98 compliance with the manufacturer's instructions. PCRs were performed using a Veriti™
99 Thermal Cycler (Applied Biosystems) in a total volume of 25 µl containing 1x reaction buffer
100 (supplied with 2.0 mM Mg^{2+}); approximately 100 ng DNA; 0.3 µM of each primer; 0.3 mM
101 of each dNTPs; and 0.5 U of KAPA HiFi HotStart DNA polymerase (KAPABIOSYSTEMS,
102 Boston, USA). The following sets of primers were used: BcF 5'-
103 GGATTAAGAGCTTGCTCTTAT-3' (forward) and 16S2 5'-
104 AAGCCCTATCTCTAGGGTTT-3' (reverse) for *16S rRNA* (Chen and Tsen, 2002); gyrB-F1
105 5'-ATGGAACAAAAGCAAATGCA-3' (forward) and gyrB-R1 5'-
106 TTAAATATCAAGGTTTTTCA-3' (reverse) for *gyr;* aroE-F1 5'-
107 ATCGGAAATCCAATTGGACA-3' (forward) and aroE-R1 (5'-
108 CCTGTCCACATTTCAAAYGC-3' (reverse) for the *aroE* gene (Soufiane and Côté, 2009);

slt-F1, 5'-ATATGCAAGCACTTCTTTTACT-3' (forward) and fliC-R6, 5'-ATTHGCDGGATTATCMGAAGC-3' (reverse) for the amplification of the flagellin sequences between *slt* and *fliC* genes (Xu and Côté, 2008). To amplify the *hag* allele internal sequences, the following primers pairs were used: BtHag-F1, 5'-AGTACATGCGCCAAAACCAAG-3' (forward) and BtHag-R1, 5'-GTTTGCTTGAGAAAGCATGCT-3' (reverse); BtHag-F2, 5'-GGGGTTCTTAATCATGAGAA-3' (forward) and BtHag-R2, 5'-TAACTCAAATGGCTTATTGT -3' (reverse) (Xu and Côté, 2006).

PCR conditions were set according to the above mentioned references and PCR products were routinely analyzed by agarose gel electrophoresis using SYBR® Safe DNA stain (Life Technologies Europe BV, Bleiswijk, The Netherlands) for DNA visualization using UV transillumination. The bands of interest were excised from gel, and the DNA was extracted using a QIAquick gel extraction kit (Qiagen). Purified amplicons were supplied to the Sanger sequencing facilities of BMR Genomics (Padova, Italy) and output sequences were analyzed with the NCBI Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

In addition to taxonomic studies, genomic DNA was enriched with the bacterial plasmid isolated with PureYield™ Plasmid Midiprep System (Promega®, Madison, USA) in compliance with manufacturer's instructions, and used as a template for the detection of possible *cry* genes, using the PCR primer system designed by Noguerra and Ibarra (2010) on conserved regions of the *cry* family. These analyses were conducted using *B. thuringiensis kurstaky* HD1 as a positive control.

2.2. Bacterial preparations

Bacteria were routinely grown in conical flasks containing 1 l T3 medium (Travers et al., 1987) incubated at 30 °C with shaking at 180 rpm. An aliquot (10 ml) of an overnight LB pre-culture inoculated with 1 ml heat-activated spore suspension (70°C for 30 min) was used as *inoculum* to facilitate culture synchronization. In these conditions, culture sporulation and sporangia lysis usually occurred within 72 hrs, as monitored by phase microscopy. Sporulated cultures were harvested by centrifugation at 10,000 x g for 10 min at 4 °C, washed three times in sterile water to eliminate main cell debris, and quantified using a Thoma chamber (E. Hartnack, Berlin, Germany). Culture supernatants and these spore suspensions at a concentration of 2 x 10^9 spores/ml were stored at -20 °C until use in bioassays. As confirmed by phase microscopy observations, no parasporal crystals were detected in these spore suspensions employed in bioassays.

At a later stage, an additional cleaning step was included in preparation procedures, in order to separate spores from other insoluble components that could be harvested by centrifugation. For this purpose, whole spore suspensions obtained as previously described, were submitted to five consecutive centrifugation cycles at 5,000 x g for 3 min at 4 °C. The resulting supernatant was ultra-centrifuged for 1 h at 50,000 x g to collect the insoluble extra-spore (ES) fraction, while the remaining pellet was represented by pure spores.

2.3. Bioassays

Experiments were conducted with insects from a *C. capitata* colony established in the entomology laboratory of the Dipartimento di Agraria of the University of Sassari (Italy). Insect rearing methods and conditions have been described elsewhere (Falchi et al., 2015).

Dose-response bioassays were conducted to determine the lethal effects caused by different bacterial preparations, including whole spore suspensions, culture supernatants and, at a later stage, pure spores and the insoluble extra-spore fraction. For this purpose, the bioassay design

158 involved four replications consisting of four groups of 24 second instar larvae to be
159 maintained in 96-well polystyrene microplates (one larva per well) filled with an artificial diet
160 made as described in Falchi et al. (2015). The diet was incorporated with the different
161 bacterial preparations to be assayed or was left untreated (control). Insects were routinely
162 incubated inside a growth chamber at 25 °C and 60% relative humidity, and were inspected
163 daily. In line with similar experiments conducted with fly larvae, mortality data were assessed
164 after 5 days (Floris et al., 2007; Ruiu et al., 2007).
165 In preliminary experiments, the spore suspension obtained harvesting and washing the whole
166 sporulated culture was assayed at a standard concentration of 10^9 spores per g of diet, while
167 culture supernatant was mixed with the diet at a 1:1 rate. Subsequently, bioassays were
168 conducted with a range of 7 concentrations used to estimate the median lethal concentration
169 (LC_{50}) value: $1x10^9$, $7.5x10^8$; $5x10^8$, $2.5x10^8$, $1.2x10^8$, $0.6x10^8$ and $0.3x10^8$ spores per g of
170 diet. This experiment was repeated three times with different batches of flies and bacterial
171 preparations.
172 Additional observations were conducted to study the sub-lethal effects of sporulated cultures
173 on *C. capitata* larvae exposed to a selected range of concentrations ($5x10^8$ and $1x10^8$ spores/g
174 of diet). In this case, four replicated groups of 24 larvae were individually maintained until
175 pupation in 96 wells microplate as previously described. The pupal development time, the
176 pupal weight and the percentage of adult emergence calculated on the number of pupated
177 insects were determined.
178 In a separate experiment, four replicated groups of 10 adults (5 males and 5 females),
179 emerging from larvae reared on a diet treated with a concentration of $5x10^8$, $1x10^8$ spores per
180 g of diet or untreated (control), were maintained in transparent plastic boxes where food was
181 provided *ad libitum* as a solid powder containing sugar and brewer's yeast hydrolysate (1:1),
182 while water was supplied by a cotton thread soaked with water. On a lateral face, each box

had a window (3 x 2 cm) covered with gauze that allowed females to insert the ovipositor and to lay eggs that were collected into a water-containing plate underneath. From the 5^{th} day on, the number of laid eggs were recorded for a 3 weeks period. During the same period and until death of all flies, insect mortality was assessed daily.

Egg viability for each treatment group and replicate was determined counting the number of larvae emerging from 20 eggs collected in three different dates during the bioassay (after 6, 10 and 15 days). For this purpose eggs were kept at 25 °C in a plate onto wet filter paper and at 90% relative humidity until hatching.

Further dose-response experiments were conducted to assay pure spores and the ES fraction obtained from the sporulated culture as previously described. This fraction was assayed at a concentration of 1 mg/g of diet, which was in line with the amount of proteins normally associated to $10^{\wedge 9}$ spores. Each experiment involved four replications (24 larvae each) and was repeated three times with different batches of insects and of bacterial preparations.

2.3. Statistical analysis

Data were analyzed using SAS software (version 9.1) with significance level set at $\alpha = 0.05$ (SAS Institute, 2004).

Probit procedure (Finney, 1971) was used to analyze dose-response data to determine the median lethal concentration (LC_{50}).

General Linear Models (GLM) of ANOVA were used to analyze data on immature development (larval and pupal development time, pupal weight and percentage of adult emergence). Data on larval mortality, on adult longevity and fecundity, and on egg viability, were compared across treatments using one-way ANOVA (factor: concentration). When significant treatment effects were detected, ANOVAs were followed by Least Significant Difference (LSD) tests for post-hoc comparison of means.

208

209 *2.3. Proteomic studies*

210 Proteins extracted from the ES fraction were analyzed by polyacrylamide gel electrophoresis
211 and subsequent mass spectrometry. Protein concentration was routinely determined using the
212 Folin-phenol reagent (Lowry et al., 1951) and bovine serum albumin (Sigma) as a standard.
213 For protein extractions, ES was resuspended in 1% SDS and 0.01% 2-mercaptoethanol,
214 followed by boiling for 5 min before centrifugation for 5 min at 10,000 x g. The supernatant
215 was resuspended in Laemmli buffer and analyzed by 10% SDS-PAGE (Laemmli and Favre,
216 1973). Gels were routinely stained with Coomassie and digitized with an ImageScanner III
217 (GE Healthcare). Three biological replicates were analyzed for each group.
218 Individual regions including major protein bands (molecular weight ranging between 35 and
219 65 kDa) obtained from the extra-spore fraction were manually excised from the gels,
220 destained and subjected to *in situ* tryptic digestion before analysis by MS/MS (Biosa et al.,
221 2011). Tryptic peptides were supplied to the proteomic facilities of Porto Conte Ricerche Srl
222 (Tramariglio, Alghero, Italy) where samples were analyzed using an LTQ-Orbitrap Velos
223 (Thermo Scientific, San Jose, CA, USA) interfaced with an UltiMate 3000 RSLCnano LC
224 system (Dionex, Sunnyvale, CA, USA, now part of Thermo Scientific) as described in Tanca
225 et al. (2013). Mass spectrometry output data were analyzed on a protein discoverer (v.1.4i,
226 Thermo Scientific, Bremen, Germany) for protein identification employing an in-house
227 Mascot server (Matrix Science, London, UK) and processed against the NCBI database
228 (http://www.ncbi.nlm.nih.gov).

229

230 **3. Results**

231

232 *3.1. Bacterial strain identification and preliminary characterization*

233 The *16S rRNA* gene sequence showed the highest percentage sequence similarity (99%) for
234 bacterial strains belonging to the *B. cereus* cluster including *B. thuringiensis*, *B. cereus sensu*
235 *stricto*, *B. mycoides*, and *B. weihenstephanensis*. The *gyrB* and *aroE* gene nucleotide
236 sequences confirmed the highest percentage similarity (99%) for *B. thuringiensis* and *B.*
237 *cereus sensu stricto*. DNA sequences of these genes were deposited in the GenBank database
238 (National Centre for Biotechnology Information, Bethesda, Maryland) under accession
239 numbers KP297917, KP318802, KP318803, respectively.
240 Complementary observations on flagellin sequences showed at most 95% similarity with
241 different *B. thuringiensis* serovars including *jegathesan*, *monterrey*, *canadensis*,
242 *darmstadiensis*, *londrina*, and *galleriae*, and 94 % with *B. cereus*.
243 Besides, no *cry* genes were detected by PCR and no parasporal bodies were observed by
244 phase or electron microscopy (Fig. 1).
245 Given the results of our observations and in line with the taxonomic relationships among
246 members of the *B. cereus* cluster, the new isolate is considered as *B. cereus sensu lato (s.l.).*
247
248 *3.2. Bioassays*
249 As a result of preliminary observations, significant levels (> 80%) of larval mortality were
250 observed when 24-hr-old *C. capitata* larvae were reared on an artificial diet incorporated with
251 a sporulated culture (10^9 spores/g of diet) of the *B. cereus s.l.* isolate ($F_{2,33}$ = 385.27, P <
252 0.0001). In the same bioassays, no significant lethal effects were detected for the culture
253 supernatant. The lethal effects were concentration dependent with a LC_{50} of 4.48 x 10^8
254 spores/g of diet (FL = 4.03-4.96, Slope ± SE = 1.91 ± 0.12, χ^2 = 235.66, P < 0.0001).
255 The sub-lethal effects of *B. cereus s.l.* on immature development are shown in table 1.
256 Development time from egg hatching to pupation significantly increased in larvae reared on

257 treated diets ($F_{2,225}$ = 66.96, P < 0.0001), with higher effects on larvae exposed to the highest
258 concentration assayed.
259 Average pupal weight was affected by treatments at the highest concentration assayed ($F_{2,9}$ =
260 4.98, P = 0.0349), with a 27.3% reduction in comparison to the control, but it was not
261 significantly reduced for the low concentration treatment.
262 Pupal development time was not significantly influenced by treatments ($F_{2,186}$= 1.82, P =
263 0.1656), while the percentage of adult emergence was reduced in pupae from treated diets
264 ($F_{2,9}$ = 11.93, P = 0.0029) with a significant decrease (25.3%) for the highest concentration
265 assayed.
266 The sub-lethal effects observed on *C. capitata* adults emerging from larvae reared on *B.*
267 *cereus s.l.* treated diets are shown in table 2. Differences between treated and control groups
268 were not significant for male ($F_{2,57}$ = 1.85, P = 0.1664) and female ($F_{2,57}$ = 0.35, P = 0.7097)
269 longevity, and for egg viability ($F_{2,33}$ = 0.13, P = 0.8754). A significant decrease in fecundity
270 was observed in the group treated with the highest bacterial concentration in comparison to
271 the control ($F_{2,9}$= 8.22, P = 0.0093).
272 Significant mortality was determined by both pure spores and the ES fraction even if at a
273 lower degree compared to the whole spore suspensions ($F_{3,44}$ = 174.27, P < 0.0001) (Table 3).

274

275 *3.3. Proteomic studies*
276 The proteome of the insoluble ES fraction obtained from sporulated cultures of the *B. cereus*
277 *s.l.* strain showed major proteins within the 35-65 kDa range (Fig. 2).
278 Based on the results of mass spectrometry, we successfully analyzed more than 700 internal
279 peptide sequences from 5 main gel regions (Fig. 2). The most significant results of protein
280 identification are summarized in Table 4. These proteins include metalloproteases, aldehyde
281 dehydrogenases, molecular *chaperones* (GroEL), peptidases, and other enzymes.

Discussion

The present study demonstrated that the Mediterranean fruit fly larvae are susceptible to a newly isolated *B. cereus s.l.* strain. The potential of *B. thuringiensis* against the Mediterranean fruit fly was formerly reported by Gingrich (1987), followed by different studies highlighting the susceptibility of other Tephritid fruit flies such as the Olive fruit fly *Bactrocera oleae* (Gmelin) (Karamanlidou et al., 1991; Alberola et al., 1999) and the Mexican fruit fly *Anastrepha ludens* (Loew) (Robacker et al., 1996) to different *Bt* isolates. In a screening program involving a wide variety of *B. thuringiensis* strains from citrus orchards in Spain, Vidal-Quist et al. (2009) observed a maximum toxicity against *C. capitata* adults of barely 30%. Other studies reported a higher susceptibility of larvae (Floris et al., 2007; Aboussaid et al., 2011). In these studies the insecticidal action against fruit flies was associated to both spore-crystal mixtures or to the soluble and heat-stable β-exotoxin, a metabolite certain *Bt* strains produce and release in the culture broth. Robacker et al. (2000) demonstrated the effectiveness of pure spore-crystal suspensions against *A. ludens*, which supported a possible direct action of Cry toxins in the gut. Later on, Vidal-Quist et al. (2010) for the first time demonstrated the activity of Cyt1Aa delta-endotoxin from *B. thuringiensis* serovar *israelensis* against *C. capitata* larvae, thus envisioning possible future biotechnological approaches for the development of new management strategies for this pest. In this case, authors showed that *Bti* spores and crystal mixtures were not active, while solubilized and activated toxins were significantly toxic. In the case of our *B. cereus s.l.* strain showing toxicity against *C. capitata* larvae, no parasporal crystals were observed by electron microscopy, and neither Cry proteins nor *cry* genes were detected through proteomic and genomic analyses, respectively. The insecticidal action was never associated to the culture supernatant, thus excluding a major implication of possible soluble metabolites produced by the bacterium. On the contrary,

toxicity was associated to spores and to the insoluble extra-spore protein fraction harvested from sporulated cultures. This is different from the results obtained by Molina et al. (2010) in bioassays with a *Bacillus pumilus* strain toxic to *C. capitata* larvae. In that case, a low temperature treatment of the whole bacterial culture (cell fractions and supernatant) is required to activate virulence factors.

In line with other studies involving the entomopathogenic bacterium *Brevibacillus laterosporus* and the house fly *Musca domestica* L. (Ruiu et al. 2007), the insecticidal effects of the *B. cereus s.l.* isolate were concentration dependent with a median lethal concentration of spores in larval diet comparable to values observed in studies with environmental isolates of *Bacillus thuringiensis* against Diptera (Lonc et al., 2001).

When administered at sub-lethal concentrations, sporulated cultures of this strain significantly reduced immature survival, extended larval development time, reduced pupal weight, adult emergence rate, and fecundity. Also these effects were concentration dependent, which corroborates the outcomes of former studies with diptera such as the house fly (Ruiu et al., 2006) and the mosquito *Aedes aegypti* L. (Hare and Nasci, 1986). The mechanism causing such reduction in adult reproduction fitness has not been explained, however it is known that a lower fecundity of fly females emerging from smaller pupae is associated to a reduced number of ovarioles (Bennetova et al., 1981).

Effects on survival and reproduction of *C. capitata* were also observed in studies with the insect endosymbiont *Wolbachia pipientis* Hertig (Sarakatsanou et al., 2011). Transinfection events with some strains of this bacterium reduced survival in egg and pupal stage and caused a slight decrease in female fecundity on specific Mediterranean fruit fly lines.

Both the lethal and sub-lethal effects caused by *B. cereus s.l.* on *C. capitata* can provide an important contribution to the prospect of the eventual use of the bacterium or its virulence factors as pest management active ingredients.

Toxic effects of sporulated cultures against *C. capitata* larvae are partly associated to a proteinaceous extra-spore fraction produced by the bacterium, which contains putative virulence factors. The characterization of this fraction focused on the identification of major proteins using electrophoresis combined with mass spectrometry. These useful proteomics tools allowed us to identify the most abundant proteins that were represented by functional molecules like *chaperones* (GroEL), proteins with primary metabolic functions like aldehyde dehydrogenases, and degradative enzymes like metalloproteases and various peptidases. Some of these proteins have been found also as component of *B. cereus* exosporium (Charlton et al., 1999) and extracellular proteome (Gohar et al., 2005), and belong to families that have already been associated to the insecticidal action of diverse entomopathogens. Interestingly, toxicity of the *B. cereus s.l.* was associated to both pure spores and the extra-spore fraction, even if at a lower degree compared to the whole sporulated culture. This might be in relation to the presence of the same virulence factors in the exosporium and in the extra-spore proteome, in addition to a synergistic action of spore germination (Liu et al., 1998). Besides, it is not excluded that the insoluble extra-spore fraction may contain fragments of the exosporium.

Among putative virulence factors are bacterial metalloproteases, with special regard to zinc containing proteases, that are known to act occasionally as toxins in many pathogenic bacteria or they may play indirect roles in pathogenicity (Miyoshi and Shinoda, 2000). This is the case of the 51.8 kDa metalloprotease released by the nematode symbiont *Photorhabdus luminescens* and showing oral toxicity against the diamondback moth *Plutella xylostella* L. (Chang et al., 2013). A significant role in virulence of zinc metalloproteases produced by acrystalliferous *B. thuringiensis* strains when the host is infected via the oral route, was also observed (Fedhila et al., 2002). Although specific investigations are required to demonstrate the actual role of the different proteases produced by the *B. cereus s.l.* strain, their possible

implication in the observed effects on *C. capitata* is in line with a well known potential of a variety of microbial proteases to act as insect toxins targeting different sites like the cuticle, the peritrophic matrix in the gut, the hemocoel, haemocytes and fat bodies (Harrison and Bonning, 2010; Manachini et al., 2013; Castagnola and Stock, 2014). Similarly, other bacteria showing pathogenicity against *C. capitata* larvae, like *B. pumilus*, are known to produce abundant extracellular proteases (Yu et al., 2014).

Among other proteins identified in the ES fraction produced by the *B. cereus s.l.* strain there is a 53 kDa protein showing high homology with a *B. cereus* betaine-aldehyde dehydrogenase whose insecticidal potential still need to be demonstrated, even though the contribution of this group of proteins to bacterial virulence and pathogenesis has recently been reported (Lee et al., 2014).

The extra-spore proteome of our strain contained a patatin-like phospholipase homolog, which may display insect inhibitory properties, as demonstrated against larvae of Coleoptera (Alibhai et al., 2006).

Among other major proteins identified in the protein profile of the *B. cereus s.l.* strain there are molecular *chaperones* (GroEL), normally involved in protein folding. Some studies show the implication of chaperone-like proteins in enhancing Cry toxins synthesis and crystallization (Crickmore and Ellar, 1992; Diaz-Mendoza et al., 2012). More recent studies highlighted an insecticidal 58-kDa GroEL homolog with chitin binding activity, secreted by *Xenorhabdus nematophila* in the culture medium through outer membrane vesicles (Joshi et al., 2008). This might not be our case, but we could speculate that these proteins could help the synthesis of other bacterial virulence factors.

In conclusion, the present study determined the lethal and sub-lethal effects caused by sporulated cultures of a new *B. cereus s.l.* strain against *C. capitata* larvae. As a result of bioassays combined with proteomic analysis, bacterial toxicity is associated both to spores

and to the extra-spore proteome. The characterization of the latter fraction highlighted major proteins, whose possible implication in the pathogenesis mechanisms has been discussed. Further studies are needed to determine the specific role of each of these compounds and to evaluate their possible use for the development of new bio-based pest management ingredients.

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Table 1 **Means (± SEM) of larval development time, pupal weight, pupal development time and percentage of emergence of *Ceratitis capitata* larvae reared on *Bacillus cereus s. l.* treated diets.**

Treatment concentration (spore/g)	n[a]	Larval development time[b] (days)	Pupal weight (mg)	n[c]	Pupal development time[d] (days)	Adult emergence (%)
$5x10^8$	52	12.18 ± 0.76 a[e]	7.48 ± 0.76 a	43	5.84 ± 0.36 a	66.68 ± 1.65 a
$1x10^8$	83	10.26 ± 0.72 b	9.58 ± 0.65 b	73	5.83 ± 0.39 a	85.41 ± 1.06 b
Control	93	9.73 ± 0.41 c	10.29 ± 0.52 b	83	6.03 ± 0.31 a	89.21 ± 0.81 b

[a] Number of surviving pupae from 96 treated larvae.

[b] Days from egg hatching to pupation.

[c] Number of emerged *C. capitata* adults.

[d] Days from pupation to adult emergence.

[e] Means followed by different letters are significantly different (GLM followed by LSD test: $P < 0.05$).

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Table 2 **Means (± SEM) of longevity, fecundity, percentage of egg hatching of ovipositing females of *Ceratitis capitata* emerging form larvae reared on *Bacillus cereus s. l.* treated diets.**

Treatment concentration (spore/g)	Longevity (days)[a]		Fecundity (min-max)	Egg hatching[b]
	Male	Female	n	%
$5x10^8$	19.95 ± 2.31 a[c]	26.35 ± 1.06 a	164.5 ± 21.02 a	96.67 ± 0.42a
$1x10^8$	23.45 ± 1.03 a	27.10 ± 1.08 a	258.2 ± 22.97 b	97.50 ± 1.25 a
Control	24.45± 1.82 a	28.15 ± 1.13 a	296.3 ± 26.60 b	97.08 ± 1.10 a

[a] Days from adult emergence to adult death.

[b] Mean values of egg hatching recorded after 6, 10 and 15 days from adult emergence.

[c] Means in each column followed by different letters, are significantly different (ANOVA followed by LSD test: $P < 0.05$).

Table 3

Mortality (mean ± SEM) of *Ceratitis capitata* larvae reared on a diet incorporated with different fractions of the *Bacillus cereus s. l.* strain.

Treatment[a]	Mortality (%)
Whole spore suspension	82.64 ± 2.61 a[b]
Pure spores	55.56 ± 3.64 b
Extra-spore fraction (ES)	40.63 ± 1.99 c
Control (untreated)	3.47 ± 1.00 d

[a] Whole spore suspensions and pure spores were applied at a concentration of 10^9 spores/ g of diet, while the extra-spore fraction was applied at a concentration of 1 mg/g of diet.

[b] Means in each column followed by different letters are significantly different (LSD test, $P < 0.05$).

Table 4

Major proteins of the *B. cereus s. l.* insoluble extraspore fraction (ES).

Gel region No.[a]	Accession number	Protein	Coverage (%)	Peptides	MOWSE Score	PI	MW (kDa)
1	218235093	Molecular chaperone GroEL	99.63	84	21382.08	4.84	57.4
	446746191	Peptide ABC transporter substrate-binding protein	78.37	75	4395.26	8.46	63.4
	434374375	Oligoendopeptidase F	68.97	56	2997.81	4.94	66.1
	218231104	Succinate dehydrogenase flavoprotein subunit	80.74	35	1924.00	6.02	65.9
2	449090484	Aldehyde dehydrogenase	98.18	66	8162.62	5.66	53.7
	30020418	2-methylcitrate dehydratase	94.98	53	3813.71	5.63	53.4

	445956496	Penicillin-binding protein	62.47	39	2618.60	5.64	54.6
	446410102	Aminopeptidase A	58.50	30	1251.16	4.88	53.6
3	296501054	Homogentisate 1,2-dioxygenase	94.10	43	6997.79	6.68	45.1
	446773703	Aminotransferase	89.09	44	3291.32	6.00	50.1
	30021879	Zinc protease	91.04	44	2347.43	5.36	48.9
4	402559902	Acetoin dehydrogenase E1 component	85.17	46	5057.10	5.19	37.0
	446954252	Peptidase M28	94.40	32	2117.55	6.55	39.2
5	446457953	Formamidase	95.48	31	1970.29	5.30	36.7
	449090436	TPR domain protein	93.80	39	1699.33	5.85	43.9
	218231626	Patatin phospholipase	51.26	13	512.15	6.16	40.7

[a] Numbers indicate regions that were excised from SDS-polyacrylamide gels for Mass Spectrometry analysis.

Figure legends

Fig. 1 – Electron micrograph showing a sporangium of the *Bacillus cereus s.l.* isolate. SW, sporangium wall; Sp, spore; SC, spore coat; Ex, exosporium.

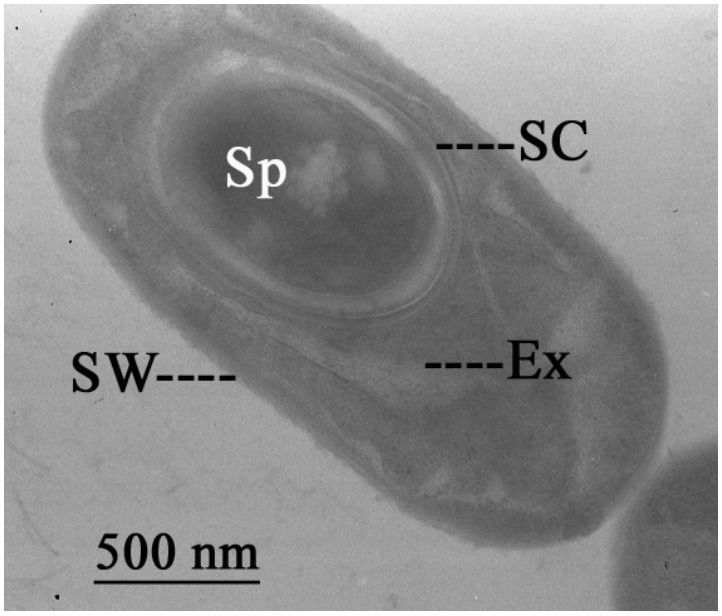
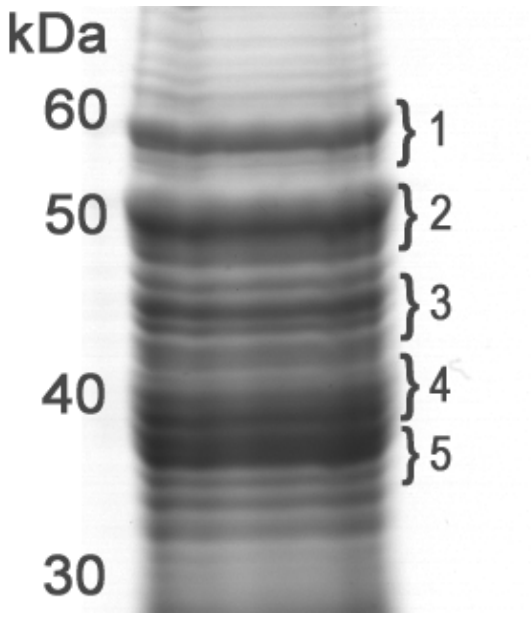
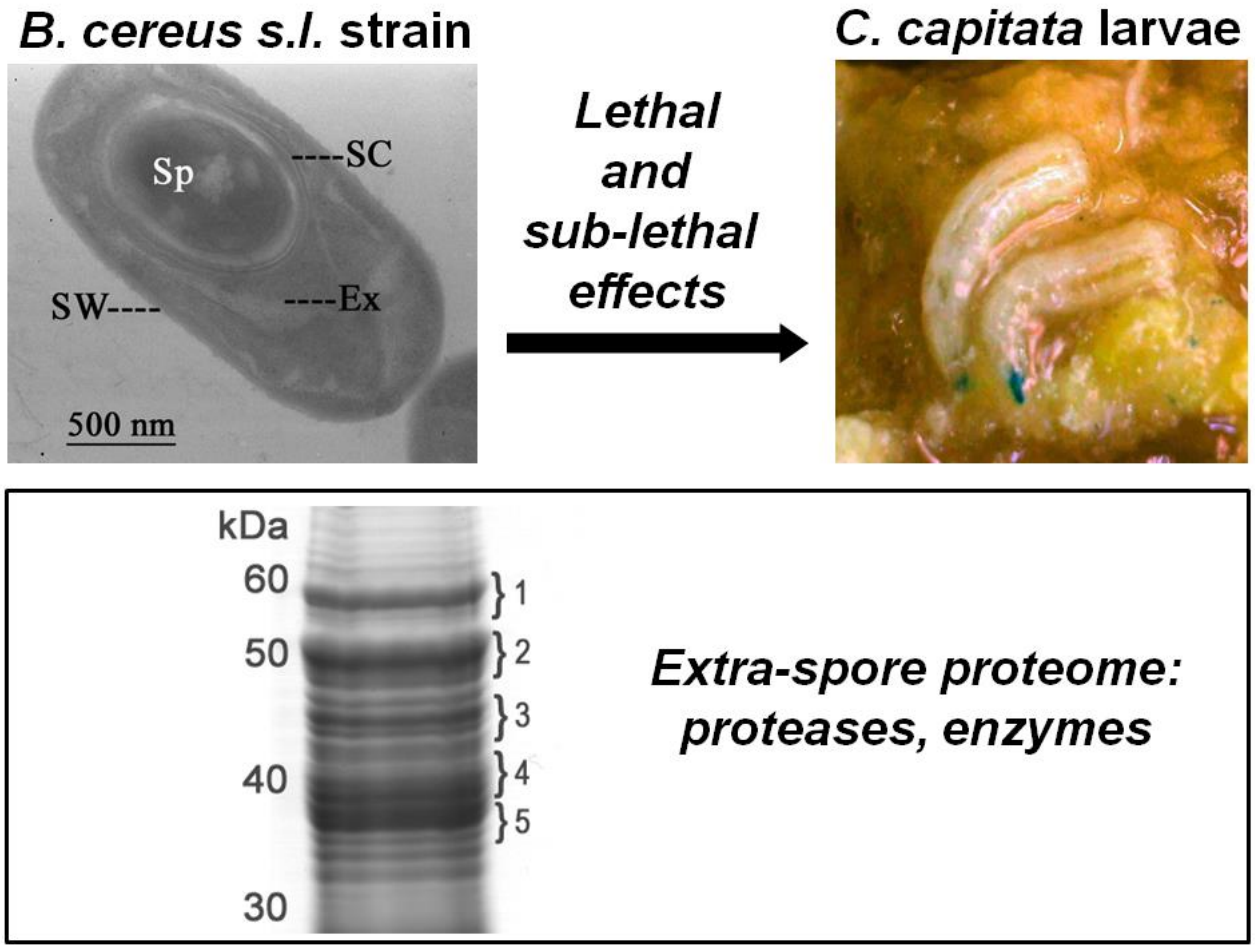


Fig. 2 – Representative protein profile on 10% SDS-PAGE of the *Bacillus cereus s.l.* isolate ES fraction. Numbers indicate gel regions that were analyzed by mass spectrometry.



Graphical abstract



Highlights

- A new *Bacillus cereus s.l.* isolate is toxic to *Ceratitis capitata* larvae.
- Spores of *B. cereus s.l.* (10^9/g of diet) cause sub-lethal effects on the Medfly.
- The insecticidal action is partly associated to an extra-spore fraction.
- The extra-spore proteome contains putative virulence factors.