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ORIGINAL ARTICLE

Selection of *Bacillus thuringiensis* strains in citrus and their pathogenicity to *Diaphorina citri* (Hemiptera: Liviidae) nymphs

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Abstract *Bacillus thuringiensis* (Bt) toxins are effective in controlling insect pests either through the spraying of products or when expressed in transgenic crops. The discovery of endophytic Bt strains opened new perspectives for studies aimed at the control of sap-sucking insects, such as the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), a vector of "*Candidatus* Liberibacter spp.," associated with citrus *huanglongbing* (HLB). In this study, translocation of endophytic Bt strains in citrus seedlings inoculated with Bt suspension delivered by soil-drench, and their systemic pathogenicity to *D. citri* nymphs were investigated. The pathogenicity of three wild-type Bt strains against *D. citri* third-instar nymphs was demonstrated. Among the 10 recombinant strains tested (each of them harboring a single *cry* or *cyt* gene), 3 can be highlighted, causing 42%–77% and 66%–90% nymphal mortality at 2 and 5 d after inoculation, respectively. The isolation of Bt cells from young citrus shoots and dead nymphs, and PCR performed with specific primers, confirmed the involvement of the Bt strains in the psyllid mortality. This is the first report showing the translocation of Bt strains from citrus seedling roots to shoots and their potential to control *D. citri* nymphs that fed on these soil-drench inoculated seedlings. The Bt strains that caused the highest mortality rates have the potential to be used as bioinsecticides to control *D. citri* and the identified genes can be used for the production of transgenic Bt citrus.

Key words Asian citrus psyllid; *Citrus sinensis*; Cry toxins; endophytic Bt; huanglongbing; young shoots

Introduction

Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is a serious threat to citrus production worldwide, since it is a vector of the phloem-limited bacteria "*Candidatus* Liberibacter asiaticus" and "*Ca.* L. americanus" associated with *huanglongbing* (HLB)

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disease (Gottwald, 2010). The five nymphal instars of *D. citri* have little mobility and feed on young citrus shoots, whereas adults exhibit greater mobility, feeding on leaves of all developmental stages and also on stems (Hall *et al.*, 2012). Females lay eggs only in the presence of young shoots (Cifuentes-Arenas *et al.*, 2018), which are nutritionally richer compared to the mature ones (Sétamou *et al.*, 2016). Nymphs and adults can acquire and inoculate the bacteria by feeding on the sap of infected plants (Inoue *et al.*, 2009; Pelz-Stelinski *et al.*, 2010). The preconized HLB management is based on the use of healthy vegetative material produced under protected environment, reducing the source of inoculum by eliminating symptomatic trees from the field, and *D. citri* chemical control (Belasque Jr. *et al.*, 2010; Bassanezi *et al.*, 2013).

Among *D. citri* management tactics, the chemical control through spraying and/or application of insecticides via soil-drench or trunk have been widely used by growers to reduce the insect vector population and HLB spread in citrus groves (Grafton-Cardwell *et al.*, 2013; Qureshi *et al.*, 2014; Boina & Bloomquist, 2015; Miranda *et al.*, 2016). However, the overuse of pesticides can cause mortality of natural enemies and pollinators, favor the occurrence of secondary pest outbreaks, and accelerate the selection process of *D. citri* populations resistant to the main insecticides (Tiwari *et al.*, 2011; Monzo *et al.*, 2014). For this reason, several research groups have been searching for alternatives to control *D. citri*, including RNA interference (El-Shesheny *et al.*, 2013; Galdeano *et al.*, 2017) and biological control with natural enemies such as the ectoparasitoid *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae) (Parra *et al.*, 2010; Flores & Ciomperlik, 2017) and entomopathogenic agents (Conceschi *et al.*, 2016; Ausique *et al.*, 2017).

Among the entomopathogens, *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (Bt) has been widely used in biopesticide formulations containing bacterial spores and crystals (Schnepf *et al.*, 1998; Jouzani *et al.*, 2017). Bt has high activity against immature stages of several pest insects belonging to different orders, such as Lepidoptera, Coleoptera, Diptera (Xu *et al.*, 2014), Hemiptera (Porcar *et al.*, 2009; Torres-Quintero *et al.*, 2016), Hymenoptera, and Orthoptera (Bravo *et al.*, 2017). During the sporulation phase, it produces crystalline protein inclusions, named δ-endotoxins or parasporal crystals (Cry or Cyt) (Aronson *et al.*, 1986), which are solubilized in the gut of the immature stages of insects under alkaline (Lepidoptera and Diptera) or acid (Coleoptera and Hemiptera) pH (Bravo *et al.*, 2012). Also in the gut, proteases will activate the toxin, causing lysis of the insect epithelial cells leading to its death (Bravo *et al.*, 2012; Jurat-Fuentes & Crickmore, 2016). This bacterium is easy to handle, does not pollute the environment (Monnerat & Bravo, 2000), and is safe for mammals (Whiteley & Schnepf, 1986). Few studies of active Bt strains or toxins have been reported in hemipterans (Porcar *et al.*, 2009) since they are rarely exposed to Bt toxins present on the plant surface (Chougule & Bonning, 2012). However, endophytic Bt was recently reported in cotton (Monnerat *et al.*, 2009), coffee (Miguel *et al.*, 2013) and wheat (Tao *et al.*, 2014). Furthermore, several studies on the colonization and translocation of Bt have been performed in beans (Maduell *et al.*, 2007; García-Suárez *et al.*, 2017), cabbage (Monnerat *et al.*, 2009; Praça *et al.*, 2012), many different legumes (Tanuja *et al.*, 2013; Mishra *et al.*, 2017) and bryophytes (Lin *et al.*, 2017). These studies open new perspectives for the use of this bacterium as a biocontrol agent for sap-sucking insects (Jurat-Fuentes & Jacksony, 2012).

For this reason, the ability of the spore-crystal complex of Bt strains to translocate from the root to the upper parts of the citrus seedlings and, most importantly, their pathogenicity against *D. citri* were assessed. To the best of our knowledge, this is the first study that reports the ability of the spore-crystal complex of Bt strains to translocate and cause mortality in *D. citri* nymphs feeding on citrus young shoots. These results may contribute to the development of new alternatives for *D. citri* management, through bioinsecticide formulations or production of genetically modified citrus plants expressing Cry toxins.

Materials and methods

Insect colony and citrus seedlings

For all assays, *D. citri* third-instar nymphs from a healthy non-*Ca*. Liberibacter spp. exposed and susceptible colony was collected on *Murraya paniculata* (L.) Jack (Sapindales: Rutaceae) in the municipality of Piracicaba, São Paulo (SP), Brazil, and maintained for several generations (5 years) in a climate-controlled room (25 $\pm$ 2°C, 60% $\pm$ 10%, RH and 14 L : 10 D h photoperiod) at Department of Entomology and Acarology at the University of São Paulo, Luiz de Queiroz College of Agriculture (ESALQ/USP), Piracicaba, SP, Brazil. The colony was proven to be CaLas-free by qPCR using the primers and conditions described in Lin *et al.* (2010). The rearing was performed on seedlings of *M. paniculata* as described by Parra *et al.* (2016).

"Pera" sweet orange [*Citrus sinensis* (L.) Osbeck] 3-month old seedlings were grown in citrus containers (120 mL) with pine bark substrate (Multiplant Citrus®, Holambra, SP, Brazil) for assessment of systemic translocation and pathogenicity assays conducted at Centro de

Citricultura Sylvio Moreira (IAC) and Fund for Citrus Protection (Fundecitrus) located in Cordeirópolis and Araraquara, SP, Brazil, respectively.

Bacillus thuringiensis strains and growth conditions

For all assays, 3 wild-type and 10 recombinant strains, in addition to water as negative control, were used. Preliminary assays showed that recombinant strain S2210, carrying *cry1Ab*, had no effect on the psyllid and, therefore, it was used as a mock control. The wild-type strains were *B. thuringiensis* subsp. *kurstaki* HD-1 (Btk) (S1450), a standard strain obtained from the collection of *B. thuringiensis* and *Lysinibacillus sphaericus* at Pasteur Institute, Paris, France, and *B. thuringiensis* (S1302) and *B. thuringiensis* subsp. *israelensis* (S1989) from the collection of bacteria of invertebrates at Embrapa Genetic Resources and Biotechnology, Brasília, DF, Brazil. The recombinant strains S2211, S2210 (here used as mock), S2209, S2396, S2212, S2036, S2037, S2492, S2038, and S2035 express the individual genes *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B*, *cry2Aa*, *cry4A*, *cry4B*, *cry10*, *cry11*, and *cyt1A*, respectively, and are deposited at the collection of bacteria of invertebrates at Embrapa Genetic Resources and Biotechnology. In addition, strain S2032 expressing *gfp* (green fluorescent protein) gene (Btk::GFP) was used to investigate the uptake and translocation of the bacterium into citrus seedlings (Monnerat *et al.*, 2009). Bt strains were cultivated in Embrapa medium containing specific antibiotics to recombinant strains (Monnerat *et al.*, 2007) in a rotating incubator at 28°C and 200 r/min, for 24 h (for Btk::GFP) or up to 72–76 h (for other strains used in the assays). Prior to use, each strain was observed under a phase-contrast microscope (Leica Microsystems Co., Wetzlar, Germany) with 1000× magnification to observe vegetative cells or spore-crystal complex.

Assessment of the Bt translocation into citrus seedlings

In order to assess the Bt endophytic translocation into citrus seedlings, 10 mL of a 2.5 : 10 Btk::GFP suspension : water was applied to the pine bark substrate, close to the roots of each seedling. The translocation of Bt from the rhizosphere to the upper young shoots and their acquisition by *D. citri* nymphs feeding on those shoots were assessed 2 d after inoculation (DAI). Citrus young shoots for both treatments (Btk::GFP and negative controls) were surface sterilized for 2 min for each solution described: sodium hypochlorite (2.5%), ethanol (70%), water (2×), and macerated in 1 mL of NaCl saline solution (8.5 g/L). For each treatment, five replicates were used. For isolation of Bt from the insects, pools of up to five nymphs were collected with the aid of a soft brush, macerated in 200 μL of water, and incubated for 12 min at 80°C and 5 min in ice (Praça *et al.*, 2012). After that, 100 μL of both macerated materials were placed in Petri dishes containing Embrapa medium, and the Bt colonies were observed in a fluorescence microscope (Olympus Co., Tokyo, Japan). A similar method was used for nymphs from the control treatment.

Later, the translocation of Bt into "Pera" sweet orange seedlings was assessed with each wild-type, nine recombinant strains, and two negative controls (water without spores and crystals and one recombinant strain *cry1Ab*) used on the pathogenicity assays, as described previously. However, the isolation of these Bt strains from young shoots and dead nymphs were assessed 5 DAI after maceration, plating, and observation of typical Bt colonies. Individual young shoots (from 6–21 replicates per treatment) and a pool of dead nymphs (containing 2–8 nymphs per tube and 3–10 replicates per treatment) were used for maceration. From the CFU obtained by isolation, a single colony was selected from each replicate/treatment/condition (plant or insect) for the confirmation of the identity and translocation in citrus by PCR using specific primers for each strain (Table 1). The PCR analysis was carried out on a Veriti 96-Well Thermal Cycler (Applied Biosystems Inc., California, USA) with 6.25 μL of DreamTaq PCR Master Mix (2×) (Thermo Scientific Inc., Massachusetts, USA), 0.3 μL of each primer (0.2 μmol/L), and 1 μL of the total DNA solution as template. For PCR cycling, the same procedure and analysis previously described were used (Céron *et al.*, 1994; 1995; Bravo *et al.*, 1998; Ibarra *et al.*, 2003; Mendoza *et al.*, 2012). PCR products were visualized in agarose gel electrophoresis (1%) with ethidium bromide (0.5 ng/μL) in ultraviolet light using a PhotoQuant 300 Imager photodocumentator (GE Healthcare Co., Chalfont, UK).

Pathogenicity of Bt strains against Diaphorina citri third-instar nymphs

A pilot screening with three Bt wild-type strains, nine recombinant strains, and two negative controls (water and strain *cry1Ab*) was performed. For this purpose, 10 *D. citri* third-instar nymphs were transferred from *M. paniculata* to each seedling and used as an experimental unit. Then, 10 mL of a bacterium suspension : water (2.5 : 10) was applied to the substrate of each seedling. Water and strain *cry1Ab* were used as controls. For each treatment, 10–35 replicates (seedlings) were used. The assay was kept in a climate-controlled chamber at 24 ± 2°C, 70% ± 10% RH, and 14 L : 10 D h photoperiod.

Table 1 Sequences of the primers used in the validation and molecular characterization of the Bt strains.

Sequence name	5′–3′ sequence	Expected fragment size (bp)	Reference
Cry1Aa	TGTAGAAGAGGAAGTCTATCCA TTGGAGCTCTCAAGGTGTAA	246	Cerón *et al.* (1994)
Cry1Ab	AACAACTATCTGTTCTTGAC CTCTTATTATACTTACACTAC	216	Cerón *et al.* (1994)
Cry1Ac	GTTAGATTAAATAGTAGTGG TGTAGGTGGTACTGTTATTG	180	Cerón *et al.* (1994)
Cry2Aa	CAAGCGAATATAAGGGAGT TAGCGCCAGAAGATACCA	470	Mendoza *et al.* (2012)
Cry1B	CTTCATCACGATGGAGTAA CATAATTTGGTCGTTCTGTT	367/369	Cerón *et al.* (1994)
Cry4A	TCAAAGATCATTTCAAAATTACATG CGGCTTGATCTATGTCATAATCTGT	459	Ibarra *et al.* (2003)
Cry4B	CGTTTTCAAGACCTAATAATATAATACC CGGCTTGATCTATGTCATAATCTGT	321	Ibarra *et al.* (2003)
Cry10	TCAATGCTCCATCCAATG CTTGTATAGGCCTTCCTCCG	348	Ibarra *et al.* (2003)
Cry11	TTAGAAGATACGCCAGATCAAGC CATTTGTACTTGAAGTTGTAATCCC	305	Bravo *et al.* (1998)
Gral-cyt	AACCCCTCAATCAACAGCAAGG GGTACACAATACATAACGCCACC	Cyt1Aa 522/Cyt1Ab 525	Bravo *et al.* (1998)
Cry3A	CAATCCCAGTGTTTACTTGGAC CCCCGTCTAAACTGAGTGT	285	Cerón *et al.* (1995)
Cry2Ab	CACCTGGTGGAGCACGAG GTCTACGATGAATGTCCC	771	Mendoza *et al.* (2012)
Cyt1	CCTCAATCAACAGCAAGGGTTATT TGCAAACAGGACATTGTATGTGTAATT	cyt1Aa 477/cyt1Ab480/cyt1Ba 477	Ibarra *et al.* (2003)

First line of each primer: forward primer; second line: reverse primer.

A second assay was carried out in order to assess the effect of the most promising Bt strains, following the same procedures and criteria described above. For each treatment, 10 replicates (seedlings) were used. The nymph mortality in each experimental unit and assay was recorded daily for 5 DAI. The dead nymphs from each treatment were collected during the assessments for Bt isolation to confirm their acquisition by the nymphs through the PCR amplification of CFU using specific primers for each *cry* and *cyt* gene described in Table 1.

Experimental design and data analysis

For all assays, a fully randomized design was used. In order to verify the effect of different Bt strains on the mortality of *D. citri* third-instar nymphs at different times (DAI), they were subjected to a repeated measure analysis using generalized linear mixed models (GLMM) of the "*lme4*" package (Bates *et al.*, 2015), with a binomial distribution. For this purpose, the effect of explanatory variables ("Pera" sweet oranges treated with Bt strains and control) and the time were considered as fixed factors, whereas the repeated measure in each seedling in time was considered as random. The effect of treatment and time was assessed by likelihood-ratio tests ($P < 0.05$) between a full and a reduced model. The same test was used to verify the significance of the treatment interaction by time, comparing two models: one with interaction and another without interaction. Furthermore, the effect of Bt strains on the mortality of *D. citri* nymphs for each time, generalized linear models (GLM) (Nelder & Wedderrbun, 1972), with quasi-binomial distribution, were used to analyze the proportion of mortality. Goodness-of-fit was assessed through half-normal plots with simulation envelope using the "*hnp*" package (Demétrio *et al.*, 2014). In

case of significant differences among treatments, multiple comparisons with the Tukey–Kramer test ($P < 0.05$) were performed using the "*glht*" function of the "*multcomp*" package, with adjusted P values (Hothorn *et al.*, 2008). All analysis were performed using the statistical software "*R*," version 3.4.4 (R Core Team, 2018).

Results

Bt strains and growth conditions

Phase contrast microscopy with a 1000 fold increase allowed confirming the presence of large numbers of spores and crystals of different forms produced in bacterial suspensions from all Bt strains after 72 h of culture (Figs. S1A and B). Vegetative cells were rarely observed.

Assessment of the Bt endophytic translocation into citrus seedlings

The uptake of the bacteria in citrus seedlings and their acquisition by the *D. citri* nymphs that fed on those seedlings were confirmed by the isolation of Btk::GFP from both citrus shoots and insects, yielding variable numbers (between 30 and 100) of CFU with typical whitish cream coloration and irregular borders in Petri dishes containing Embrapa medium (data not shown). There were no colonies observed on the negative control (water) plates. The identity of the bacterium was confirmed by fluorescence microscopy with the presence of fluorescent bacilliform cells (Figs. 1B–C).

Subsequently to the proof of concept for the Btk::GFP strain, the translocation of practically all Bt strains from the rhizosphere to the citrus young shoots and their acquisition by the *D. citri* nymphs were unequivocally confirmed 5 DAI with the recovery of typical Bt colonies (Fig. S2), followed by PCR assays with specific primers for each *cry* and *cyt* gene, detected in all treatments (except for strain S2396) (Table 2 and Fig. S3). Single fragments of expected sizes, ranging from 180 to 522 bp, were obtained for the corresponding Bt strains (Fig. S3). The number of CFU recovered from the Bt isolation from citrus young shoots and dead nymphs in both assays were variable, and they were classified as follows: (a) low: 1–10 CFU, (b) intermediate: 11–30 CFU, and (c) high: more than 30 CFU (Table 2). Twelve out of 13 Bt strains were able to translocate from the roots to the young shoots of citrus and to be acquired by the psyllid nymphs that fed on those shoots. The only strain that failed to translocate properly was S2396, containing *cry1B*. The others were detected in both conditions (plants and insects),

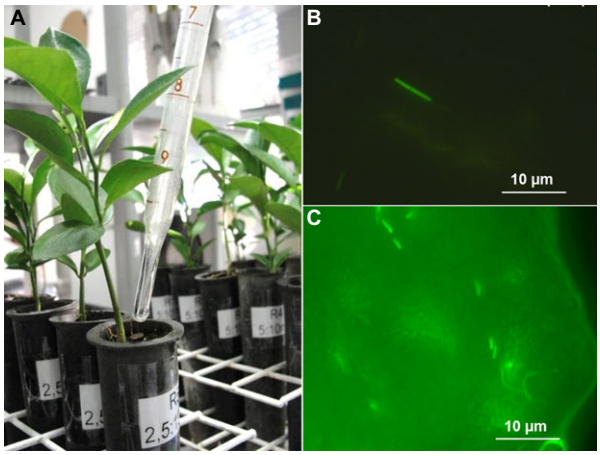

Fig. 1 Inoculation of Btk::GFP suspension in "Pera" sweet orange seedlings (A), recovery and visualization of soil-drench inoculated Btk::GFP vegetative cells in young citrus shoots by fluorescence microscopy under magnifications of 1000× (B) and 400× (C).

although most of the strains yielded a low number of colonies (S1302, S1450, S1989, S2210, S2212, S2037, S2492). Bt strains with intermediate numbers of colonies were S2211, S2209, S2036, and S2035, and only S2038 yielded a high number of colonies from both plants and insects (Figs. S2B–S2G). The identities of the Bt strains from the colonies were confirmed by PCR (Table 2 and Fig. S3).

Pathogenicity of Bt strains against Diaphorina citri third-instar nymphs

A significant interaction between treatment and time (DAI) was recorded for the initial screening ($\chi^2 = 27.03$; df = 13; $P < 0.0001$) and also for the second assay ($\chi^2 = 58.23$; df = 8; $P < 0.0001$), indicating that the mortality rates provided by different Bt strains to *D. citri* third-instar nymphs increased over time (Tables 3 and 4).

In the initial screening, water and mock strain *cry1Ab* caused mortality from 14.4% to 33% at 5 DAI, respectively. Strains S1302, S1450, and S1989 caused 55%–77% mortality of *D. citri* nymphs at 2 DAI, and 77%–90% mortality at 5 DAI (Table 3). The recombinant strains containing the genes *cry1Aa*, *cry2Aa*, *cry4B*, *cry10*, *cry11*, or *cyt1A* were also pathogenic to *D. citri* nymphs, reaching 50%–65% mortality at 5 DAI (Table 3). Strain S1302 harbors the genes *cry1Ab* and *cry3A*, strain S1450 carries genes *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ac*, *cry2Aa*, and *cry2Ab*, whereas strain S1989 contains the genes *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1A*, and *cyt1B*. Ten out of those

Table 2 Confirmation of Bt identity by isolation followed by PCR with each *cry*/*cyt* genes.

Treatments/ initial screening	†UFC range obtained in isolation of young shoots (6–21 replicates)/dead nymphs pool (2–8 nymphs/tube—3–10 replicates per treatment)	PCR for young shoots/dead nymphs pool
S1302	Few/few	+/+
S1450	Few/few	+/+
S1989	Few/few	+/+
cry1Aa	Few/mean	+/+
cry1Ac	Many/few	+/+
cry2Aa	Few/few	+/+
cry1B	Without growth/poor growth	–/+
cry4A	Few/many	+/+
cry4B	Few/few	+/+
cry10	Few/few	+/+
cry11	Many/many	+/+
cyt1A	Few/many	+/+
cry1Ab	Few/few	+/+
Negative Control	Without growth/without growth	–/–
Treatments/assay II	†UFC range obtained in isolation of young shoots (6–21 replicates)/dead nymphs pool (2–8 nymphs/tube—3–10 replicates per treatment)	PCR for young shoots/ dead nymphs pool
S1302	Mean	+/+
S1450	Mean	
S1989	Mean	+/+
cry2Aa	Mean	+/+
cry4A	Mean	+/+
cry10	Mean	+/+
cry11	Mean	+/+
cyt1A	Mean	+/+
Negative control	Without growth	–/–

†UFC number range (average). Low: 1–10, intermediate: 11–30, and high: more than 30.

13 toxin genes were represented in the recombinant strains and tested individually, causing different toxicity levels to the citrus psyllid nymphs. The strains containing genes *cry4B*, *cry10*, *cry11*, and *cyt1A* caused 60%–65% mortality of *D. citri* nymphs at 5 DAI. These genes are present in strain S1989, which was responsible for the death of up to 79% and 88% of the *D. citri* nymphs at 2 DAI and 5 DAI, respectively (Table 3 and Fig. S4). Strains Cry1Aa and Cry2Aa caused ~50% mortality, while strains Cry1Ab, Cry1Ac, and Cry4A were able to cause mortality from 36% to 44% of insects at 5 DAI (Table 3). Although statistical analyses detected significant differences between these strains and the control treatment, from the biological point of view, they are not effective in controlling the *D. citri* nymphs, and therefore, they were not further assessed. Strains Cry1Ab and Cry1B did not differ from the water control (Table 3).

With the results obtained in the screening of 13 strains tested, the best wild-type and recombinant strains (S1302, S1450, S1989, Cry2Aa, Cry10, Cry11, and Cyt1A) that yielded mortality above 50% and at least one strain with a mortality of less than 50% (Cry4A) were selected for a new assessment (Table 4). Similar results were obtained in assay 2. In this case, the mortality observed in *D. citri* were lower than those originally observed in the wild-type strains (S1302, S1450, and S1989), and the recombinant strains (Cry2Aa, Cry4A, Cry11, and Cyt1A) at 5 DAI, but the pathogenicity of the Bt strains was confirmed (Table 4). Moreover, strains S1450, S1302, and Cry10 confirmed their ability to cause mortality to the psyllids in the second assay as well (Table 4).

The association between Bt strains and nymphal mortality was confirmed by the recovery of typical Bt colonies from dead nymphs and PCR amplification with specific

Table 3 Initial screening of mortality of *Diaphorina citri* third-instar nymphs when "Pera" sweet orange seedlings were systemically treated with a suspension of *Bacillus thuringiensis*.

Treatment	Mortality[†] (%)/days after Bt application and nymph transfer (DAI)				
	1	2	3	4	5
S1302	56.0 ± 7.33 a	77.0 ± 7.61 a	85.0 ± 6.01 a	88.0 ± 6.29 a	90.0 ± 5.96 a
S1450	29.0 ± 9.24 ab	55.0 ± 9.46 a	64.0 ± 10.02 ab	74.0 ± 7.02 ab	77.0 ± 6.67 ab
S1989	57.0 ± 11.55 a	69.0 ± 11.00 a	75.0 ± 9.69 ab	81.0 ± 7.06 ab	82.0 ± 6.96 ab
Cry1Aa	24.7 ± 8.56 ab	36.7 ± 8.82 ab	44.0 ± 9.09 ab	46.0 ± 8.83 ab	50.0 ± 8.94 ab
Cry1Ac	25.0 ± 10.25 ab	33.0 ± 10.55 ab	35.0 ± 10.88 b	40.0 ± 10.00 b	44.0 ± 9.91 b
Cry1B	14.0 ± 4.76 b	15.0 ± 5.00 bc	17.0 ± 4.96 bc	21.0 ± 5.86 bc	26.0 ± 5.81 bc
Cry2Aa	30.0 ± 9.31 ab	38.7 ± 9.90 ab	43.3 ± 9.64 ab	44.7 ± 9.85 ab	51.3 ± 9.35 ab
Cry4A	14.5 ± 4.00 b	21.0 ± 5.71 b	26.0 ± 6.04 bc	31.0 ± 6.28 bc	36.0 ± 5.82 b
Cry4B	43.5 ± 7.99 ab	54.5 ± 7.80 a	56.0 ± 7.66 ab	59.5 ± 7.38 ab	62.0 ± 7.06 ab
Cry10	50.0 ± 6.45 a	56.5 ± 7.04 a	60.0 ± 6.96 ab	62.0 ± 6.59 ab	65.0 ± 5.83 ab
Cry11	35.1 ± 5.22 ab	46.6 ± 5.90 ab	53.7 ± 6.14 ab	56.3 ± 6.20 ab	60.0 ± 5.94 ab
Cyt1A	44.0 ± 9.80 ab	51.3 ± 9.10 a	52.7 ± 8.97 ab	58.7 ± 8.22 ab	62.0 ± 8.00 ab
Cry1Ab	9.0 ± 3.48 b	17.0 ± 4.96 bc	25.0 ± 6.87 bc	28.0 ± 8.14 bc	33.0 ± 8.70 bc
Control	1.1 ± 0.50 b	3.3 ± 0.90 c	8.0 ± 1.57 c	11.1 ± 1.92 c	14.4 ± 2.06 c
F	11.26	13.073	11.863	12.189	12.351
df	13, 241	13, 241	13, 241	13, 241	13, 241
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[†]Data (mean ± SE) followed by the same letter in a column do not differ statistically (GLM with a quasi-binomial distribution, followed by *post hoc* Tukey–Kramer test; $P < 0.05$).

primers for the *cry* or *cyt* genes present in the tested strains (Table 2). The exception was strain *cry1B*, which exhibited very poor translocation within the plant, yielding no colonies from the seedlings macerate and with a single colony from the psyllid macerate. For the other strains, few but consistent CFUs were obtained from the isolation of Bt colonies from dead nymphs and citrus shoots, and single fragments of expected sizes, ranging from 180 to 522 bp (see Table 1) were observed after PCR with specific primer pairs (Fig. S3). In most cases, data unambiguously confirmed the identity of the Bt strains and their ability to translocate within citrus seedlings.

Discussion

The morphological characterization of the Bt inocula just prior to setting up the assays was important to confirm the presence of Bt spores and crystals, recommended for a good inoculum (Johnson *et al.*, 1998; Rabinovitch *et al.*, 2017; Mukhija & Khanna, 2018). The capacity of Bt strains to reach young shoots of citrus after being inoculated to the seedling roots and be acquired by *D. citri* nymphs that fed on them was demonstrated by the isolation and plating of Bt and recuperation of CFU followed by the detection of *cry* or *cyt* gene present in each strain by PCR with specific primers, except in the control treatment. The detection of the *cry* or *cyt* genes present in each of the analyzed strains was consistent for all PCR assays and replicates (from 6 to 21) selected for each strain isolated from either young shoots or dead nymphs. Therefore, although the amount of toxin ingested by the insects may be variable, the proof of concept was obtained in all of the treatments, which means that, although standardization can be improved, the possibility of using Bt for the control of *D. citri* is promising.

These results are consistent with studies that confirmed Bt translocation and colonization in cotton seedlings (Monnerat *et al.*, 2009; Melatti *et al.*, 2010), cabbage (Monnerat *et al.*, 2009; Praça *et al.*, 2012), legumes (Tanuja *et al.*, 2013), beans (García-Suárez *et al.*, 2017), *Arabidopsis* (Vidal-Quist *et al.*, 2013; García-Suárez *et al.*, 2017), and bryophytes (Lin *et al.*, 2017), and their acquisition by insects feeding on those plants (Monnerat *et al.*, 2009; Melatti *et al.*, 2010; García-Suárez *et al.*, 2017). In these studies, the Bt structures (vegetative cells, spores, and crystals) were recovered from the plants by the isolation of one to several tissues (leaves, petioles, stems, roots). At least in cotton, cabbage, beans, and bryophytes, the bacteria were visualized by fluorescence and electron

Table 4 Evaluation of mortality of *Diaphorina citri* third-instar nymphs, in assay II, when "Pera" sweet orange seedlings were systemically treated with *Bacillus thuringiensis* strains.

Treatment	Mortality[†] (%)/days after Bt application and nymph transfer (DAI)				
	1	2	3	4	5
S1302	26.0 ± 4.00 ab	42.0 ± 1.33 b	56.0 ± 3.40 a	66.0 ± 3.40 a	68.0 ± 3.27 a
S1450	34.0 ± 2.67 ab	52.0 ± 2.49 a	56.0 ± 1.63 a	66.0 ± 3.40 a	70.0 ± 2.11 a
S1989	30.0 ± 2.98 ab	34.0 ± 1.63 b	36.0 ± 1.63 b	38.0 ± 1.33 bc	42.0 ± 2.49 c
Cry2Aa	16.0 ± 1.63 bc	18.0 ± 2.49 c	22.0 ± 2.49 c	34.0 ± 2.67 c	36.0 ± 2.67 cd
Cry4A	28.0 ± 3.89 ab	32.0 ± 3.89 b	34.0 ± 3.40 b	34.0 ± 3.40 c	40.0 ± 2.11 cd
Cry10	40.0 ± 2.11 a	54.0 ± 1.63 a	56.0 ± 2.67 a	58.0 ± 2.49 a	66.0 ± 1.63 a
Cry11	26.0 ± 2.67 ab	32.0 ± 2.49 b	34.0 ± 2.67 b	34.0 ± 2.67 c	41.0 ± 3.14 cd
Cyt1A	24.0 ± 3.40 b	34.0 ± 3.40 b	38.0 ± 2.49 b	48.0 ± 3.89 b	54.0 ± 3.40 b
Control	10.0 ± 2.11 c	24.0 ± 1.63 c	30.0 ± 2.11 b	30.0 ± 2.11 c	30.0 ± 2.11 d
F	9.515	21.392	22.910	23.424	31.770
df	8, 81	8, 81	8, 81	8, 81	8, 81
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[†]Data (mean ± SE) followed by the same letter in a column do not differ statistically (GLM with a quasi-binomial distribution, followed by *post hoc* Tukey test; $P < 0.05$).

microscopy (Monnerat *et al.*, 2009; Praça *et al.*, 2012; García-Suárez *et al.*, 2017). In cabbage, it was suggested that the entry of Bt occurred through cracks present in the roots and migrated to the leaves through the xylem (Monnerat *et al.*, 2009; Praça *et al.*, 2012; García-Suárez *et al.*, 2017).

In citrus, it is likely that this also occurs. It has been reported that *D. citri*, after periods of fasting, can feed on the xylem vessels in search of water (Bonani *et al.*, 2010). With the detection of Bt translocation in citrus seedlings, prospects of its use in the control of phloem-feeding insects are now opened. A total of 13 Bt strains were tested against *D. citri* nymphs in two large assays and strains S1450 and S1302 provided high mortality rates (68%–90%) of *D. citri* nymphs at 5 DAI, followed by recombinant Cry10 and Cyt1A strains, which caused 54%–66% nymphal mortality.

When recombinant strains containing single *cry* or *cyt* gene were inoculated and tested individually, they tended to exhibit lower mortality rates when compared to the wild-type strains. For example, strain S1302 harbors only two genes, *cry1Ab* and *cry3A*, and the mortality of *D. citri* nymphs reached up to 90% at 5 DAI. The recombinant *cry1Ab* strain was able to kill only 33% of the nymphs. Hence, the mortality observed in *D. citri* nymphs may be likely attributed to the action of the *cry3A* gene (not available for individual testing) or the synergistic interaction between *cry3A* and *cry1Ab*. These two genes have been shown to cause negative effects on *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) survival and development (Porcar *et al.*, 2009), and on *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae) (Walters & English, 1995). In addition, an increase in activity and toxicity of these (Cry1Ab and Cy3A) and other (Cry11 and Cry4) toxins was observed in the presence of activated trypsin *in vitro* and when the aphids were exposed to 500 μg/mL of the solubilized protein, from 3 d of exposure (Porcar *et al.*, 2009).

The results obtained for strain S1450, when compared to those observed for the recombinant strains harboring the single genes *cry1Ab*, *cry1Ac*, *cry1B*, *cry1Aa*, or *cry2Aa*, were analogous to the data obtained for strain S1302. While the wild-type strain caused up to 77% mortality in *D. citri* nymphs at 5 DAI, the individual genes tested did not have a significant effect on the psyllid. Unfortunately, *cry2Ab* was not available for individual testing and, therefore, that may be the main toxin responsible for the insect high mortality rate observed in the wild-type strain harboring that gene. Interestingly, *cry1Ab* and *cry1Ac* genes are among the most commonly used in the production of transgenic Bt cotton and Bt corn, respectively, for the control of caterpillars (Sheikh *et al.*, 2017), but not for hemipterans.

In this study, mortality rates from 60% to 65% of *D. citri* nymphs were obtained at 5 DAI, in both assays, when strains harboring individual *cry4B*, *cry10*, *cry11*, or *cyt1A* genes were tested. The *cry4A* strain, also present in the wild-type strain S1989, was responsible for causing the

death of only 36%–40% of the nymphs at 5 DAI. Since strain S1989 was able to control up to 82% of the *D. citri* nymphs at five DAI, this effect may be the sum or even the synergistic interaction between *cry4A*, *cry4B*, *cry10*, *cry11*, and *cyt1A*. Particularly for the wild-type strains S1302, S1989, and the recombinant strain *cry11*, a change in mortality was observed between the first and the second assays. This variation may be attributed to the standardization of the number of spores and crystals of the inoculum, or the amount of Bt acquired by the insect during feeding, which can be variable due to its systemic distribution within the plant. It is important to notice that, as expected, the number of colonies recovered by the Bt isolation in Petri dishes did not correlate directly with nymphal mortality, suggesting that mortality rates are not related with the sole presence of the bacteria, but likely with specific toxins they produce. It should be also noted that preliminary Cry toxins binding assays with *D. citri* brush border membrane vesicle (BBMV) obtained from nymphs showed binding between toxins present in Bt strains used in the pathogenic assays and receptors present in BBMV from the psyllid (data not shown).

In summary, this is the first effort to identify and characterize Bt strains and genes able to cause mortality in *D. citri* nymphs. Up till now, the few studies on the use of Bt to control phloem-feeding hemipteran insects were concentrated on aphids (Melatti *et al.*, 2010; Chougule *et al.*, 2013). For the first time, we confirmed the ability of Bt strains to translocate from roots to shoots in citrus seedlings, and cause high mortality of *D. citri* nymphs feeding on them. Furthermore, PCR allows rapid identification of Bt strains and toxin genes, and it has been successfully used in this and other studies (Monnerat *et al.*, 2009; Praça *et al.*, 2013; Nair *et al.*, 2018). PCR of Bt colonies with the specific primers used in this work allowed to unequivocally confirm the identity of the strain, and also confirmed the cause of nymphal mortality observed in our assays. The confirmation of the endophytic capacity of Bt in citrus, and the identification of Bt strains and toxins pathogenic to *D. citri* suggest that either classical biological control or transgenic Bt citrus could be employed as one more tool in the management of HLB, the most important disease of the citriculture worldwide.

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Author contributions

SD, JB, and JA contributed with the conception and design of the study; SD, JB, and TC performed the experiments; SD, TC, OZ, MP, and JA analyzed the data; OZ performed the statistical analysis; JA, RM, MP, JL, and MM provided guidance and contributed with reagents, materials, and infrastructure; SD, TC, OZ, MP, and JA wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

Disclosure

The authors declare that there is no conflict of interest on both sides.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Image of phase contrast microscopy showing spores, crystals, and endospore structures present in Bt suspensions after 3 d of cultivation: recombinant strain *cry4B* exhibiting spherical crystals (A) and strain S1302 showing bipyramidal crystals (B).

Fig. S2 Detection of recombinant Bt strains with each cry/cyt gene. (M) indicates the 100 bp ladder, Invitrogen; H_2O and N indicate negative controls from the isolation of young shoots; *cry1A*, *cry1Ab*, *cry1Ac*, *cry2Aa*, *cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1A*, and *cyt1B* represent the genes present in the strains evaluated.

Fig. S3 Morphology of Bt colonies from the isolation of fresh leaves of citrus and dead nymphs. Bt colonies isolated from leaves: (A) control negative, (B) UFC from Cry11-treated seedlings, (C) Bt colony enlargement on Cry11-treated seedlings, (D) UFC from seedlings treated with S1450, and (E) colony enlargement of Bt on seedlings treated with S1450.

Fig. S4 Assessment of *Diaphorina citri* nymph survival after feeding on (A) and (B) control, noninoculated "Pera" sweet orange seedlings, (C) and (D) citrus seedlings inoculated with Bt strain S1989 (dead nymphs).