Chromobacterium Csp_P biopesticide is toxic to larvae of three Diabrotica species including strains resistant to Bacillus thuringiensis

Adriano E. Pereira1,2,3, Man P. Huynh2,3, Kyle J. Paddock2, José L. Ramirez4, Eric P. Caragata5, George Dimopoulos5, Hari B. Krishnan1, Sharon K. Schneider6, Kent S. Shelby7 & Bruce E. Hibbard1

The development of new biopesticides to control the western corn rootworm (WCR), Diabrotica virgifera virgifera LeConte, is urgent due to resistance evolution to various control methods. We tested an air-dried non-live preparation of Chromobacterium species Panama (Csp_P), against multiple corn rootworm species, including Bt-resistant and -susceptible WCR strains, northern (NCR, D. barberi Smith & Lawrence), and southern corn rootworm (SCR, D. undecimpunctata howardi Barber), in diet toxicity assays. Our results documented that Csp_P was toxic to all three corn rootworms species based on lethal (LC50), effective (EC50), and molt inhibition concentration (MIC50). In general, toxicity of Csp_P was similar among all WCR strains and ~ 3-fold less toxic to NCR and SCR strains. Effective concentration (EC50) was also similar among WCR and SCR strains, and 5-7-fold higher in NCR strains. Molt inhibition (MIC50) was similar among all corn rootworm strains except NCR diapause strain that was 2.5–6-fold higher when compared to all other strains. There was no apparent cross-resistance between Csp_P and any of the currently available Bt proteins. Our results indicate that Csp_P formulation was effective at killing multiple corn rootworm strains including Bt-resistant WCR and could be developed as a potential new management tool for WCR control.

The western corn rootworm (WCR), Diabrotica virgifera virgifera LeConte, and the northern corn rootworm (NCR), Diabrotica barberi Smith & Lawrence, are the most devastating maize pests in the U. S. Corn Belt1–4, causing yield losses and control costs of up to $2 billion per year5. The southern corn rootworm (SCR), Diabrotica undecimpunctata howardi Barber, is a multivoltine and polyphagous pest that feeds on several crops including maize, whereas WCR and NCR are univoltine and feed almost exclusively on maize1. Corn rootworm larvae cause significant damage to the maize roots compromising the uptake of water and nutrients, as well as plant stability in heavy infestations6,7. Although SCR beetles can migrate northward, they usually do not overwinter in most parts of the Corn Belt; thus, damage by SCR larvae is typically not a concern in those areas.

WCR and NCR management tactics rely on crop rotation primarily with soybeans and maize hybrids expressing Bacillus thuringiensis (Bt) proteins. The extraordinary ability to rapidly evolve resistance to management tactics has made corn rootworms one of the most challenging maize pests in North America. Recent cases of resistance evolution to Bt maize hybrids in both species8,9,11–14 and to RNA interference (RNAi) in WCR15 have highlighted the urgent need for insecticides with new modes of action that do not confer cross-resistance to current control methods including Bt and RNAi16.
Bacterial insecticidal proteins have been used against insect pests for many years, especially lepidopteran, coleopteran, and dipteran pests, with Bt being the most widely used. New insecticidal proteins produced by soil bacteria other than Bt such as *Photorhabdus luminescens*, *Chromobacterium piscinae*, *Pseudomonas chlororaphis* and *P. mosselii*, *Alkaligenes faecalis*, and *Brevibacillus laterosporus*, and even those produced by mushrooms from the genus *Pleurotus* have been explored as future venues for corn rootworm management and resistance management. In addition, recent studies have documented the toxicity of modified or engineered Bt proteins such as *Cry6Aa1*, *CytAa3*, *Cry8Hb3*, and the first vegetative insecticidal protein *Vpb4Da* against WCR larvae. Finding new molecules with distinct modes of action without cross-resistance to current technologies should be the goal for corn rootworm discovery programs, given the pest’s ability to evolve resistance to multiple control tactics with possible multiple resistance mechanisms.

*Chromobacterium* is a Gram-negative soil bacterium that produces insecticidal metabolites documented to be toxic not only to mosquitoes, but also to WCR larvae. The *Chromobacterium* species *Panama* (Betaproteobacteria: Neisseriaceae), referred to as *Csp_P* from hereafter, was isolated from mosquito vectors *Aedes aegypti* midguts, which were collected in dengue-endemic areas in Panama. *Csp_P* has been demonstrated to have high insecticidal activity against the larvae from three mosquito vector species. The objective of the present study was to evaluate the susceptibility of multiple WCR strains including Bt-resistant, -susceptible and diapause strains, diapause and non-diapause NCR strains, and an SCR strain, to an air-dried non-live preparation of *Csp_P*. In 7-d diet overlay toxicity assays, we estimated the lethal concentration (LC50) based on survival data, the effective concentration (EC50) based on dry weight of surviving larvae, and the molt inhibition concentration (MIC50) based on the number of larvae reaching second instar. Our results indicated that *Csp_P* was effective at killing multiple corn rootworm strains including WCR larvae resistant to Bt toxins.

**Results**

**Diet assays.** After 7 d, the highest concentration of the *Csp_P* preparation used in the assays (40 µg/cm² for WCR, 160 µg/cm² for NCR, and 320 µg/cm² for SCR) killed between 78 and 92% of the WCR larvae across strains (Fig. 1), 100% of the NCR larvae and 90% of the SCR larvae (Fig. 2). The LC50 values ranged from 12.9 µg/cm² (WCR Texas) to 61.1 µg/cm² (NCR diapause and non-diapause) (Table 1), the EC50 values ranged from 1.30 µg/cm² (WCR eCry3-R and Texas) to ~10 µg/cm² (NCR diapause and non-diapause) (Table 1), and the MIC50 values ranged from <1.25 µg/cm² (WCR Texas) to 8.46 µg/cm² (NCR diapause) (Table 1). The WCR eCry-R strain exhibited the highest LC50 among all but WCR mCry3A-R strain, with no overlapping 95% confidence intervals (C.I.), whereas WCR Texas exhibited the lowest LC50 and MIC50 values of all the strains (Table 1). The EC50 values were similar among all WCR strains (except for WCR Texas) as were MIC50 (Table 1). The NCR strains exhibited the highest LC50, EC50, and MIC50 values, that ranged from 2-fold to 18-fold higher than the WCR strains (Table 1). The SCR strain exhibited higher LC50, similar EC50 and similar MIC50 values than all the WCR strains, except WCR Texas (Table 1).
Histologic pathogenesis. Normal gut ultrastructure was observed in the WCR feeding for 72 h on a diet supplemented with sucrose alone (Fig. 3A). Columnar cells with an intact brush border membrane lined with microvilli surrounded the gut lumen. However, 72 h of feeding on Csp_P significantly affected the gut structure (Fig. 3B). In the treated insects, the microvilli on the apical side of the gut lumen were dissolved. The columnar cells were multilayered as evidenced by cell nuclei along the posterior portion of the midgut. Stem cells could be seen in high numbers toward the anterior portion of the midgut. A dark purple staining observed in the gut lumen of the treated insects may have been commensal bacterial (e.g. bacilli) altered due to the infection by Csp_P.

Discussion
In the present study, we have used diet overlay toxicity assays to evaluate and demonstrate the toxicity of a Csp_P formulation against Bt-resistant and susceptible WCR strains, diapause and non-diapause NCR strains, and an SCR strain. We found no evidence of cross-resistance with the WCR Bt-resistant strains, especially with the
in the WCR larvae following Bt exposure. The identity of the insecticidal component(s) of mCry3A proteins, but not to Gpp34/Tpp35Ab1, has been reported previously. The WCR continues to be reporting an LC50 of ~ 60 µg/cm2; this value is two to five-fold higher than the LC50 values reported in our study for WCR control, or any other bacterial toxin, including but not limited to Bt, could help delay resistance evolution in rootworms. Therefore, in those areas where the WCR have evolved complete or incomplete resistance to Cry3Bb1 or to Gpp34/Tpp35Ab1 maize hybrids in North Dakota, Iowa, and Nebraska. In addition, Calles-Torrez et al. have reported the first case of NCR field-evolved resistance to Bt maize. The EPA has implemented a framework to delay resistance evolution in rootworms. The addition of a different mode of action, such as the DvSnf7 double stranded RNA pyramided with Bt in SmartStax Pro for WCR control, or any other bacterial toxin, including but not limited to Bt, could help delay resistance evolution in the field. Based on its toxicity and lack of cross-resistance with Bt proteins reported in this study (Table 1; Fig. 1), the biopesticide Csp_P could potentially kill those WCR with homozygous Bt-resistance as well as heterozygotes and delay the development of resistant populations, depending on the concentration and if delivered properly against WCR larvae.

The lack of cross-resistance with the Bt-resistant WCR strains after exposure to Csp_P is indicated not only by the similar LC50 values we found (except for WCR Texas), but also by the EC50 and MIC50 values (except for the WCR-SUS, mCry-R, and Texas strains), based on overlapping 95% C.I. values (Table 1). It is important to note that even the lowest concentration of Csp_P (1.25 µg/cm2) caused significant growth inhibition in all WCR strains, based on lower dry weight and lower percent of 2nd instar larvae when compared to control (Fig. 2). It was not surprising that the field-derived WCR from Texas exhibited the lowest LC50 and MIC50 values when compared to all other WCR strains (Table 1). It is possible that inbreeding depression could explain the strain’s increased susceptibility to Csp_P, or it could be related to the genetics of this specific strain collected from maize fields in Texas. It is not unusual that some of the WCR strains exhibit slow growth when compared to other strains. The WCR diapause larvae (and adults) are usually smaller than most diapausing strains (Pereira, personal observation), and this likely explains the lower larval dry weight observed in control (Fig. 1).

We exposed the WCR Texas larvae to Csp_P at 80 µg/cm2 for 72 h to evaluate histologically and note any symptoms to identify any possible mechanisms involved. It is unclear whether Csp_P produces pore-forming toxins, but it appeared to collapse the midgut entirely and dissolve the microvilli in those treated larvae. Cellular debris is visible within the gut lumen. The portion of the midgut displays dissolved CC and MV and an increased abundance of stem cells (SC). The CC appear multilayered in some places in the treated insects. Cellular debris is visible within the gut lumen.

Cry3Bb1 and Gpp34/Tpp35Ab1 proteins (Table 1; Fig. 1). Cross-resistance between Cry3Bb1, eCry3.1Ab and mCry3A proteins, but not to Gpp34/Tpp35Ab1, has been reported previously. The WCR continues to be a challenging maize pest given the recent cases of Bt resistance evolution to Cry3Bb1 and Gpp34/Tpp35Ab1 maize hybrids in North Dakota, Iowa, and Nebraska. In addition, Calles-Torrez et al. have reported the first case of NCR field-evolved resistance to Bt maize. The EPA has implemented a framework to delay resistance evolution in rootworms. The addition of a different mode of action, such as the DvSnf7 double stranded RNA pyramided with Bt in SmartStax Pro for WCR control, or any other bacterial toxin, including but not limited to Bt, could help delay resistance evolution in the field. Based on its toxicity and lack of cross-resistance with Bt proteins reported in this study (Table 1; Fig. 1), the biopesticide Csp_P could potentially kill those WCR with homozygous Bt resistance as well as heterozygotes and delay the development of resistant populations, depending on the concentration and if delivered properly against WCR larvae.

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Sampson et al. have evaluated the toxicity of purified C. piscinae protein against WCR larvae in diet assays, reporting an LC50 of ~ 60 µg/cm2; this value is two to five-fold higher than the LC50 values reported in our study for WCR (Table 1), although they did not report 95% C.I. values in their study. In addition, Sampson et al. reported less root damage in transformed maize plants expressing C. piscinae protein when compared to control plants that did not express C. piscinae protein. However, toxicity against SCR was not observed nor were NCR larvae tested in Sampson et al., as opposed to our study that confirmed activity of Csp_P to both species (Table 1).

Even though they are not directly comparable due to different formulations and purity, the LC50 values reported in this study for Csp_P in WCR larvae were higher than those values reported for purified Bt proteins, using similar diet assays. Depending on the Bt protein, LC50 values can vary from 1.60 µg/cm2 for Gpp/TppAb1 to 6.39 µg/cm2 for mCry3A. These values are approximately 3- to 18-fold lower than those found for Csp_P.
formula, which was not purified. Csp_P formulation was also offered to WCR adults mixed with Cucurbitacin in powder or diluted in sucrose, but no mortality was observed at the concentration tested (data not shown).

In summary, the biopesticide *Chromobacterium Csp_P* was efficient in killing WCR, NCR, and SCR larvae from different strains including WCR resistant to differing Bt toxins with varying levels of susceptibility among the three species and no cross-resistance with Bt toxins. Resistance management practices, such as rotating crops and the use of products that do not confer cross-resistance, should be implemented to avoid the spread of resistance alleles in those areas with resistance. The next steps should focus on determining the corn rootworm active components, testing Csp_P as a seed treatment in plant assays for corn rootworm larvae, on selected natural enemies for an initial risk assessment for integrated pest management, and also on non corn rootworm pests to determine if Csp_P is a broad-spectrum insecticide.

### Materials and methods

**Insects.** Adults of the non-diapausing WCR susceptible and resistant strains were maintained in 30 × 30 × 30 cm BugDorm® cages (BioQuip Products, Rancho Dominguez, CA) at the Plant Genetics Research Unit, USDA/ARS in Brookings, SD. The eggs were collected in 9-cm Petri dishes (Fisher Scientific, Pittsburgh, PA) containing 80-mesh sieved soil and kept at ~ 25 °C for prompt use (colony or assays) or at ~ 9 °C for later use. The rearing techniques are described elsewhere. The diapausing NCR and WCR strain eggs were received from USDA/ARS in Brookings, SD. SCR eggs were purchased from Crop Characteristics Inc. (Farmington, MN). All egg dishes were kept in a dark chamber at 25 °C until bioassays were initiated.

The WCR colonies resistant to Cry3Bb1 and Gpp34/35Ab1 are described in Ludwick et al. These insect strains initially evolved some level of resistance in the field, were crossed to a non-diapausing strain from Brookings, SD, and thereafter were continuously selected on seeds expressing Cry3Bb1 or Gpp34/35Ab1 at the USDA-ARS facilities in Columbia, MO. The WCR eCry3.1Ab-R strain was generated from a population collected in 2008 by French Agricultural Research Inc. (Lamberton, MN), and has been maintained in the lab for over 40 generations. The mCry3-R strain was generated from beetles collected at three different sites in Missouri in 2006. The WCR Texas strain was generated from a population collected from a mCry3A maize field in 2018 near Thr, Texas, and was suspected to be evolving resistance to mCry3A based on large number of beetles that emerged from the field. Once brought to the lab, the F1 offspring of this colony were crossed with WCR non-diapause beetles from the USDA-ARS in Brookings, SD, to reduce generation time (non-diapause). Root damage data from greenhouse single plant assays indicated no resistance to mCry3A when compared to susceptible strain (data not shown). The strain has been reared on untreated non-Bt corn hybrid (Viking 42–92; Albert Lea Seed, Albert Lea, MN, USA), and is considered susceptible non-diapause field strain.

**Air-dried Chromobacterium formulation.** A Csp_P non-live air-dried preparation was generated by using the method ‘nonlive_1’ in a previously described protocol. In brief, live Csp_P were inoculated onto 400 cm² Luria Bertani agar plates and then cultured at 30 °C for 2 days. The plates were covered in Luria Bertani broth and incubated for a further 5 days at room temperature. The liquid phase was then removed, and the Csp_P biofilm was collected and air dried before being crushed to a fine powder with a mortar and pestle. The ‘biofilm’ is the bacterial cell growth and debris on the agarose plates after 7 days of growth. It is not a direct growth on the solid agarose surface, since liquid media was added prior to the 7 days growth (see Fig. 1 in Caragata et al. ). This preparation contains the insecticidal factor(s) that is/are currently being investigated. This Csp_P powder was stored at 4 °C and used in the assays described below.

**Diet assays.** The artificial diet used in this research for WCR was an improved diet described by Huyhn et al. The NCR artificial diet was developed specifically for NCR and the SCR diet was purchased from Frontier Agricultural Sciences (Newark, DE). Rootworm egg sterilization and assays were conducted using methodology similar to that in Ludwick et al. The Csp_P was diluted in sucrose (50 mg/ml, pH 8.8; 99% purity, MP Biomedicals, Inc., Solon, OH) and six concentrations (1.25; 2.5; 5; 10; 20; and 40 µg/cm²) plus sucrose alone were inoculated onto 400 µl Luria Bertani agar plates and then cultured at 30 °C for 2 days. The plates were covered in Luria Bertani broth and incubated for a further 5 days at room temperature. The liquid phase was then removed, and the Csp_P biofilm was collected and air dried before being crushed to a fine powder with a mortar and pestle. The ‘biofilm’ is the bacterial cell growth and debris on the agarose plates after 7 days of growth. It is not a direct growth on the solid agarose surface, since liquid media was added prior to the 7 days growth (see Fig. 1 in Caragata et al. ). This preparation contains the insecticidal factor(s) that is/are currently being investigated. This Csp_P powder was stored at 4 °C and used in the assays described below.

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Histology of exposed and non-exposed WCR larvae. WCR-Texas larvae were exposed to either 80 µg/cm² of Csp_P diluted in 50 mg/ml of sucrose or to sucrose alone for 72 h in 96-well plates containing artificial diet, similar as described for diet assays. Larvae were collected after 72 h and placed in 1.5 ml centrifuge tubes containing FAA (50% ethyl alcohol, 5% glacial acetic acid and 10% formaldehyde) for 14 h at 4 °C. Larvae were dehydrated sequentially in a graded ethanol/xylene series and infiltrated with paraffin as described in Kim and Krishnan. Paraffin-embedded larvae were sectioned with a microtome to a thickness of 5 µm and were stained with hematoxylin and eosin. Images of standard larvae sections were acquired to visualize the midgut region and were obtained at 10 × and 40 × with a Leica DM550B widefield microscope (Leica Microsystems, Buffalo Grove, IL, USA) equipped with a Leica DFC290 color camera.

Statistical analysis. The mortality (as a percentage) and the percentage of larvae reaching second instar were calculated by dividing the number of surviving or second instar larvae, respectively, by the total number of larvae transferred to the plates for each treatment. The LC₅₀ and MIC₅₀ data were generated using PROC PROBIT with Distribution = Logistic in SAS 9.4 (SAS Institute, Cary, NC). For LC₅₀ and MIC₅₀, the number of dead larvae and the number of larvae that reached the second instar, respectively, were used in the analysis to generate the values. Average larval weight was recorded as the pooled larval weight/number of surviving larvae in each treatment. A nonlinear regression model using PROC NLIN in SAS 9.4 was adopted to generate the EC₅₀ values by using dry weight data, following the methods described in Marçôn et al. Control mortality in all plates was < 7%.

Data availability
All pertinent data are found in the figures and tables. Requests for data and additional information should be submitted to the corresponding author.

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Author contributions

Conceptualization: A.E.P., J.L.R.; data curation: A.E.P., M.P.H., K.J.P.; formal analysis: A.E.P.; funding acquisition: B.E.H., J.L.R., G.D.; investigation: A.E.P., M.P.H.; Methodology: A.E.P.; project administration: A.E.P., J.L.R.; resources: B.E.H., J.L.R., G.D., E.P.C., K.S.S., S.K.S.; supervision: A.E.P.; validation: A.E.P.; visualization: A.E.P., J.L.R.; roles/writing—original draft—A.E.P.; writing—review and editing: A.E.P., J.L.R., S.K.S., B.E.H. All co-authors read and approved the final draft of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to A.E.P.

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