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Research Article

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Evidence of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) field-evolved resistance to Cry3Bb1 + Cry34/35Ab1 maize in Nebraska

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Abstract

BACKGROUND: Western corn rootworm (WCR; *Diabrotica virgifera virgifera*) field-evolved resistance to transgenic maize expressing the Cry3Bb1 protein derived from *Bacillus thuringiensis* (Bt) has been confirmed across the United States Corn Belt. Although use of pyramided hybrids expressing Cry3Bb1 + Cry34/35Ab1 has increased in recent years to mitigate existing WCR Bt resistance, susceptibility of Nebraska WCR populations to this rootworm–Bt pyramid has not been assessed. Plant-based bioassays were used to characterize the susceptibility of WCR populations to Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 maize. Populations were collected from areas of northeastern Nebraska with a history of planting Bt maize that expressed Cry3Bb1 and Cry34/35Ab1.

RESULTS: Significant differences in mean corrected survival among populations within Bt hybrids indicated a mosaic of WCR susceptibility to Cry3Bb1 + Cry34/35Ab1 and Cry3Bb1 maize occurred in the landscape. All field populations exhibited some level of resistance to one or both Bt hybrids when compared to susceptible laboratory control populations in bioassays. Most WCR populations exhibited incomplete resistance to Cry3Bb1 + Cry34/35Ab1 maize (92%) and complete resistance to Cry3Bb1 maize (79%).

CONCLUSION: The present study confirms the first cases of field-evolved resistance to Cry3Bb1 + Cry34/35Ab1 maize in Nebraska and documents a landscape-wide WCR Cry3Bb1 resistance pattern in areas characterized by long-term continuous maize production and associated planting of Cry3Bb1 hybrids. Use of a multi-tactic integrated pest management approach is needed in areas of continuous maize production to slow or mitigate resistance evolution to Bt maize.

Supporting information may be found in the online version of this article.

Keywords: *Diabrotica virgifera virgifera*; western corn rootworm; *Bacillus thuringiensis*; transgenic maize; field-evolved resistance; resistance management

1 INTRODUCTION

The western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte) is a functionally monophagous, univoltine insect pest of maize (*Zea mays* L.)[1] that is annually responsible for US $1–2 billion in management costs and yield losses in the United States.[2,3] Initial larval eclosion occurs from late May to early June in most areas of the US Corn Belt with subsequent root feeding during June and July.[1] This coincides with the most rapid period of maize vegetative growth.[4] Significant larval feeding injury can reduce plant growth by interfering with water and nutrient uptake, decreasing plant stability, and reducing grain yield.[5–11] A 15–17% reduction in grain yield can occur with each full node of root injury.[12,13]

Historically WCR management programs have relied upon two main strategies: (1) annual rotation between maize and a non-host crop, and (2) soil- or foliar-applied insecticides in continuous maize (two or more successive years of cultivation). Crop rotation remains a recommended management strategy[14–16] because WCR females exhibit a strong affinity to oviposit in maize fields[17,18] and larvae can survive only on specific grass species.[19–21] However, a rotation-resistant strain that lays sufficient eggs in non-host crops to cause injury to first-year maize has evolved in the eastern US Corn Belt, limiting effectiveness of this strategy in some areas.[22,23] Insecticides have been used in

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continuous maize since the late 1940s to manage both larval and adult stages of WCR.[24–27] Long-term use of specific modes of action has facilitated WCR field-evolved resistance to four insecticide classes in areas of the western US Corn Belt.[27–31]

The introduction of transgenic maize hybrids expressing rootworm-active insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) Berliner provided growers with an additional management tactic to combat this pest in continuous maize. Initial rootworm-active proteins were registered in the 2000s [Cry3Bb1 in 2003,[32] Cry34/35Ab1 (now reclassified as Gpp34Ab1/Tpp35Ab1)[33] in 2005,[34] and mCry3A in 2006[35]] and marketed as single-protein hybrids. A fourth protein, eCry3.1Ab, was registered in 2012 but was not sold as a single trait product.[36] The high efficacy and convenience of transgenic maize facilitated widespread adoption by US growers.[37,38] Use of Bt technology over time led to WCR field-evolved resistance to the Cry3Bb1 protein in Iowa,[39–41] Illinois,[42,43] Nebraska,[44,45] Minnesota[46] and North Dakota.[47] Variable levels of cross-resistance among the Cry3 proteins (Cry3Bb1, mCry3A and eCry3.1Ab) have been reported,[39,43–45,48–50] limiting efficacy in areas of field-evolved resistance to any Cry3 protein. Field-evolved resistance to Cry34/35Ab1 also has been documented in Iowa[41,51] and Minnesota.[46]

Transgenic hybrids expressing two Bt proteins (i.e. pyramid) with unique modes of action have been utilized by maize growers to mitigate resistance evolution in recent years.[38] The following rootworm-active Bt pyramids have been commercialized in the United States: Cry3Bb1 + Cry34/35Ab1,[52] mCry3A + Cry34/35Ab1[53–55] and mCry3A + eCry3.1Ab.[56] Initial field trials with the first rootworm–Bt pyramid (Cry3Bb1 + Cry34/35Ab1) documented that significantly greater root protection and WCR density reduction could be obtained with the pyramid compared to single-protein CryBb1 or Cry34/35Ab1 hybrids.[57,58] Pyramids expressing Cry3Bb1 + Cry34/35Ab1 have been adopted widely in Nebraska over the past decade.[44,45]

Resistance of Nebraska WCR populations to Cry3Bb1 was first documented in Chase and Cuming counties from WCR collections made in 2011–2012.[44] Subsequently, resistance to Cry3Bb1 has been confirmed in various counties across the state.[44,45,59] By contrast, Nebraska WCR populations collected in 2012 were susceptible to Cry34/35Ab1.[44] By 2015, greater than expected injury[38,60] was recorded from Cry3Bb1, Cry34/35Ab1 and Cry3Bb1 + Cry34Ab1/35Ab1 treatments at a location in Cuming County, Nebraska,[61] which suggested that resistance was evolving to both Cry3Bb1 and Cry34/Cry35Ab1. These data also raised concerns about the durability of pyramids planted in areas where WCR resistance occurred to one or more proteins included in the pyramid.

A single-plant larval bioassay, developed by Gassmann *et al*.,[39] is one resistance monitoring technique widely used by entomologists across the US Corn Belt to detect shifts in WCR susceptibility to Bt maize.[40–47,51] Field-evolved resistance is confirmed when WCR field populations exhibit significantly higher survival on Bt maize compared to susceptible laboratory control populations. WCR resistance also can be classified as incomplete or complete based upon proportional survival and larval development data from single-plant bioassays.[62] Incomplete resistance (i.e. significantly higher survival on Bt maize than laboratory control populations and significantly greater survival and/or development metrics on non-Bt than Bt maize[63]) often is associated with earlier stages of selection in the field when the frequency of resistant WCR individuals is still low-to-moderate in a population.[39,44] By contrast, WCR populations exhibiting complete resistance (i.e. no significant difference in survival and development metrics between Bt and non-Bt hybrids[41]) include a high frequency of resistant individuals and often are associated with three or more years of continuous selection with a specific Bt trait.[62,64] In susceptible WCR populations, sublethal larval exposure to Cry3Bb1, Cry34/35Ab1 and Cry3Bb1 + Cry34/35Ab1 maize has contributed to delays in development and later timing of mean adult emergence.[65–70] The delays in development decrease as corrected survival increases[71] and disappear in populations exhibiting complete resistance.[41,62]

During the last five years, anecdotal reports of reduced WCR control with Cry3Bb1 + Cry34/Cry35Ab1 pyramids have increased, underscoring the need to evaluate the susceptibility of Nebraska WCR populations to Cry3Bb1 + Cry34/35Ab1 maize. Therefore, to evaluate current susceptibility of WCR populations to Cry3Bb1- and Cry3Bb1 + Cry34/35Ab1-expressing hybrids, F_1 progeny from WCR populations collected in northeastern Nebraska were bioassayed using the Gassmann single-plant technique.[39] Mean fresh weight and head capsule width also were measured and used to characterize differences in larval development rate resulting from sublethal exposure to Bt proteins.

2 MATERIALS AND METHODS

2.1 Insect populations

Adult WCR were collected from fields located in Cuming (C), Stanton (S), Polk (P) and Colfax (Cx) counties in northeastern Nebraska during 2017 and 2018 (Fig. 1). Each collected population was given a unique number to accompany the county identifier in parentheses shown above (i.e. alphanumeric code; Fig. 1). This study area was chosen owing to the concentration of continuous maize (three to >10 consecutive years) associated with the large confined livestock industry, the long history of planting hybrids expressing single or pyramided rootworm-active Bt traits, and anecdotal reports of performance issues with rootworm–Bt hybrids. Cry3Bb1 resistance previously had been confirmed in Cuming County[44] and bioassay data from 2011 to 2014 indicated that clusters of Cry3 WCR resistance can occur across the landscape.[59,62]

A minimum of 50 gravid females (range 50–2000) were collected from each field to obtain a subset of the natural variation present. A total of 15 WCR populations were collected from Cuming County between 2017 and 2018 (Fig. 1). Eight WCR populations were collected from different fields in 2017; four of these fields were re-sampled (C3, C4, C5, C7) and seven new populations were collected in 2018. One WCR population was collected from Stanton County in 2017 and 2018 (Fig. 1). One WCR population was collected from Polk County in 2017 and Colfax County in 2018 (Fig. 1). An additional population was collected from the Eastern Nebraska Research and Extension Center in Saunders County (Sa) in 2018 as a field control (Fig. 1). Large areas of continuous maize without rootworm–Bt traits serve as a refuge around the Sa collection area characterized by only periodic small-plot use of rootworm–active Bt maize hybrids.

Diapausing WCR colonies reared and maintained at the USDA-ARS North Central Agricultural Research Laboratory in Brookings, South Dakota, were used as laboratory control populations (LABC) during each bioassay year. Each LABC population was collected before the initial commercialization of Bt proteins in 2003 and has been reared continuously without the addition of wild-type genes, preserving susceptibility to rootworm–active transgenic maize. Two cohorts of a population collected from Moody

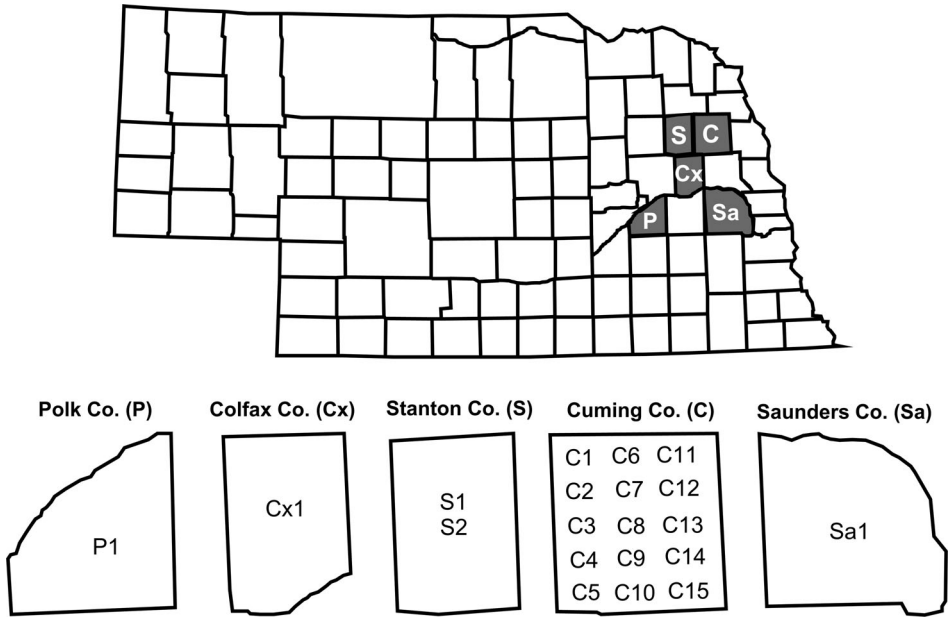

Figure 1. Nebraska state map showing counties from which WCR populations were collected: Cuming (C), Stanton (S), Polk (P), Colfax (Cx) and Saunders (Sa). Field codes within each county refer to a population sampled from an individual field.

County, South Dakota (1987), were used in 2018 bioassays. Individual populations collected from Butler County, Nebraska (1990), Potter County, South Dakota (1995), Finney County, Kansas (2000) and Centre County, Pennsylvania (2000) were used in 2019 bioassays.

2.2 Single-plant larval bioassays

Field-collected adult WCR were transported to the Department of Entomology at the University of Nebraska-Lincoln and maintained by population in 28cm^3 plexiglass cages under laboratory conditions during the summer and fall of 2017 and 2018 to collect eggs for use in single-plant larval bioassays. The procedural steps used to maintain adults, collect eggs and the temperature regimens used to facilitate egg diapause and post-diapause development are described in Wangila *et al.*[44] A subset of ~10,000 eggs from each WCR population was placed at 25°C for 14 days to promote egg hatch. Randomly selected neonate progeny of the F_1 generation from each population then were used in bioassays as described by Gassmann *et al.*[39] and adapted by UNL researchers.[44,45] Bioassays were conducted during the summer and fall of 2018 (2017 collections) and 2019 (2018 collections).

Three maize hybrids without seed treatments were used in bioassays: DKC 64–69 GENVT3P (single-protein Cry3Bb1, 'VT3P'), DKC 64-34 GENSS (Cry3Bb1 + Cry34/35Ab1 pyramid, 'SSX'), and DKC 66-87 GENVT2P (non-rootworm Bt, 'VT2P'). Single plants were grown in individual 1-L plastic containers (Johnson Paper & Supply Co., Minneapolis, MN, USA) and maintained to the V4–V5 growth stage.[72] Twelve replications of each hybrid were included in each bioassay. Twelve neonate F_1 larvae were infested onto the roots of each plant using a size 20/0 soft hair brush. Plants then were placed in growth chambers (Percival Scientific Inc., Perry, IA, USA) maintained at 24°C with a 14h:10 h, light:dark photoperiod for 17 days to promote larval feeding and development. After 17 days, plants were placed in Berlese funnels (40W, 120 V bulbs; Philips Lighting Company, Worcester, MA, USA) and larval survivors were collected in jars of 70% ethyl alcohol. Larval development of bioassay survivors was indirectly characterized by measuring head capsule width and fresh weight; both metrics increase as larvae progress through each instar.[73]

Individual larval head capsule widths were measured to the nearest 0.01 mm using an AmScope 3.5×–90× Simul-Focal Trinocular Stereo Zoom microscope with an attached 18MP USB3 Camera (United Scope LLC, Irvine, CA, USA). Larval fresh weight was determined on a per-plant basis. Larval survivors from each plant were air-dried on a Kimwipe (Kimberly-Clark Worldwide, Inc., Roswell, GA, USA) for 3 min before weighing using an OHAUS Voyager PRO VP413CN precision balance (OHAUS Corporation, Pine Brook, NJ, USA) to measure collective fresh weight.

2.3 Statistical analysis

Proportional survival was calculated on a per-plant basis by dividing the number of larval survivors by 12 (i.e. number of larvae infested per plant). Proportional survival on each maize hybrid was evaluated using a generalized linear mixed model (GLMM; implemented using PROC GLIMMIX, SAS 9.4 software[74]) with the number of larval survivors following a binomial distribution with a logit link function and a trial size of 12 (i.e. number of larvae infested per plant).[75,76] Separate analyses were conducted for each bioassay year. Initial analyses indicated no significant difference in survival among LABC populations on Cry3Bb1 or Cry3Bb1 + Cry34/35Ab1 maize within a bioassay year (Table S1 in Appendix S1). Therefore, data from LABC populations were pooled within a given year to create a composite sample. Population, maize hybrid and the population-by-maize hybrid interaction were included in the model as fixed factors and plant observation nested within the population-by-maize hybrid interaction was included as a random factor to control for an overdispersion of variance because the binomial distribution belongs to a one-parameter exponential family and does not include a natural residual.[76] To ensure all sources of variation were accounted for, model fit was evaluated by examining the generalized chi-square/df value (i.e. ≈1) and conditional residual plots.[76] The

SLICE statement was used to identify significant differences in proportional survival between the Bt and non-Bt hybrids within a population using Tukey's multiplicity adjustment to control for type I error rates. Comparisons within a hybrid were made among populations relative to the LABC using Dunnett's adjustment (i.e. used for multiplicity comparisons against a control). Statistical significance was reported at $\alpha = 0.05$.

Corrected survival was calculated as the complement of corrected mortality using Abbott's correction.[77] Corrected survival on the Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 hybrids was calculated as survival on each Bt bioassay plant divided by mean survival on the non-Bt hybrid for each population.[41] A linear model (implemented using PROC GLIMMIX[74]) following a normal distribution with unequal variances between populations was used to evaluate corrected survival on each rootworm–Bt maize hybrid. Population, maize hybrid and the population-by-maize hybrid interaction were included in the model as fixed factors. After assessing normality assumptions, heterogenous variance between populations was allowed to control for nonconstant variance by specifying GROUP = Population in the random statement. The SLICE statement was used to identify significant differences in corrected survival between Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 maize within a population. Tukey's multiplicity adjustment was used to identify significant differences in corrected survival among populations within each rootworm–Bt hybrid. Statistical significance was reported at $\alpha = 0.05$.

Head capsule width was averaged for larval survivors on each bioassay plant using PROC SQL[74] and larval fresh weight was evaluated on a per-plant basis. Fresh weight per larva was calculated by dividing the weight of all larvae from a specific plant by the number of survivors on the same plant. The two larval developmental factors were analyzed separately using the same analysis approach. A linear model was used to determine the mean head capsule width or mean larval fresh weight of survivors per hybrid for each population. Population, maize hybrid and the population-by-maize hybrid interaction were included in the model as fixed factors. Residual plots were used to evaluate normality assumptions and model fit. The population-by-maize hybrid LSMEANS are reported in this manuscript. The SLICE statement was used to identify significant differences in larval development metrics between the Bt and non-Bt hybrids within a population using Tukey's multiplicity adjustment to control for type I error rates. Comparisons within a hybrid were made among populations relative to the LABC using Dunnett's test. Statistical significance was reported at $\alpha = 0.05$.

For both Bt hybrids evaluated (i.e. Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1), each population was classified as completely resistant, incompletely resistant or susceptible based upon mean proportional survival, larval head capsule width and larval fresh weight criteria. WCR populations were classified as having complete resistance to a rootworm–Bt hybrid if: (i) proportional larval survival on the Bt hybrid was significantly greater than the LABC, (ii) proportional survival on the Bt and non-Bt hybrids were not significantly different, and (iii) both larval development metrics were not significantly different between the Bt and non-Bt hybrids. By contrast, WCR populations were classified as having incomplete resistance if: (i) proportional larval survival on the Bt hybrid was significantly greater than the LABC, and (ii) proportional survival on the non-Bt hybrid was significantly greater than survival on the Bt hybrid, and/or (iii) either larval development metric was significantly lower on the Bt than non-Bt hybrid. WCR populations were classified as susceptible if proportional survival was not significantly different than the LABC. Fisher's exact test[78] was used to test the equality of the proportion of WCR populations exhibiting complete resistance to Cry3Bb1 versus the proportion of WCR populations exhibiting complete resistance to the Cry3Bb1 + Cry34/35Ab1 pyramid.

3 RESULTS

3.1 Proportional survival from bioassays

A significant effect of population, maize hybrid and population-by-maize hybrid interaction on mean proportional survival occurred in both bioassay years (Table S2 in Appendix S1). A significant difference in mean larval survivorship between the Cry3Bb1 and non-Bt hybrids was observed in two of ten field populations in 2018 [Fig. 2(A)] and three of 14 field populations in 2019 [Fig. 3(A)]. Mean survivorship of most WCR populations (eight of ten in 2018; 13 of 14 in 2019) was significantly different between the Cry3Bb1 + Cry34/35Ab1 and non-Bt hybrids in both bioassay years [Figs 2 (A) and 3(A)]. All field populations exhibited significantly higher larval survival on the Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 hybrids in the 2018 and 2019 bioassays compared to the LABC, except for Sa1 on Cry3Bb1 maize in 2019 [Figs 2(A), 3(A)]. The composite LABC was highly susceptible to Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 compared to non-Bt maize in both bioassay years.

3.2 Corrected survival

A significant effect of population, maize hybrid and population-by-maize hybrid interaction on corrected survival occurred in both bioassay years (Table S2). Within field populations, five of ten assayed in 2018 and ten of 14 assayed in 2019 had significantly higher corrected survival values on Cry3Bb1 compared to the Cry3Bb1 + Cry34/35Ab1 pyramid (Table 1). In both 2018 and 2019, LABC corrected survival was not significantly different when reared on the Cry3Bb1 or Cry3Bb1 + Cry34/35Ab1 maize hybrids (Table 1). Significant variation in corrected survival was observed among populations within the Cry3Bb1 hybrid in 2018 (range 0.044 to 1.094) and 2019 (range 0.019 to 0.980) (Table 1). Corrected survival on Cry3Bb1 + Cry34/35Ab1 maize also was significantly different among populations in 2018 (range 0.037 to 1.208) and 2019 (range 0.010 to 0.753) (Table 1). The LABC exhibited significantly lower corrected survival on Cry3Bb1 compared to all field populations in the 2018 and 2019 bioassays (Table 1). However, LABC corrected survival on Cry3Bb1 + Cry34/35Ab1 maize was not significantly different than S1 in 2018 and C5 and Sa1 in 2019 (Table 1).

3.3 Larval head capsule width

A significant effect of population, maize hybrid and population-by-maize hybrid interaction on mean head capsule width occurred in both bioassay years (Table S2). Within field populations, no significant differences in mean larval head capsule width between Cry3Bb1 and non-Bt survivors were observed in the 2018 [Fig. 2 (B)] and 2019 [Fig. 3(B)] bioassays, except for the field control (Sa1) in 2019. Mean head capsule width of Cry3Bb1 survivors from the LABC was significantly smaller than mean head capsule width of LABC non-Bt survivors in each bioassay year [Figs 2(B) and 3(B)]. Comparisons within the Cry3Bb1 hybrid indicated larval survivors from all field populations assayed in 2018 and eight of 14 populations assayed in 2019 had significantly larger mean head capsule width compared to survivors from the LABC [Figs 2(B) and 3(B)].

Significant differences in mean head capsule width between Cry3Bb1 + Cry34/35Ab1 pyramid and non-Bt survivors were observed in four of ten field populations in the 2018 bioassays

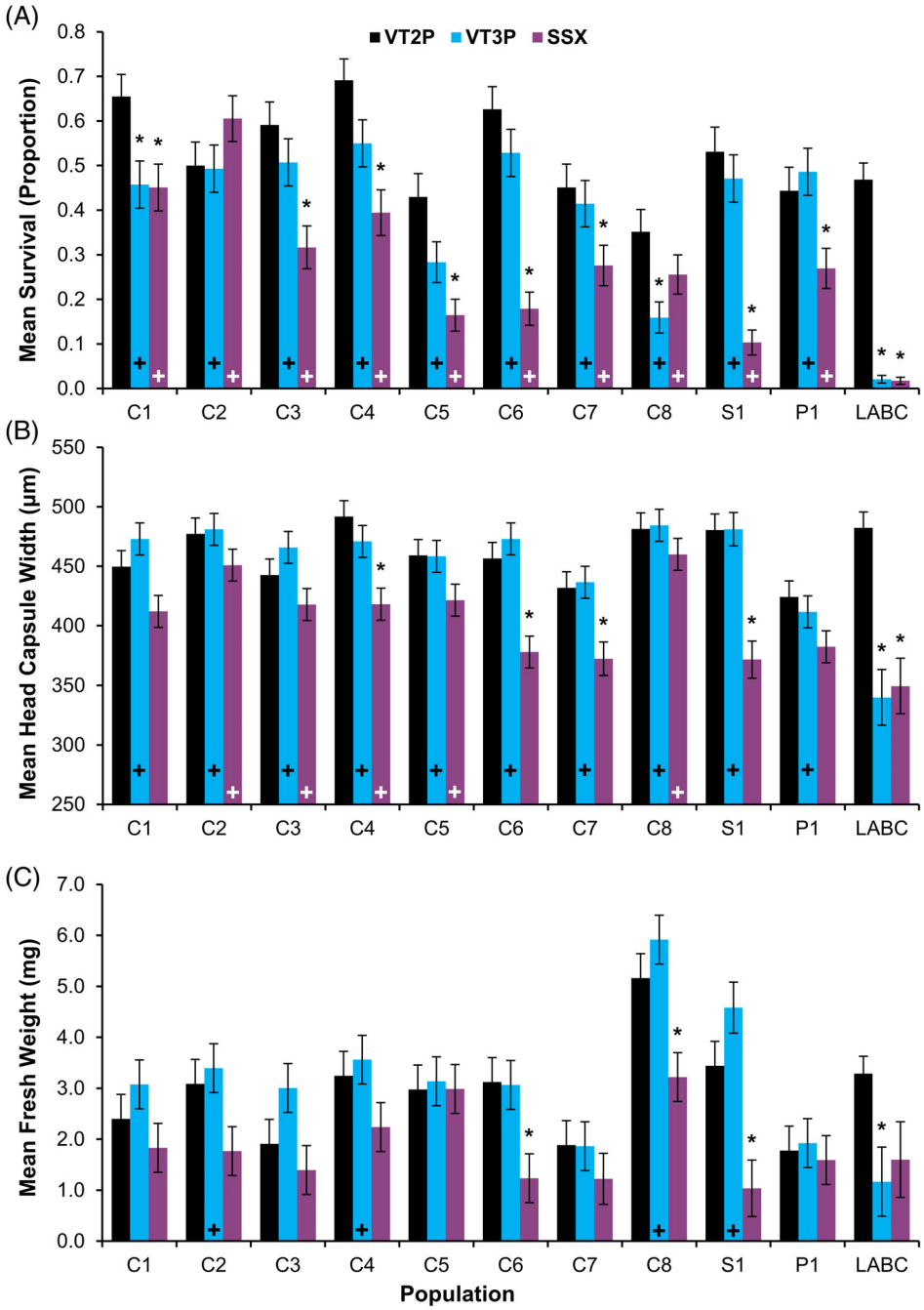

Figure 2. Survival and development of WCR populations bioassayed in 2018. (A) Mean proportional survival ± SE (out of 12 larvae infested per plant, 12 plants infested per hybrid), (B) mean head capsule width ± SE, and (C) mean fresh weight ± SE. Populations were assayed from fields in Cuming (C), Stanton (S) and Polk (P) counties and laboratory-reared control populations (LABC). Asterisks above individual bars indicate significant differences in survival or development metric within a population when reared on Bt and non-Bt hybrids (Tukey's multiplicity adjustment, $P < 0.05$). A '+' within rootworm–Bt bars indicates significant differences between a population compared to the lab control on the corresponding hybrid (Dunnett's test, $P < 0.05$).

[Fig. 2(B)] and five of 14 field populations in 2019 bioassays [Fig. 3 (B)]. Within the LABC, the mean head capsule width of pyramid survivors was significantly smaller than the mean size of LABC non-Bt survivors during the 2018 [Fig. 2(B)] but not the 2019 bioassays [Fig. 3(B)]. The latter result may be an artifact of the LABC (2019) population having one of three total survivors developing to 3rd instar, which may explain the higher mean head capsule width and associated large SE [Fig. 3(B)]. Comparisons of survivors within the Cry3Bb1 + Cry34/35Ab1 hybrid indicated five of ten field populations assayed in 2018 exhibited significantly larger mean head capsule width compared to LABC survivors [Fig. 2 (B)]. No significant differences were observed between mean survivor head capsule width on Cry3Bb1 + Cry34/35Ab1 maize versus the LABC in 2019 bioassays [Fig. 3(B)].

3.4 Larval fresh weight

A significant effect of population, maize hybrid and population-by-maize hybrid interaction on mean larval fresh weight

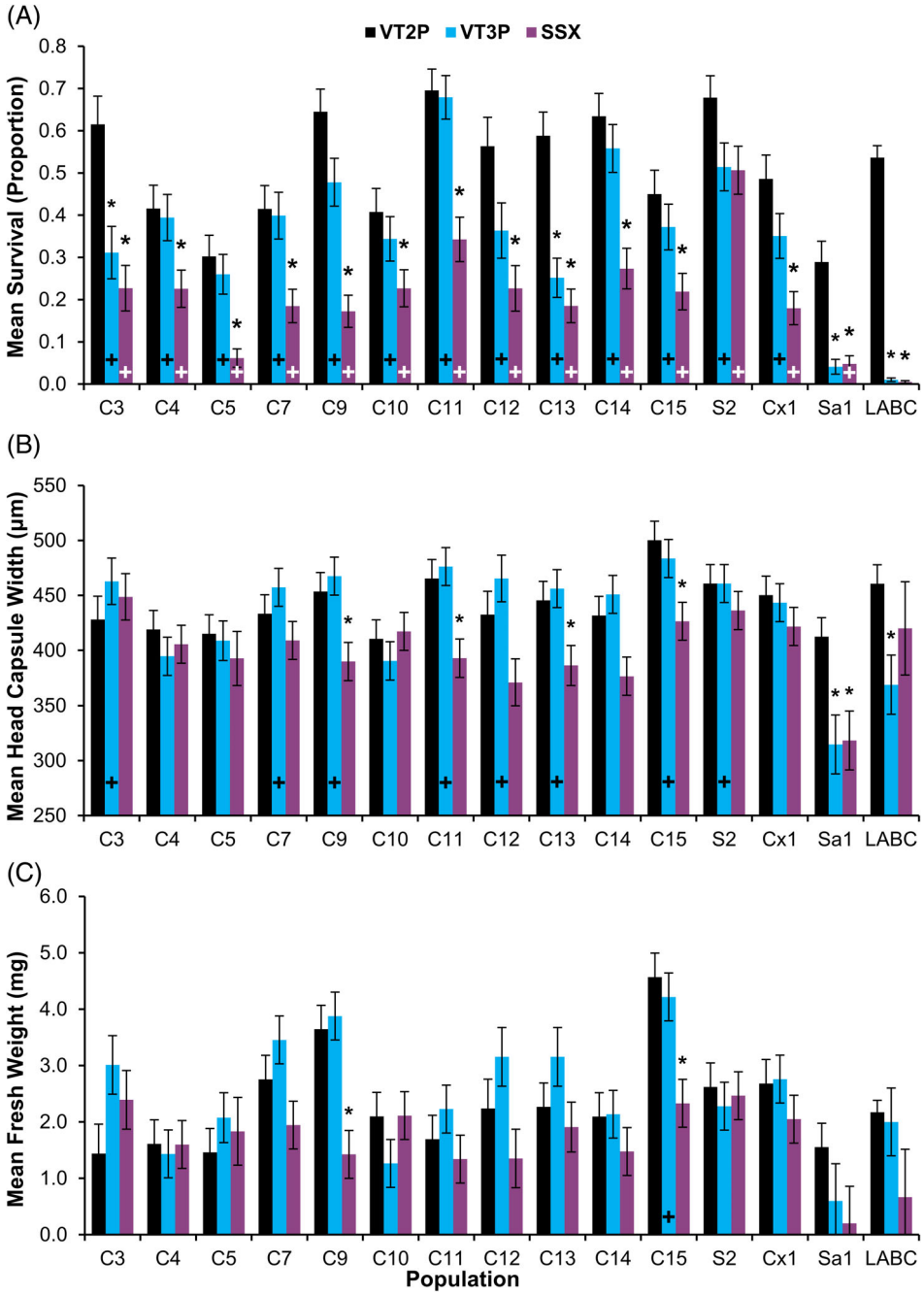

Figure 3. Survival and development of WCR populations bioassayed in 2019. (A) Mean proportional survival ± SE (out of 12 larvae infested per plant, 12 plants infested per hybrid), (B) mean head capsule width ± SE, and (C) mean fresh weight ± SE. Populations were assayed from fields in Cuming (C), Stanton (S), Colfax (Cx) and Saunders (Sa) counties and laboratory-reared control populations (LABC). Asterisks above individual bars indicate significant differences in survival or development metric within a population when reared on Bt and non-Bt hybrids (Tukey's multiplicity adjustment, $P < 0.05$). A '+' within rootworm–Bt bars indicates significant differences between a population compared to the lab control on the corresponding hybrid (Dunnett's test, $P < 0.05$).

occurred in both bioassay years (Table S2). Within populations, no significant differences in mean larval fresh weight between Cry3Bb1 and non-Bt survivors were observed in the 2018 [Fig. 2(C)] and 2019 bioassays [Fig. 3(C)]. Mean larval fresh weight of Cry3Bb1 survivors was significantly lower than the mean fresh weight of non-Bt survivors within the LABC during the 2018 bioassays [Fig. 2(C)]. Within the Cry3Bb1 hybrid, mean fresh weight of survivors in four of ten and one of ten field populations was significantly greater than mean weight of LABC survivors in the 2018 and 2019 bioassays, respectively [Figs 2(C) and 3(C)].

Significant differences in mean larval fresh weight between the Cry3Bb1 + Cry34/35Ab1 pyramid and non-Bt survivors were observed in three of ten field populations in the 2018 bioassays [Fig. 2(C)] and two of 14 field populations in the 2019 bioassays [Fig. 3(C)]. Within the Cry3Bb1 + Cry34/35Ab1 hybrid, significant differences in mean fresh weight were not observed between field populations and the LABC in either bioassay year [Figs 2(C)

Table 1. Corrected survival (± SE) of WCR populations on Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 maize from bioassays conducted in 2018 and 2019

County	Population	Cry3Bb1 corrected survival ± SE†	Cry3Bb1 + Cry34/35Ab1 corrected survival ± SE†	*P*-value‡
2018 Bioassays				
Cuming	C1	0.702 ± 0.09bc	0.692 ± 0.09b	0.9343
Cuming	C2	0.986 ± 0.11ab	1.208 ± 0.11a	0.1847
Cuming	C3	0.859 ± 0.09bc	0.541 ± 0.09bc	**0.0223**
Cuming	C4	0.798 ± 0.08bc	0.576 ± 0.08bc	0.0688
Cuming	C5	0.661 ± 0.08 cd	0.387 ± 0.08 cd	**0.0296**
Cuming	C6	0.844 ± 0.07bc	0.289 ± 0.07d	**<0.0001**
Cuming	C7	0.923 ± 0.11abc	0.615 ± 0.11bc	0.0618
Cuming	C8	0.451 ± 0.07d	0.726 ± 0.07b	**0.0150**
Stanton	S1	0.829 ± 0.10bc	0.183 ± 0.10de	**<0.0001**
Polk	P1	1.094 ± 0.07a	0.609 ± 0.07bc	**<0.0001**
Laboratory Control	LABC	0.044 ± 0.02e	0.037 ± 0.02e	0.7379
2019 Bioassays				
Cuming	C3	0.509 ± 0.06de	0.373 ± 0.06bc	0.1248
Cuming	C4	0.950 ± 0.09ab	0.550 ± 0.09ab	**0.0058**
Cuming	C5	0.864 ± 0.11abc	0.205 ± 0.11cde	**0.0004**
Cuming	C7	0.967 ± 0.13ab	0.450 ± 0.13bc	**0.0086**
Cuming	C9	0.750 ± 0.07bc	0.271 ± 0.07 cd	**<0.0001**
Cuming	C10	0.848 ± 0.10abc	0.559 ± 0.10ab	**0.0472**
Cuming	C11	0.980 ± 0.09a	0.505 ± 0.09ab	**0.0015**
Cuming	C12	0.648 ± 0.07ab	0.407 ± 0.07bc	**0.0247**
Cuming	C13	0.441 ± 0.07e	0.321 ± 0.07bcd	0.2483
Cuming	C14	0.879 ± 0.11abc	0.440 ± 0.11bc	**0.0075**
Cuming	C15	0.831 ± 0.11abc	0.492 ± 0.11abc	**0.0348**
Stanton	S2	0.763 ± 0.08abc	0.753 ± 0.08a	0.9278
Colfax	Cx1	0.729 ± 0.08bc	0.371 ± 0.08bc	**0.0060**
Saunders	Sa1	0.143 ± 0.06f	0.167 ± 0.06de	0.7841
Lab Control	LABC	0.019 ± 0.01 g	0.010 ± 0.01e	0.2985

† Corrected survival values followed by the same lowercase letter within a hybrid column and bioassay year are not significantly different among populations (Tukey's multiplicity adjustment, $P > 0.05$).
‡ *P*-values from the linear model comparing mean corrected survival on Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 maize within populations. Significant differences in corrected survival between hybrids within a population are shown in bold ($P < 0.05$).

and 3(C)]. Within the LABC, mean fresh weight of survivors was not significantly different between the rootworm–Bt pyramid and non-Bt treatment in either year [Figs 2(C) and 3(C)]. This may have been an artifact of the low sample sizes (low survival) contributing to the LABC means and large SEs.

3.5 Resistance classification

Fisher's exact test indicated that there was a significantly higher proportion of WCR field populations with complete resistance to Cry3Bb1 compared to Cry3Bb1 + Cry34/35Ab1 maize in 2018 ($P = 0.0055$) and 2019 ($P < 0.0001$) bioassays. In the 2018 bioassays, eight of ten and one of ten field populations exhibited complete resistance to Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 maize, respectively (Table 2). No populations were classified as susceptible to Cry3Bb1 or Cry3Bb1 + Cry34/35Ab1 maize in the 2018 bioassays. Complete resistance to Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 was observed in 11 of 14 and one of 14 WCR populations assayed in 2019, respectively (Table 2). One WCR field population (Sa1) was susceptible to Cry3Bb1.

4 DISCUSSION

This study confirms the first cases of field-evolved resistance to the Cry3Bb1 + Cry34/35Ab1 pyramid in Nebraska with plant-based bioassays, adding to previous reports from North Dakota[47] and Iowa.[41] Many growers initially responded to Cry3 resistance by planting rootworm–Bt pyramids containing Cry3Bb1 and Cry34/35Ab1 proteins,[38,79,80] which has been effective at reducing root injury and population densities in past Cry3Bb1 problem fields.[79] The relative advantage of the Bt pyramid over single-trait Cry3Bb1 is still apparent in this study, although evidence of WCR adaptation to the pyramid is clear. Most (92%) WCR populations from the northeastern Nebraska study area exhibited incomplete resistance to Cry3Bb1 + Cry34/35Ab1 maize (Table 2, Figs 2 and 3) and significant differences in corrected survival among populations document the mosaic of WCR susceptibility to the pyramid in the landscape (Table 1). Based on the criteria measured in this study, complete resistance to Cry3Bb1 + Cry34/35Ab1 maize was evident in two WCR populations [C2 (2018) and S2 (2019)] and pyramid-corrected survival >0.60 in six populations suggests that WCR resistance to Cry34/35Ab1 also is present in the study area. Additional bioassays are needed to evaluate the susceptibility of Nebraska WCR populations to single-trait Cry34/35Ab1 maize.

In contrast to Cry3Bb1 + Cry34/35Ab1, results from the 2018 and 2019 larval bioassays confirmed complete resistance to Cry3Bb1 in 79% of the WCR populations collected from the northeastern Nebraska study area, particularly in Cuming County (Table 2, Figs 2 and 3). The significant difference between the

Table 2. Classification of resistance to Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 maize in each WCR field population based on proportional survival and larval development metric criteria

County	Population	Classification of Cry3Bb1 resistance	Classification of Cry3Bb1 + Cry34/35Ab1 resistance
2018 bioassays			
Cuming	C1	Incomplete†	Incomplete
Cuming	C2	Complete‡	Complete
Cuming	C3	Complete	Incomplete
Cuming	C4	Complete	Incomplete
Cuming	C5	Complete	Incomplete
Cuming	C6	Complete	Incomplete
Cuming	C7	Complete	Incomplete
Cuming	C8	Incomplete	Incomplete
Stanton	S1	Complete	Incomplete
Polk	P1	Complete	Incomplete
2019 bioassays			
Cuming	C3	Incomplete	Incomplete
Cuming	C4	Complete	Incomplete
Cuming	C5	Complete	Incomplete
Cuming	C7	Complete	Incomplete
Cuming	C9	Complete	Incomplete
Cuming	C10	Complete	Incomplete
Cuming	C11	Complete	Incomplete
Cuming	C12	Complete	Incomplete
Cuming	C13	Incomplete	Incomplete
Cuming	C14	Complete	Incomplete
Cuming	C15	Complete	Incomplete
Stanton	S2	Complete	Complete
Colfax	Cx1	Complete	Incomplete
Saunders	Sa1	Susceptible§	Incomplete

Within years, a significantly higher proportion of WCR field populations exhibited complete resistance to Cry3Bb1 compared to Cry3Bb1 + Cry34/35Ab1 maize (Fisher's exact test, $P < 0.05$).
† Criteria for complete resistance: (i) proportional larval survival on the Bt hybrid was significantly greater than the laboratory control, (ii) within populations, proportional survival and larval development metric comparisons on the Bt and non-Bt hybrids were not significantly different.
‡ Criteria for incomplete resistance: (i) proportional larval survival on the Bt hybrid was significantly greater than the laboratory control, (ii) within populations, proportional survival and/or either larval development metric was significantly greater on the non-Bt hybrid than the Bt hybrid.
§ Criteria for susceptible: no significant difference in proportional survival compared to the laboratory control.

proportion of WCR populations exhibiting complete resistance to Cry3Bb1 versus Cry3Bb1 + Cry34/35Ab1 (Table 2) suggests that populations are in more advanced stages of field-evolved resistance to Cry3Bb1 than the pyramid. The long-term cultivation of continuous maize and associated use of Cry3Bb1 has placed selection pressure on WCR populations over an extended period in this area.[44,59] An example of this is field C7, which was planted to a single-trait Cry3Bb1 hybrid from 2007 to 2011 and in 2013[44,59] and Cry3Bb1 + Cry34/35Ab1 during the years of this study. Complete resistance to Cry3Bb1 was confirmed after single-trait use[59] and after WCR dietary exposure to the pyramid (Table 2, Figs 2 and 3). The persistence of Cry3Bb1 resistance in WCR populations even after rotation to a Cry34/35Ab1-expressing hybrid[59] and gene flow of resistance alleles through adult WCR movement[45,64] have probably contributed to the landscape-level Cry3Bb1 resistance pattern observed within Cuming County. Widespread Cry3Bb1 resistance also has been reported in numerous Iowa counties.[81,82] The neighborhood clusters of Cry3Bb1 resistance evident in Nebraska reinforce the role of localized selection pressure and WCR population dynamics as key contributors to resistance evolution within the landscape.[45,79–82]

Larval development metrics such as head capsule width and fresh weight complement survival as potential indicators of the level of WCR resistance present within a population. The Sa1 and LABC populations assayed with Cry3Bb1 (2018, 2019) and Cry3Bb1 + Cry34/35Ab1 (2018) in this study exhibited mean extended development (as measured by head capsule width) typically observed in Bt-susceptible populations,[65,66,71,83] whereas WCR populations exhibiting incomplete resistance to Cry3Bb1 or Cry3Bb1 + Cry34/35Ab1 had variable mean larval development (i.e. significant or non-significant mean development comparisons occurred within populations between Bt and non-Bt hybrids or in comparison to the LABC; Figs 2 and 3). Significant differences in development between Bt and non-Bt hybrids within populations observed in some susceptible or incompletely resistant populations disappeared in all populations exhibiting complete resistance to Cry3Bb1 or Cry3Bb1 + Cry34/35Ab1 (Figs 2 and 3). This follows the general inverse relationship between mean larval development time and corrected survival that has been reported for WCR populations selected with Cry3Bb1.[71]

Bioassays are important tools to characterize changes in susceptibility to toxins in field or laboratory populations.[84] A number of

studies have documented that proportional survival measured in laboratory bioassays can be used to detect practical resistance, which refers to a decrease in product efficacy that can impact pest control in the field.[84,85] Relatively low resistance ratios (three-to six-fold range) obtained from on-plant bioassays have been correlated with greater than expected WCR root injury and practical resistance in the field.[39,40,44,48,51] Measuring practical resistance was not a goal of this study, but the high mean corrected survival of most populations reared on Cry3Bb1 (many >0.70) and some populations reared on Cry3Bb1 + Cry34/35Ab1 (i.e. C2, C8, S2 each >0.70) is similar or greater than that reported in previous studies where corrected survival was correlated with a high level of root injury in the field.[39,41,44] To date, most published studies have focused on detection and confirmation of resistance or associated inheritance of resistance and have not formally documented the potential impact of resistance on yield in the field.[40,41,44,80] In this study, the range of WCR corrected survival values suggests that populations will vary in their potential to cause significant root injury to Bt maize and associated yield loss. The interaction of resistance level with other factors such as WCR density and environmental conditions will ultimately determine the impact of resistance in the field.[84] Therefore, more research comparing laboratory bioassay data with field performance (i.e. specifically yield) of Bt hybrids is needed to gain a more complete understanding of the potential effect of different levels of resistance.

Significant variation in mean WCR corrected survival within populations between Bt hybrids indicates there was not a consistent relationship between Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 susceptibility (Table 1). For example, mean corrected survival values were significantly different between Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 in population C5 2019 (Cry3Bb1: 0.86, Cry3Bb1 + Cry34/35Ab1: 0.21) and C8 2018 (Cry3Bb1: 0.45, Cry3BB1 + Cry34/35Ab1: 0.73). However, Cry3Bb1-corrected survival was higher than the pyramid in C5 and lower in C8 (Table 1). This suggests that efficacy of Cry3Bb1 + Cry34/35Ab1 maize is influenced by the relative susceptibility of a population to each trait in the pyramid, which can vary among populations in the landscape.[41,48,86] The lack of structural similarity,[87] different midgut binding sites,[88] and no evidence of cross-resistance between Cry34/35Ab1 and Cry3 proteins[39,40,43,44,48,50] supports an additive function of each trait within the pyramid. Because past use of rootworm–Bt hybrids is a key driver of WCR susceptibility at the local level[45,79–81] and variability can occur in the landscape, it is important to understand the Bt trait history and relative WCR susceptibility to both Cry3Bb1 and Cry34/35Ab1 maize at the farm and field level to develop appropriate IPM and resistance management strategies when deploying a Cry3Bb1 + Cry34/35Ab1 hybrid.

In conclusion, plant-based bioassays from this study provide the first formal confirmation of WCR field-evolved resistance to the Cry3Bb1 + Cry34/35Ab1 pyramid in Nebraska and document that complete resistance to Cry3Bb1 is widespread in Cuming County. Data support previous work that has documented long-term history of continuous maize production coupled with use of rootworm–Bt hybrids can create the selection pressure that leads to WCR evolution of resistance in the field.[40,44,45,89] The significant variability in corrected WCR survival between Cry3Bb1 and Cry3Bb1 + Cry34/35Ab1 maize among populations suggests different levels of WCR resistance to Cry3Bb1 versus Cry34/35Ab1 exist in northeastern Nebraska. Additional research is needed to understand the relationship between bioassay results and practical resistance in Nebraska. Results from this study provide a snapshot of the existing WCR resistance landscape that is duplicated in some parts of the US Corn Belt where the latest rootworm technology to be registered in the US[90] [pyramid that includes Cry3Bb1, Cry34/35Ab1 and DvSnf7 dsRNA (RNA interference technology)] could be planted upon commercialization. Resistance levels identified in this study indicate that a more holistic approach is needed to mitigate WCR resistance to Cry3Bb1 + Cry34/35Ab1 maize or slow resistance evolution to RNA interference technology when introduced. A key will be to manage WCR densities and injury at the local level using multiple tactics within an integrated pest management framework.[38,45,80,91]

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DATA AVAILABILITY STATEMENT

All relevant data are within the paper and its Supporting Information files.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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