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David S. Wangila

University of Nebraska-Lincoln, dwangila2@unl.edu

Arnubio Valencia

Universidad de Caldas, Manizales, Colombia, arnubio.valencia@ucaldas.edu.co

Haichuan Wang

University of Nebraska-Lincoln, hwang4@unl.edu

Blair D. Siegfried

University of Florida, bsiegfried1@ufl.edu

Lance Meinke

University of Nebraska--Lincoln, lmeinke1@unl.edu

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Influence of calcareous soil on Cry3Bb1 expression and efficacy in the field

David S. Wangila,¹ Arnubio Valencia J,^{1,3} Haichuan Wang,¹
Blair D. Siegfried,^{1,2} and Lance J. Meinke¹

1 Department of Entomology, University of Nebraska-Lincoln, 220 Entomology Hall, Lincoln, NE 68583, USA

2 Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611, USA

3 Departamento de Producción Agropecuaria, Universidad de Caldas, Manizales, Colombia

Corresponding author — D. S. Wangila dwangila2@huskers.unl.edu

Abstract

Greater than expected injury by western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte) to Cry3Bb1 expressing maize hybrids (*Zea mays* L.) has been reported in southwestern Nebraska. Affected areas of some fields are often associated with high pH calcareous soils where maize growth is poor and iron chlorosis is common. As part of a comprehensive study to understand potential causes of unexpected injury, experiments were conducted during 2013 and 2014 to ascertain whether the calcareous soil conditions and associated poor maize growth negatively affect the expression of Cry3Bb1. Quantitative determination of Cry3Bb1 protein expression levels in root tissues was carried out on plants at V5–V6 growth stage using the enzyme-linked immunosorbent assay. Cry3Bb1 and non-Bt near isoline maize hybrids were artificially infested with Cry3Bb1-susceptible WCR eggs to measure survival and efficacy of Cry3Bb1 maize in calcareous and noncalcareous soils. Results showed that there was not a significant difference in expression of Cry3Bb1 protein between plants from calcareous and

non-calcareous soils (18.9–21.2 µg/g fresh weight). Western corn rootworm survival was about sevenfold greater from the non-Bt isoline than Cry3Bb1 maize indicating that Cry3Bb1 performed as expected when infested with a Cry3Bb1 susceptible rootworm population. When survival from calcareous and non-calcareous soils was compared, no significant differences were observed in each soil. A significant positive correlation between soil pH and expression of Cry3Bb1 protein in roots was detected from samples collected in 2014 but not in 2013. No such correlation was found between soil pH and survival of WCR. Results suggest that Cry3Bb1 expression levels were sufficient to provide adequate root protection against WCR regardless of soil environment, indicating that lowered Cry3Bb1 expression is not a contributing factor to the greater than expected WCR injury observed in some southwestern Nebraska maize fields.

Keywords: Transgenic crop, *Bacillus thuringiensis*, Western corn rootworm, Bt expression, ELISA, Insecticide resistance management

Introduction

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is one of the most economically important insect pests of field maize (*Zea mays* L.) in North America (Rice 2004; Gray et al. 2009). Economic loss from the WCR is primarily caused by the larval stage which can cause substantial injury to maize roots. Larval injury can negatively impact maize photosynthetic rates and reduce plant vegetative biomass accumulation (Godfrey et al. 1993; Hou et al. 1997; Urías-López et al. 2000), cause plant instability (Levine and Oloumi-Sadeghi 1991; Spike and Tollefson 1991), and reduce grain yield (Urías-López and Meinke 2001; Gray and Steffey 1998; Dun et al. 2010; Tinsley et al. 2013). The invasive nature (Meinke et al. 2009) and adaptability of this species to selection pressure (Miller et al. 2009) has made management in field maize an ongoing challenge (Gray et al. 2009).

Genetically modified maize hybrids expressing insecticidal toxins from the soil bacterium, *Bacillus thuringiensis* Berliner (Bt) have been widely adopted by farmers as a primary tactic for management of corn rootworms (Andow et al. 2016). Bt-based transgenic maize provides an important alternative for farmers that has largely replaced the application of conventional soil insecticides at planting in continuous maize production. The rootworm-active protein Cry3Bb1 which was first registered in the United States as part of event MON863 and later event MON88017 has been a commercially available trait in field maize for corn rootworm control since 2003 (EPA 2003, 2012; Vaughn et al. 2005). Cry3Bb1 was initially marketed only in single rootworm-trait hybrids, but since 2009, the Cry3Bb1 × Cry34/35Ab1 pyramid has also been available to farmers in the U.S. (EPA 2009).

Greater than expected injury by WCR to single-trait Cry3Bb1 maize hybrids was initially observed in a number of locations between 2009 and 2011 (Gassmann et al. 2011, 2012, 2014; Potter and Ostlie 2011; Wright and Meinke 2011; Gray 2012, 2014; Wangila et al. 2015) and field evolved resistance to Cry3Bb1 has been confirmed in Iowa, Illinois, Minnesota, and Nebraska using laboratory bioassay techniques (Gassmann et al. 2011, 2012, 2014; Gray and Spencer 2015; Wangila et al. 2015; Zukoff et al. 2016). In recent years, to improve resistance management, single trait Cry3Bb1 maize hybrids

have been phased out of the marketplace and replaced by maize hybrids expressing the Cry3Bb1 × Cry34/35Ab1 pyramid.

In southwestern Nebraska, the agro-ecosystem presents challenges to optimal maize production. Irrigated continuous maize is the predominant crop and often the most profitable; crop rotation options are limited. High soil pH (>7.0) is common in this agroecosystem and some fields contain patches of calcareous soil (pH often >8.0) in which maize can be chlorotic (i.e., iron chlorosis, Wortmann and Hergert 2013) and stunted, exhibit slower growth, or sustain stand reduction. Continuous maize often produces high annual densities of WCR that increases the risk of root injury and yield loss in many maize fields. Finally, there are increasing weed management problems (especially Palmer amaranth, *Amaranthus palmeri*), which are especially apparent in fields with patches of calcareous soils.

Unexpected WCR injury to single-trait Cry3Bb1 maize hybrids was initially observed in some southwestern Nebraska maize fields in 2011 (Wangila et al. 2015). This injury was especially apparent in the patches of calcareous soils (L. J. M, Personal communication). Previous research documented that WCR resistance to Cry3Bb1 had evolved in isolated parts of this region (Wangila et al. 2015) but the potential impact of other factors that could lead to rootworm injury in the calcareous high pH soil environment was unknown. The chlorotic appearance and reduced growth of single trait Cry3Bb1 maize in the calcareous soil raised the question as to whether Cry3Bb1 protein expression was compromised in calcareous soil maize possibly contributing to reduced efficacy.

Many studies have investigated factors affecting the expression of lepidopteran active Bt proteins (Nguyen and Jehle 2007; Luo et al. 2008; Badea et al. 2010; Székács et al. 2010a, b, 2012; Mejia and de Polania 2012), but only a few studies have focused on coleopteran active Bt proteins, especially Cry3Bb1 (Sidhu and Brown 2004; Nguyen and Jehle 2009; Marquardt et al. 2014). In particular, information on expression of Cry3Bb1 proteins in relation to soil factors/maize iron chlorosis is lacking. Therefore, as part of a larger project to develop sustainable maize rootworm management strategies in southwestern Nebraska, this study was conducted to investigate whether the high pH calcareous soil and associated poor maize growth leads to reduced

expression of Cry3Bb1 protein which may contribute to unexpected rootworm injury. The null hypotheses tested included: (1) WCR survival is similar in calcareous soil and surrounding non-calcareous soil; and, (2) Cry3Bb1 protein expression is also similar in calcareous soil and surrounding non-calcareous soil.

Materials and methods

Experimental system and design

To address each hypothesis, irrigated first year maize fields (300–500 ha) in Chase County Nebraska that had patches of calcareous soil (size range: 0.5–3 ha) were selected as study sites. Three fields were used for experiments in 2013 and two in 2014. Within each field, there were three (2013) and five (2014) replicates of calcareous soil and non-calcareous soil (called soil types throughout remainder of paper) arranged in a completely random design. Topsoil of calcareous soils at these sites was shallow, with weathered limestone bedrock fragments distributed throughout (up to 15% CaCO₃, USDA 2016) which created a very high pH environment (often >7.8–8.0). Calcareous soil patches could be visually identified by color as they had a white chalky appearance and surrounding non-calcareous soil was darker brown to black. Within fields, soil sampling was conducted following standard sampling guidelines (USDA 2001; Ferguson et al. 2007) in the preselected replicates of each soil type to characterize soil texture and pH. Samples were collected with a 2.2 × 83.8 cm soil probe (Grainger, Inc. 100 Grainger Parkway Lake Forest, IL) and analysis was conducted at AgSource Harris Laboratories (Lincoln, NE). A pH meter (Ben Meadows Company, Janesville, WI) was also used to record additional soil pH measurements as defined in Heggenstaller (2012). Soil texture classification in the calcareous patches was a Rosebud-Canyon loam (21.8% clay, 29.4% silt, 48.8% sand) and non-calcareous areas of fields were a combination of Canyon loam (22.0% clay, 37.9% silt, 40.1% sand) and either Kuma or Goshen silt loams (22.3–23.8% clay, 61.7–66.2% silt, 10.1–16% sand) (USDA 2016). The mean pH values of calcareous and non-calcareous soils obtained from the soil analyses were significantly different (i.e., 7.89 ± 0.1 and 7.35 ± 0.1, respectively; n = 19 in each soil; t test PROC GLIMMIX in SAS 2012, P < 0.05). Both soil types were alkaline soils, but 73% of the soil

samples from the calcareous soils were >pH 7.8 which often leads to reduced availability of iron in maize (Shaver 2014). In this system, iron chlorosis was exhibited in maize grown in the calcareous soil but not the non-calcareous soil.

A maize hybrid (Stone 6021VT3) that expressed Cry3Bb1 protein and the near isoline-maize hybrid (Stone 6021RR2) that does not express Cry3Bb1 (specified as isoline in remainder of paper) were used in this study. Seed pre-coated with neonicotinoid insecticide (Clothianidin, 0.25 mg AI per kernel) was provided by Monsanto Company (St. Louis, MO). When commercially planted maize reached V1 stage (Abendroth et al. 2011), seedlings were removed in each field from one 8 m row per replicate per soil type and replaced with the maize hybrids previously mentioned. Fifteen seeds of each maize hybrid were planted at a plant spacing of 25 cm in half of the row, respectively (4 m of each hybrid per soil type per rep) with hybrid position within rows randomly assigned. Lateral flow strips, an Enzyme Linked ImmunoSorbent Assay (ELISA)—based technique (Quantiplate™ kits; EnviroLogix, Portland, ME), were used to confirm the expression of Cry3Bb1 protein in maize leaf tissue before conducting experiments to address each hypothesis.

Western corn rootworm emergence and survival

Within each replicate of calcareous and non-calcareous soil, two plants each of Cry3Bb1 and isoline maize spaced 2-m apart were artificially infested with ca. 1300 WCR eggs per plant. The WCR population was susceptible to Cry3Bb1 (obtained from USDA-ARS North Central Agricultural Research Laboratory, Brookings, SD) which enabled measurement of adult survival in each soil type and hypothesis 1 to be addressed. The eggs were suspended in 1.25% agar-water solution, drawn in a 60 ml hypodermic syringe (Becton, Dickinson and company, Franklin Lakes, NJ) and mechanically infested in two holes, 6–10 cm deep (20 ml of egg-agar water per plant) on opposite sides of each plant. Eggs were infested when maize was V2–V3 stage (Abendroth et al. 2011) to synchronize the appropriate larval rootworm-maize phenological interaction commonly observed in southwestern Nebraska. In early July, all infested plants were caged using single plant emergence cages, 76 cm long × 38 cm wide, a modified

design of Fisher (1980). Each cage was partially buried in the soil and secured around the plant stalk to prevent adult WCR escape but allowed normal plant growth throughout the season. Adults that emerged were trapped in cylindrical glass collection jars (PL2 0090, Solo Cup Company, Lake Forest, IL) placed on the cages. The jars were replaced on a weekly basis during the emergence period (July-late September) and beetles collected were taken to the laboratory and counted. This experiment was conducted in 2013 and consisted of four total cages per soil type per replicate, and three replicates per soil type in each of three fields (total $n = 72$).

Plant tissue collection

The expression of Cry3Bb1 protein in maize root tissues from calcareous and non-calcareous soil treatments (hypothesis 2) was conducted in 3 fields during 2013 and 2 fields during 2014. A subset of plants (not caged in 2013) as described above was used to quantify the expression of Cry3Bb1 proteins in root tissues at V5–V6 growth stage (Abendroth et al. 2011), a plant growth stage that matches the maize growth phenology at the study location when WCR eggs typically hatch and larvae actively colonize maize roots and an optimal maize growth stage that favors WCR larval development (Hibbard et al. 2008). Three plants expressing Cry3Bb1 protein were collected per soil type per replicate in each field during 2013 (total $n = 54$) and 2014 (total $n = 60$). Soil from each plant root was gently washed three times under running water and then dried using paper towels. Due to the fact that Bt toxin is most concentrated in root tips and young root tissue (Meissle et al. 2009), a randomly chosen young root tip (composite sample about 4 cm long) was cut using a clean scissor and placed inside a small plastic vial (5 × 5 ml graduated plastic vials), snap frozen in liquid nitrogen and then stored at -80°C until used for Cry3Bb1 expression using quantitative ELISA (Stave 2002; Grathaus et al. 2006).

Protein extraction and quantitation

Proteins were extracted from maize roots prepared as previously described by Marquardt et al. (2014) by grinding 100 mg of maize root tissue, in an ice-cold mortar with 1 ml of PBST buffer (pH

7.40). Protein extract was cleared by centrifugation at $12,000g$ for 10 min at 4°C and the supernatant containing Cry3Bb1 toxin was used for ELISA assays. Protein concentration was measured by the Bradford (1976) procedure using bovine serum albumin (BSA) as standard. The established standard ($R^2 = 0.9992$; Online Resource 1) was saved for quantification of total protein expressed in root samples prior to the ELISA assays. Three replicates per sample were tested.

Quantification of expressed Cry3Bb1 toxin using ELISA

Cry3Bb1 protein was quantified by ELISA assay following the protocol described by Marquardt et al. (2014). Briefly, protein samples were diluted from mg l^{-1} to $\mu\text{g l}^{-1}$ prior performing ELISA assays. A standard curve was generated using 97% pure and trypsin-activated Cry3Bb1 protein obtained from Monsanto Company (St. Louis, MO), using eight different concentrations of pure Cry3Bb1 toxin that were prepared with PBST-buffer (pH 7.40). Standards and maize root samples were added to a 96-well ELISA plate and the color development was measured using a 96-well microplate reader (BioTek instruments, INC, Highland Park, Winooski, VT) at 650 nm. The standard curve was established ($R^2 = 0.9929$; Online Resource 2) and saved for use in quantification of Cry3Bb1 proteins. Positive and negative controls were used in all ELISA assays. Each sample was evaluated by triplicate.

Data analysis

The WCR emergence data per cage was transformed with the function $\log(n + 1)$ and analyzed as a multi-location $3 \times 2 \times 2$ generalized mixed model ANOVA (PROC GLIMMIX) in SAS (SAS Institute 2012). The fixed factors in the model included, three fields, two soil types (calcareous and non-calcareous soil) and two maize hybrids (Cry3Bb1 and near isoline) and their interaction. The random factor in the model was the field (SAS Institute 2012). Nontransformed data are reported in this paper.

Data on expression of Cry3Bb1 proteins in root tissues was analyzed as a two-way mixed model ANOVA, using PROC GLIMMIX in SAS (SAS Institute 2012). Data for each year (2013 and 2014)

were analyzed separately. Each ANOVA included the fixed factors field and soil type. In both analyses, the Kenward–Roger adjustment was used to adjust for the standard error means for fixed effects (Littell et al. 2006).

Correlation analyses were used to measure the strength of association between soil pH and WCR emergence from Cry3Bb1 and isoline hybrids. Also, correlation was measured between soil pH and concentration of Cry3Bb1 protein in maize root tissues. The significance of correlation was determined against the null hypothesis of $P = 0$ using the Pearson correlation coefficient (PROC CORR in SAS). For all analyses, the treatment differences were determined by the LSMEANS test at $P = 0.05$ level of significance (SAS Institute 2012).

Results

Survival of WCR in 2013 field studies

Western corn rootworm emergence was not significantly impacted by the three-way or any of the two way interactions (Table 1). Also, no main effects were statistically significant except maize hybrid (Table 1; Fig. 1). The number of WCR adults recovered in emergence cages of non-Bt maize was greater (12.33 ± 1.92) than that recovered on Cry3Bb1 maize (1.81 ± 0.52 ; Fig. 1). The overall means for WCR emergence in each soil type were 6.78 ± 1.95 and 7.36 ± 1.84 rootworms per cage in calcareous and non-calcareous soil, respectively. There was not a significant correlation between soil pH and WCR emergence in either Cry3Bb1 ($r = 0.0911$; $df = 33$; $P = 0.4105$; Fig. 2a) or isoline maize ($r = 0.2128$; $df = 34$; $P = 0.2125$; Fig. 2b).

Table 1. General linear mixed model analysis of variance for adult western corn rootworm survival from Cry3Bb1 and non-Bt isoline maize hybrids, 2013

Effect	df	F	P
Field	2, 24	2.37	0.1150
Hybrid	1, 24	46.83	<0.0001
Field × hybrid	2, 24	0.98	0.3883
Soil type	1, 24	0.48	0.4956
Field × soil type	2, 24	0.19	0.8276
Hybrid × soil type	1, 24	0.50	0.4871
Field × hybrid × soil type	2, 24	0.49	0.6163

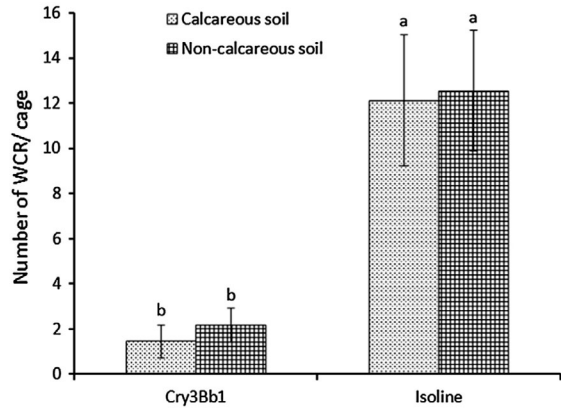


Fig. 1. Western corn rootworm (WCR) adult emergence from Cry3Bb1 and non-Bt isoline maize artificially infested with 1300 eggs per plant of a Cry3Bb1 susceptible WCR population. The design included a total of four cages per soil type per replicate, and three replicates per soil type in each of three fields, $n = 72$, conducted during 2013.

Expression of Cry3Bb1 protein in maize root tissues at V5–V6 growth stage

The interaction between field and soil type did not significantly affect the mean quantity of Cry3Bb1 protein in maize root tissues of plants in either 2013 or 2014 (Table 2). Similarly, there were no statistically significant main-effects during either year (Table 2). Mean Cry3Bb1 expression levels are presented for each soil type within years in Fig. 3. In 2013, there was not a significant correlation between soil pH and concentration of Cry3Bb1 protein ($r = 0.0374$; $df = 81$; $P = 0.7364$; Fig. 4a); however, in 2014, a significant positive correlation was detected ($r = 0.4411$; $df = 49$; $P = 0.0012$; Fig. 4b).

Discussion

Similar levels of Cry3Bb1 protein expression obtained from maize plants growing in calcareous and noncalcareous soil in 2013 and 2014 indicate that the high pH calcareous soils within the associated environment of this study did not compromise Cry3Bb1 expression (Table 2; Fig. 3). These results are similar to those reported by Badea et al. (2010), who reported no difference between expression level of Cry1Ab proteins in maize growing in

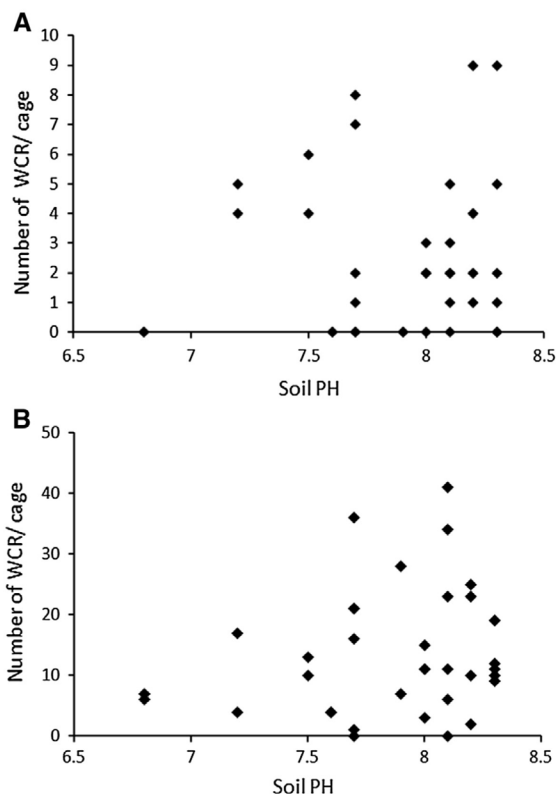


Fig. 2. Correlation of western corn rootworm (WCR) adult emergence from Cry3Bb1 (a) and non-Bt isoline maize (b) infested with 1300 eggs per plant in three fields during 2013.

three soil types under greenhouse conditions. The significant but weak correlation between soil pH and plant expression of Cry3Bb1 protein in 2014 (Fig. 4b) suggests that a small increase in expression with higher pH can occur within the pH range evaluated. This increase in plant expression at highest pH was somewhat variable and mainly observed in calcareous soil but was not large enough to cause significant differences in expression when soil types were

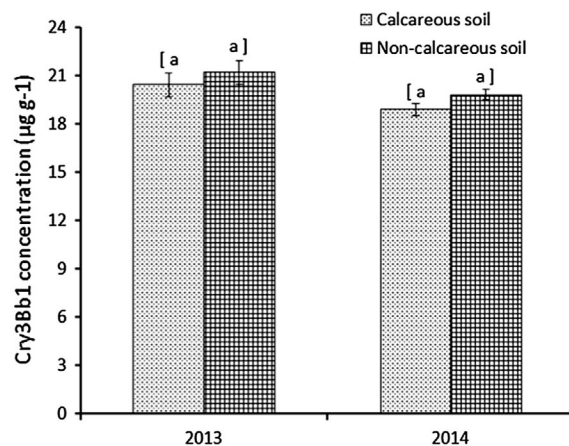


Fig. 3. Average expression of Cry3Bb1 proteins ($\mu\text{g/g} \pm \text{SE}$ of fresh weight root tissue) in Bt maize plants during 2013 ($n = 54$) and 2014 ($n = 60$) field studies. Means within brackets (year) with the same letter are not significantly different, ($P > 0.05$, LSMeans test).

compared. Therefore, the increase in Cry3Bb1 expression is probably not biologically relevant in the southwestern Nebraska system. It would be interesting to investigate if pH values lower than measured in this study would significantly contribute to lower expression.

A number of agronomic and environmental factors have been shown to affect the expression level of Bt proteins in plants (Trarore et al. 2000; Bruns and Abel 2003; Chen et al. 2005; Clark et al. 2005; Icoz and Stotzky 2007, 2008; Luo et al. 2008; Marquardt et al. 2014; Trtikova et al. 2015). In maize, Marquardt et al. (2014) found a positive linear relationship between nitrogen applied in the soil and Cry3Bb1 expression under greenhouse conditions. A similar positive correlation between the concentrations of Bt expressed and applied nitrogen rates was reported from experiments with MON-810 (Cry1Ab)

Table 2. General linear mixed model analysis of variance for the expression of Cry3Bb1 proteins in maize root tissues of plants at V5–V6 growth stage using Enzyme Linked Immunosorbent Assays (ELISA)

Effect	2013			2014		
	df	F	P	df	F	P
Soil type	1, 79	0.12	0.7288	1, 47	2.00	0.1640
Field	2, 79	0.50	0.6064	1, 47	0.15	0.7045
Soil type \times field	2, 79	0.99	0.3748	1, 47	0.23	0.6328

Data were analyzed within year from three fields in 2013 and two fields 2014.

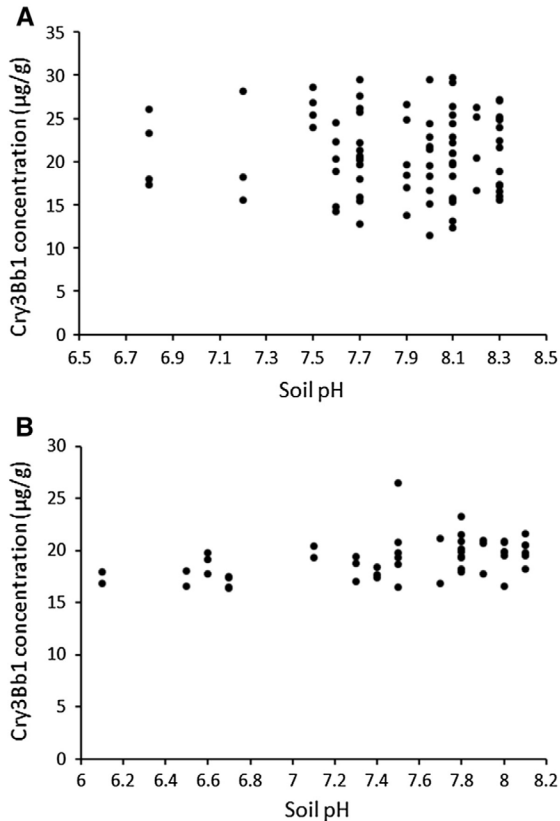


Fig. 4. Linear correlation analysis of soil pH and quantity of Cry3Bb1 proteins expressed in Bt maize plants from studies conducted during 2013 (a) and 2014 (b).

and DBT 418 (Cry1Ac) Bt maize hybrids (Bruns and Abel 2003). Variable Cry1Ab expression levels in maize have also been associated with different moisture and temperature stress conditions (Trtikova et al. 2015). In this study, a uniform rate of nitrogen was applied within each field across soil types. If differences in plant nitrogen uptake or available nitrogen per soil type were present, they were not large enough to significantly affect Cry3Bb1 expression. A key factor in our study system was irrigation. Maize in each field was grown under center pivot irrigation which may have mediated some potential differential effects of agronomic or environmental factors on plant expression of Cry3Bb1 in each soil type.

The significantly greater WCR survival (ca. sevenfold) from Cry3Bb1 versus non-Bt isolate maize in this study (Table 1) indicates that Cry3Bb1 was performing as expected when infested with a Cry3Bb1

susceptible rootworm population. The lack of a significant difference in rootworm survival between soil types suggests that potential differences in soil physical properties between soil types did not significantly influence rootworm population dynamics in this study. Soil factors that could vary by soil type that have been shown to affect WCR egg survival, larval establishment, or larval survival and movement in the soil (i.e., texture, porosity, moisture, compaction, etc., Turpin et al. 1972; MacDonald and Ellis 1990; Beckler et al. 2004; Meinke et al. 2009), did not appear to significantly impact the results of this study in an irrigated system.

In this study, Cry3Bb1 protein expression was quantified from only one maize growth stage (V5–V6). Previous studies have documented that expression of Cry3Bb1 can be variable among maize root tissues (Meissle et al. 2009, 2011) and often declines in root tissue as plants age (Vaughn et al. 2005; Nguyen and Jehle 2009). This suggests that declines in Cry3Bb1 expression may have been found in this study as well if sampling would have been extended over multiple growth stages. However, the key point to consider with any Bt technology is whether or not variability in expression adversely impacts efficacy. The decline in Cry3Bb1 expression reported by Vaughn et al. (2005) was not associated with a decline in efficacy in greenhouse or field trials. If a decline in efficacy did occur over time in our study, the adult survival data suggest that efficacy was not significantly affected. Efficacy may be preserved partly because a large proportion of mortality associated with dietary exposure to Cry3Bb1 on vegetative stage plants occurs during the first instar (Becker 2006) when Cry3Bb1 expression levels are the greatest (Vaughn et al. 2005; Nguyen and Jehle 2009).

Results of this study suggest that greater than expected WCR injury to Cry3Bb1 expressing maize hybrids in calcareous soils of southwestern Nebraska might be due to factors other than reduced Cry3Bb1 expression. The stunted growth of maize in patches of calcareous soil can lead to later pollination than found in non-calcareous soils. Also, *A. palmeri* weed growth can dominate patches of calcareous soil and bloom late in the growing season. Because the WCR is highly attracted to maize or weed pollen and younger plant tissues (Darnell et al. 2000; Campbell and Meinke 2006), the phenological contrast

between maize and weeds in calcareous soils and surrounding maize can act as a sink for immigrating beetles causing densities to rise in calcareous soil patches later in the season (Wangila 2016). Trap crop situations can lead to greater oviposition and higher larval densities the following season (Hill and Mayo 1974). Therefore, larval densities and associated injury potential in some patches of calcareous soil may be greater than densities found in the remainder of the field. Secondly, the smaller root size of stunted plants in calcareous soil compared to the root mass of plants in non-calcareous soil may cause these plants to be more susceptible to larval injury and lodging per WCR density present. A third factor that can be layered across both soil types is the evolution of resistance to Cry3Bb1 as a result of trait overuse (Wangila et al. 2015). In fields where western corn rootworm populations exhibit low levels of resistance to Cry3Bb1, (i.e., early stages of resistance evolution), an interaction of rootworm density, stunted plant size, and resistance may lead to visibly greater than expected injury in calcareous soils before it occurs in non-calcareous soils. An example of this may have been a Chase Co. NE field in 2011 where greater than expected injury was observed in patches of calcareous soil but only a low resistance level was recorded when single-plant bioassays were conducted (field PA, Wangila et al. 2015).

In summary, the Cry3Bb1 protein expression data and WCR survival data presented in this paper collectively support the conclusion that Cry3Bb1 expression levels in the southwestern Nebraska irrigated agroecosystem are sufficient to provide adequate root protection against WCR regardless of soil type. Therefore we accept the null hypotheses addressed in this paper. This study increases our understanding of Cry3Bb1 protein expression in maize plants especially in a high soil pH environment and also helps narrow down possible causes of greater than expected injury to Cry3Bb1 observed in the southwestern Nebraska agro-ecosystem.

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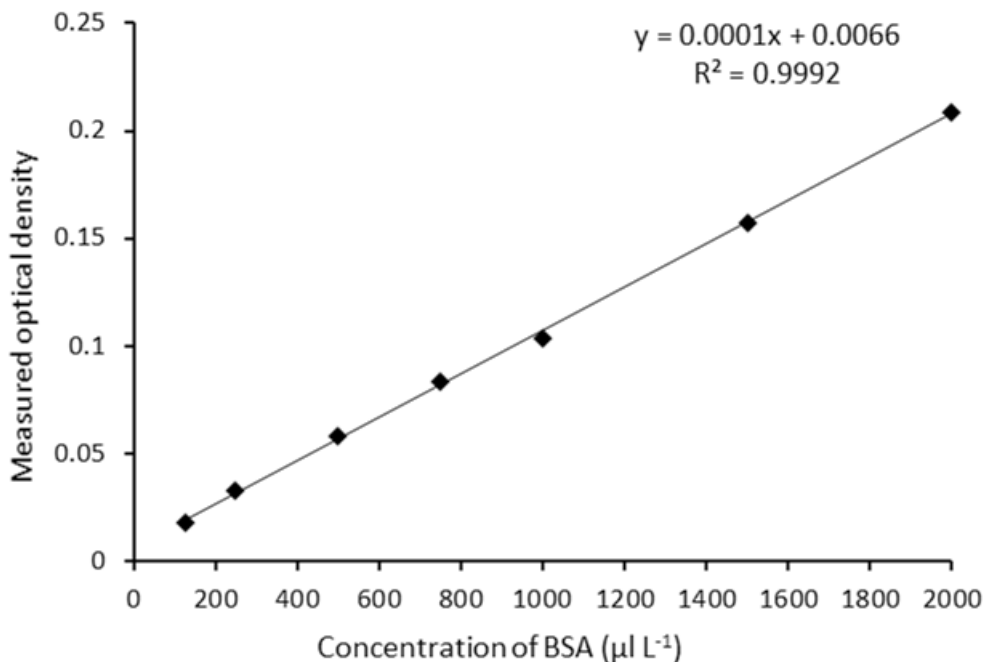


Fig. S1. Bradford's Assay standard curve using Bovine Serum Albumin (BSA) to quantify the total protein concentration in maize root samples at V5–V6 growth stage sampled from fields during 2013 and 2014.

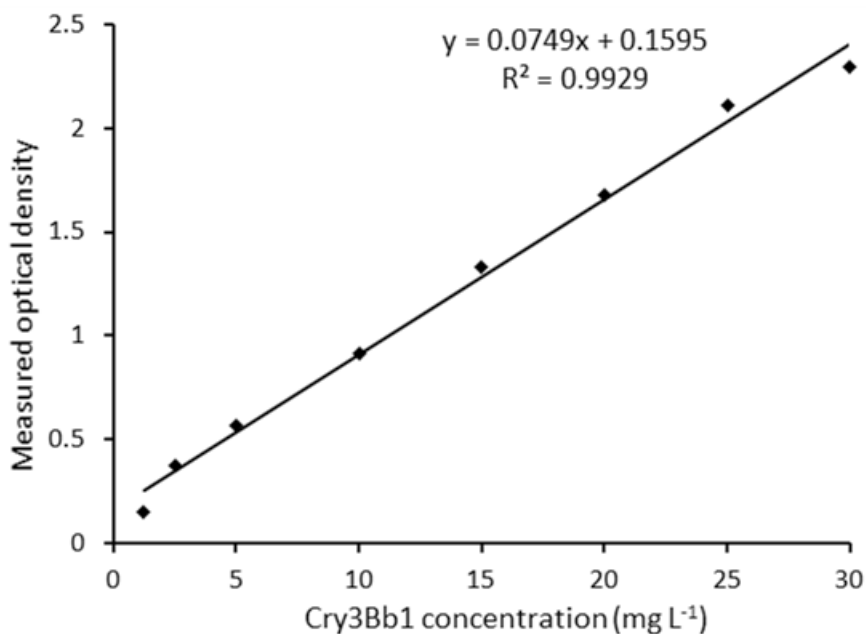


Fig. S2. The Cry3Bb1 standard curve used to quantify the concentration of Cry3Bb1 proteins in maize roots at V5–V6 growth stage sampled from the field during 2013 and 2014.