

Host Recognition Responses of Western (Family: Chrysomelidae) Corn Rootworm Larvae to RNA Interference and Bt Corn

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Abstract

Western corn rootworm *Diabrotica virgifera virgifera* LeConte is an important pest of corn whose larvae exhibit particular quantifiable patterns of locomotion after exposure to, and removal from, host roots and nonhost roots. Using EthoVision software, the behavior and locomotion of the western corn rootworm larvae was analyzed to determine the level of host recognition to germinated roots of differing corn hybrids containing either rootworm targeted Bt genes, RNA interference (RNAi) technology, the stack of both Bt and RNAi, or the isoline of these. The behavior of the rootworm larvae indicated a significant host preference response to all corn hybrids (with or without insecticidal traits) compared to the filter paper and oat roots. A weaker host response to the RNAi corn roots was observed in the susceptible larvae when compared to the resistant larvae, but not for the Bt + RNAi vector stack. Additionally, the resistant larvae demonstrated a weaker host response to the isoline corn roots when compared to the susceptible larvae. Although weaker, these host responses were significantly different from those observed in the negative controls, indicating that all hybrids tested do contain the contact cues necessary to elicit a host preference response by both Cry3Bb1-resistant and Cry3Bb1-susceptible larvae that would work to hinder resistance development in refuge in a bag fields.

Key words: *Bacillus thuringiensis*, Bt, corn, *Diabrotica virgifera virgifera*, EthoVision

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is considered to be the most important insect pest of corn (*Zea mays* L.) in major corn-producing regions (Stamm et al. 1985, Krysan et al. 1986) with crop losses and control costs estimated to be over \$2 billion annually in the United States alone (Mitchell 2011). WCR larvae are subterranean and specialize on corn roots. Although these larvae will feed on most grass roots (Family Poaceae), they can only complete their development on a select few species other than corn (Branson and Ortman 1970, Clark and Hibbard 2004). Root damage caused by intense larval feeding can severely limit the ability of the plant to absorb nutrients and water and can weaken the plant base, making the plant vulnerable to lodging (Kahler et al. 1985, Sutter et al. 1990).

WCR eggs are laid in soil near the base of the plant, and after hatching larvae use CO₂ (a volatile given off by all plants) as a long range attractant as they move through the soil in search of host roots (Branson 1982, Strnad et al. 1986, Hibbard and Bjostad 1988, Bernklau and Bjostad 1998, Miller et al. 2006). In addition to CO₂, ethylene, a volatile phytohormone in corn, is used by WCR larvae to

locate potential host plants (Robert et al. 2012). Interestingly, WCR larvae are also attracted to (*E*)- β -caryophyllene, which is an induced plant volatile given off when WCR larvae feed on the roots of certain corn varieties. Robert et al. (2012) discovered that both of these volatiles are used by the larvae to evaluate the health of the plant from a distance. Although older larvae can survive starvation for up to 96 h, neonate larvae need to locate host roots within 12–36 h or risk being too weak to burrow into the root (Strnad and Bergman 1987b).

Once the WCR larvae find potential host roots, contact cues are picked up by the maxillary palps that aid the larvae in making feeding decisions (Branson and Ortman 1969). Feeding stimulants used by the WCR larvae to identify a host have been identified as a combination of simple sugars, 30:4:4 mg/ml glucose:fructose:sucrose, and one of the free fatty acids in germinating corn roots, oleic acid, or linoleic acid (Bernklau and Bjostad 2008). Interestingly, individual components by themselves did not elicit a major feeding response by the WCR larvae, but together, they did (Bernklau and Bjostad 2008).

Larvae of the WCR have a set of behaviors that help the larvae locate food patches and stay within food patches. When WCR

larvae are exposed to a substrate that is not recognized as a host and then are removed, they exhibit a “ranging” behavior, where they crawl in a relatively straight direction and move quickly (Strnad and Dunn 1990). Until the larvae encounter host volatiles, they will continue searching in this manner. In contrast, when WCR larvae are exposed to a host root and then are removed, they exhibit a “localized searching” behavior. This behavior involves a restricted area of search with greater number of turns, path crossings and a decrease in speed (Strnad and Dunn 1990, Bell 1991). Throughout their development, WCR larvae move to higher quality, younger root whorls (Apple and Patel 1963, Strnad and Bergman 1987a), and this localized searching behavior likely helps the larvae stay in the root zone while moving around. These behaviors are important for larval survival and contribute to the highly successful nature of this pest (Strnad and Dunn 1990).

In behavioral bioassays, Strnad and Dunn (1990) analyzed the paths that WCR larvae took after exposure to germinated roots of corn and other grasses. They found that after being exposed to corn and wheat roots, the rootworms initiated localized search. The WCR larvae exposed to giant foxtail (*Setaria faberi* Herrm) and oat (*Avena sativa* L.) seedling roots showed in part localized search by having a somewhat reduced area of search and reduced velocity. However, these larvae did not show any differences in the number of turns and path crossing as the moist filter paper control. Although the rootworm larvae will feed briefly on the oats, they will abandon them due to a feeding deterrent (Branson and Ortman 1969). WCR larvae have been shown to survive on giant foxtail roots (Clark and Hibbard 2004), but Chege et al. (2005) discovered weed host phenology may affect larval survival. Bernklau et al. (2009) found that WCR larvae will initiate localized search when exposed to root extracts, corn root pieces, and corn root juice.

Transgenic corn lines with genes from *Bacillus thuringiensis* Berliner (Bt) with resistance to WCR feeding are commonly used for rootworm management in the United States. These products range from single event hybrids to pyramided hybrids that have two or more Bt genes targeting rootworms. Current commercially available Bt hybrids targeting the WCR produce one or more of the following proteins mCry3A, Cry3Bb1, eCry3.1Ab, or Cry34/35Ab1. SmartStax is a stacked corn hybrid from Monsanto/Dow Agrosciences (St. Louis, Missouri, USA/Midland, Michigan, USA), which includes two pyramided rootworm genes, Cry3Bb1 and Cry34/35Ab1 as well as three Bt toxins targeted towards above ground pests. Syngenta's (Basel, Switzerland) next generation product, Agrisure Duracade, which includes mCry3A + eCry3.1Ab, is now commercially, but was not included in this study.

RNA interference (RNAi) is a novel way of controlling target pests by using double-stranded RNA (dsRNA) to silence-specific mRNA genes by way of interference with the target pests own RNAi pathway (Fire et al. 1998, Bolognesi et al. 2012). This pathway regulates gene expression and is also used for protection against viral infections (Waterhouse et al. 2001, Wang et al. 2006, Bolognesi et al. 2012). Artificial diet incorporating dsRNA and genetically engineered plants that express dsRNA can be fed to WCR larvae that cause mortality after entering the midgut cells (Baum et al. 2007, Whyard et al. 2009, Bolognesi et al. 2012). After ingestion, these dsRNA travel to other tissues and then are diced into short interfering RNA and interfere with gene expression causing larval mortality and stunting (Baum et al. 2007, Whyard et al. 2009, Bolognesi et al. 2012). Monsanto's next-generation rootworm product contains Corn Rootworm III (more information can be found here <http://www.monsanto.com/SiteCollectionDocuments/whistle-stop-corn-pipeline.pdf>) which includes RNAi + Cry3Bb1 genes. If

approved, this product will be the first of its kind for rootworm control.

WCR host recognition behavior is unknown for these transgenic genes and the recent discovery of populations of WCR resistant to Cry3Bb1 Bt corn in the field (Gassmann et al. 2011) raises concerns about the rootworm–transgenic corn interactions. If WCR larvae were to demonstrate nonhost, ranging behavior to a corn hybrids' roots, this could be beneficial for WCR management as the corn plant would not be recognized as a host and thus not be damaged. The larvae would presumably wander away from the plant and starve to death. However, if resistant larvae perceived the isolate as nonhosts over Bt hybrids, then in refuge in a bag (RIB) hybrid planted fields, this could add to the resistant population. Therefore, the objective of this study was to investigate how mCry3A, Cry3Bb1, Cry34/35Ab1, and RNAi corn influence the host recognition behavior of neonate WCR larvae.

Materials and Methods

The study was conducted at the USDA-ARS Plant Genetics Research Unit on the University of Missouri-Columbia campus in 2010 and 2011. To assess the host recognition behavior of WCR neonates on different varieties of corn roots, we conducted two sets of bioassays. The first set of bioassays consisted of a randomized complete block of nine treatments replicated 20 times. In this set, susceptible WCR were exposed to seven types of germinated corn roots, MIR604 (mCry3A), DAS59122-7 (Cry34/35Ab1), MON88017 (Cry3Bb1), SmartStax (Cry3Bb1 + Cry34/35Ab1), and their corresponding isolines, as well as germinated oat roots (nonhost, living plant control) and filter paper (control). The second set of bioassays included a full factorial randomized complete block design of 14 treatments with 20 replicates per treatment. These treatments consisted of two sources of eggs (resistant and susceptible colonies) on one of five types of corn, MON88017 (Cry3Bb1), RNAi, the vector stack (RNAi + Cry3Bb1), their corresponding isolines, filter paper (control), or oats (nonhost control).

Insects

WCR eggs were obtained from nondiapausing (Branson 1976) colonies maintained in our laboratory. In the first set of bioassays, the egg type used was from a susceptible WCR line (Janesville control, see Meihls 2010). In the second set of bioassays, eggs from the same susceptible colony were used and eggs from a line selected for resistance to MON88017 (Janesville selected) (Meihls 2010). WCR eggs were placed in 15 cm × 10 cm oval containers (708 ml, The Glad Products Company, Oakland, CA) and filled approximately 4-cm deep with a moistened growth medium of 2:1 autoclaved soil and ProMix (Premier Horticulture Inc. Quakertown, Pennsylvania, USA). The eggs were incubated in the soil at 25 °C for approx. 2 wk before hatching. Unfed neonate larvae <24 h old were used in the bioassays.

Plant Material

All of the corn used was soaked in a 10% bleach solution for 10 min, rinsed well and allowed to dry completely prior to germination. The corn was then soaked in water at room temperature for 8 h. After soaking, corn kernels were placed onto a saturated paper towel in closed oval containers and placed in a growth chamber at 25 °C to germinate. Oats were treated with a soapy water solution, rinsed well, and placed on a saturated paper towel in oval containers for germination in the growth chamber. Upon germination, all plants were kept moist on clean, saturated filter paper in closed oval containers. Corn seedlings were used in bioassays when they reached

3–4 d old; oats were used at 4–5 d old. All roots used in the assays were approximately 1.5–2 inches in length.

Gene checks were performed on MON88017 and SmartStax roots at the end of the study using QuickStix test strips (EnviroLogix, Portland, ME), and tissue samples were also taken from RNAi and the Vector Stack seedlings and sent to Monsanto for expression studies. All plants used in the assays tested positive for the appropriate genes.

Bioassays

Assays used in this study were modified from those developed by Strnad and Dunn (1990). During the bioassays, a single, clean seedling was placed on moistened filter paper in a petri dish and one neonate larvae was placed on the root (or on the filter paper for the control) using a moistened camel's-hair paintbrush. After exposure to the root for 5 min, the larvae were immediately transferred to the center of a specially designed 12.5 cm arena on lightly moistened filter paper where the larvae's host-searching behaviors were recorded for 5 min using the EthoVision system (version 3.1; Noldus Information Technology, The Netherlands). The bioassay was terminated early if any larvae exited the arena during the 5-min trial period. Each bioassay resulted in one track file in the EthoVision program. A total of 460 unique track files were recorded for this study.

EthoVision Protocol

The EthoVision arena comprised a moist 125-mm filter paper circle mounted on a clean glass plate and replaced between each larval exposure. The arena was enclosed in a clear acrylic box (20 × 20 × 18 cm) mounted under the EthoVision system video camera (Panasonic wv BP334) positioned 0.64 cm above the box with a 15-W fluorescent light located on top for even lighting. For optimum viewing of larvae with the EthoVision system, the tracking settings were set to the following specifications: detection method, subtraction; processing settings, only detect objects that are darker than background; scan window of 50 pixels set to search the complete arena; minimum object size, one pixel; maximum object size, 20 pixels; sample rate, 5.994 samples/s. Recording began after the

larvae were placed in the arena and the door to the arena closed. The recording continued for 5 min unless the larva left the filter paper before time expired. To account for any changes in the settings due to replacing the filter paper between bioassays, the detection variables were updated before the start of each trial.

Parameters measured by the EthoVision system during bioassays included total distance moved (the distance traveled by the center of gravity of the larva), maximum distance from the origin (the farthest distance traveled by the center of gravity of the larva from the point of origin), mean velocity (mm/s), mean turn angle (the change in direction of movement between two samples), and mean meander (the change in direction of movement of an object relative to the distance it moves). To mitigate image noise and larval body wobbles being recorded as true movement, the following filters and settings were used when calculating the above parameters: total distance moved, downsize filter (1/25) and minimum distance moved (0.2 cm); maximum distance from origin, downsize filter (1/25); mean velocity, downsize filter (1/25); mean turn angle, absolute setting, and downsize filter (1/25); mean meander, absolute setting, and downsize filter (1/25). Limited larval movement coupled with the above filters sometimes resulted in no value being calculated for a specific parameter. For trials that did not last the full 5 min as a result of larvae leaving the arena during their search, total distance traveled was adjusted to reflect the distance the larvae would have traveled during the five minute period using their average velocity as calculated by the EthoVision software.

Statistical Analysis

An analysis of variance was used for these data analyses and was calculated by using the PROC MIXED of the SAS statistical package (SAS Institute 2008; Cary, North Carolina, USA). For the mean meander, total distance moved, mean turn angle, maximum distance from origin and the velocity, the linear statistical model contained the main plot effect of treatment. Data were transformed by square root ($x + 0.5$) to meet the assumptions of the analysis. Both of the experiments were run as a randomized complete block.

Table 1. Effect of treatment on each parameter measured of the movement of the western corn rootworm during bioassays from experiment one (using susceptible insects) and experiment two (using both susceptible and resistant insects)

Assay set	Analysis		df	<i>f</i>	<i>P</i>
One	Distance moved	Medium	4, 149	9.80	<0.0001
	Mean velocity	Medium	8, 149	19.16	<0.0001
	Mean turn angle	Medium	8, 147	41.56	<0.0001
	Mean meander	Medium	8, 147	36.91	<0.0001
	Maximum distance from origin	Medium	8, 147	22.29	<0.0001
Two	Distance moved	Medium	6,226	56.29	<0.0001
		Colony	1,226	0.37	0.5411
		Medium × colony	6,226	1.71	0.1195
	Mean velocity	Medium	6,233	61.71	<0.0001
		Colony	1,233	0.07	0.7961
		Medium × colony	6,233	1.99	0.0683
	Mean turn angle	Medium	6,231	277.34	<0.0001
		Colony	1,231	1.02	0.3134
		Medium × colony	6,231	4.2	0.0005
	Mean meander	Medium	6,235	100.85	<0.0001
		Colony	1,235	0.03	0.8732
		Medium × colony	6,235	2.02	0.0643
	Maximum distance from origin	Medium	6,227	104.4	<0.0001
		Colony	1,227	3.84	0.0513
		Medium × colony	6,227	3.47	0.0027

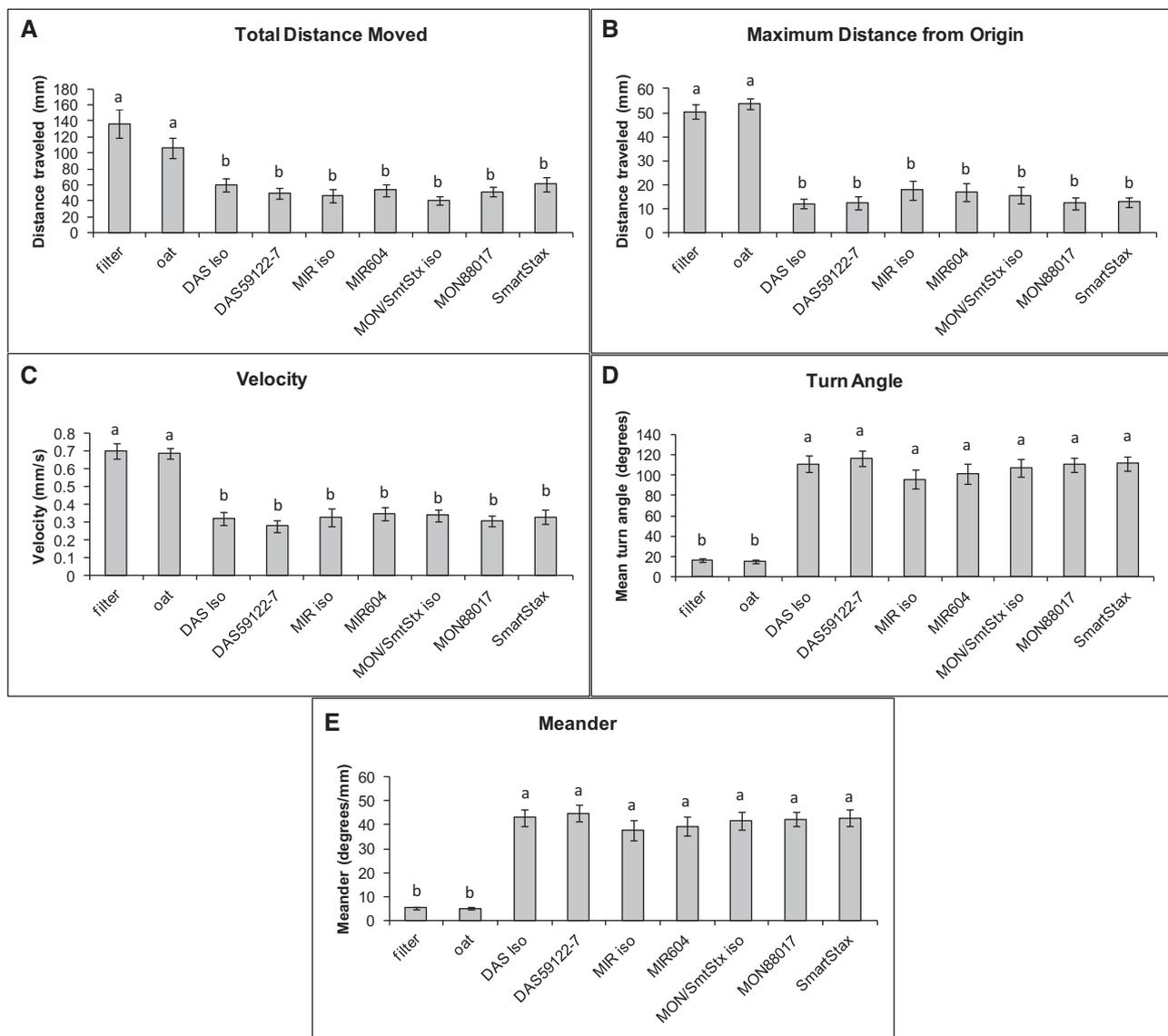


Fig. 1. The total distance moved (A), maximum distance from origin (B), velocity (C), turn angle (D), and meander (E) of the western corn rootworm larvae in 5 min after exposure to a different plant seedlings or filter paper for experiment one. Letters indicate significant differences between corn types ($P \leq 0.05$). Analysis was done with square root transformed data; figures represent untransformed data.

Results

For all parameters that were measured in the first set of bioassays using only susceptible colonies, the two negative controls (moist filter paper and germinated oat seedlings) were significantly different than all corn treatments (Table 1; Fig. 1). The larvae that were exposed to the controls had significantly longer paths and traveled farther from the distance from the origin than the larvae exposed to corn plants including the Bt plants (Fig. 1a and b). The larvae exposed to the negative controls traveled significantly faster, turned less, and crossed their paths less than the larvae exposed to the corn plants (Fig. 1c-e).

In the second set of assays using both susceptible and resistant colonies, the two negative controls were also significantly different from all of the corn treatments for all parameters measured (Table 1; Fig. 2). After exposure to the RNAi roots, susceptible larval behavior was significantly different than behavior for resistant larvae for all parameters measured (Fig. 2). The susceptible larvae had a significantly longer path on the RNAi corn than the resistant larvae (Fig. 2a). Also, the susceptible larvae moved farther from the

origin, moved faster, turned less often and crossed their own path less than the resistant larvae after RNAi corn exposure (Fig. 2b-e). The resistant larvae moved farther from the origin after exposure to the isolate than the susceptible colonies (Fig. 2b). Despite differences in every parameter measured between the two colonies on the RNAi, there were no differences between the susceptible and the resistant colonies for the vector stack exposure treatment (Fig. 2). The magnitude of the difference between the resistant and the susceptible colony on RNAi was small compared to the susceptible colony on the controls (Fig. 2).

Discussion

There were no dramatic differences between the localized search responses of WCR larvae exposed to any of the corn lines tested; however, the rootworm larvae consistently demonstrated a ranging behavior after contact with the filter paper and oats, indicating that they did not recognize the controls as hosts. This was expected since

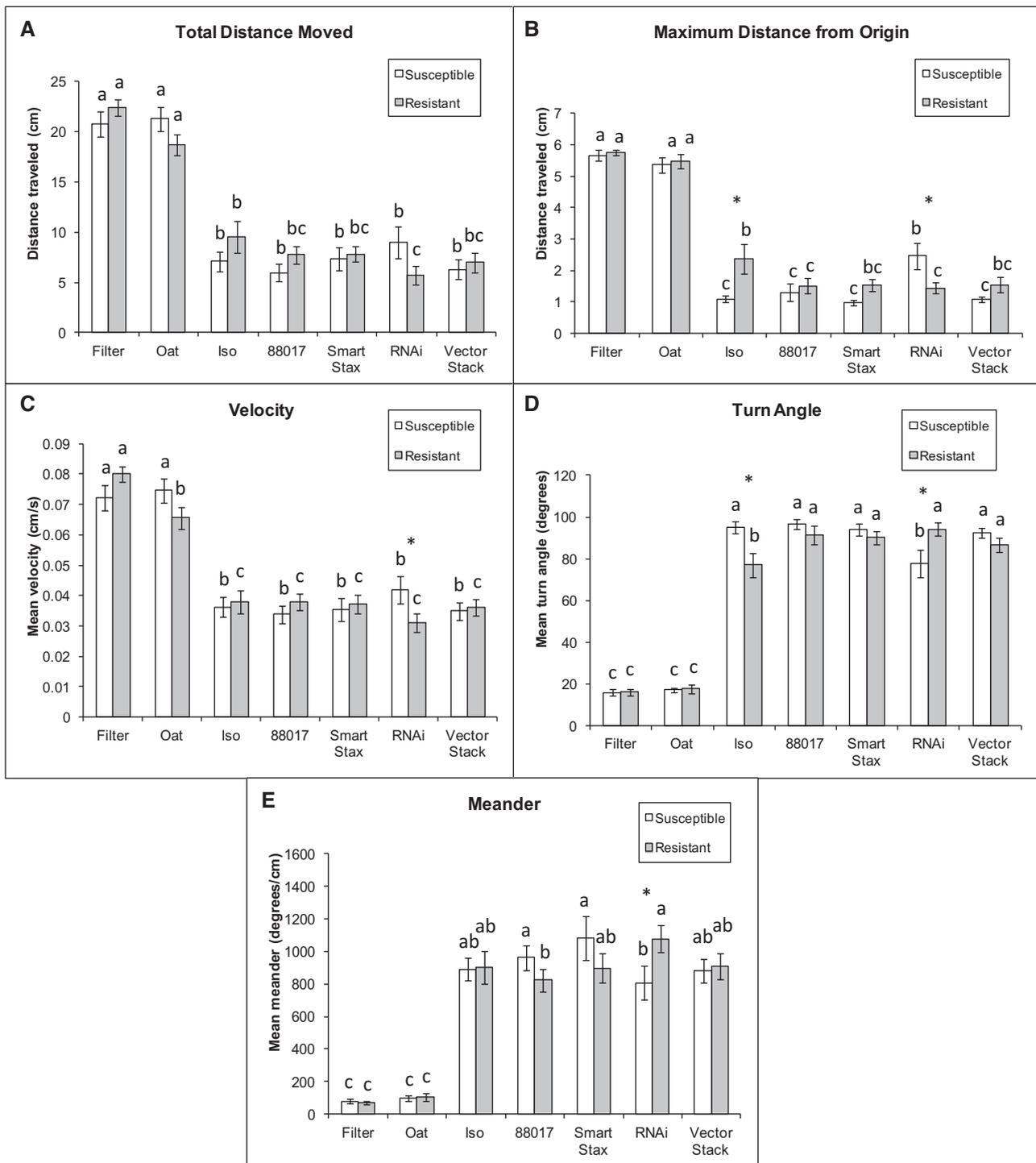


Fig. 2. The total distance moved (A), maximum distance from origin (B), velocity (C), turn angle (D), and meander (E) of the western corn rootworm larvae in 5 min after exposure to different plant seedlings or filter paper for experiment B. Letters indicate significant differences between corn types within colony ($P \leq 0.05$). *Significant differences between resistant and susceptible colonies with seed type ($P \leq 0.05$). Analysis was done with square root transformed data; figures represent untransformed data.

larvae exposed to germinated oat roots had showed a ranging behavior in previous studies (Strnad and Dunn 1990) and oats may contain a larval feeding deterrent (Branson and Ortman 1969).

Binning et al. (2005) conducted assays that were somewhat similar to the current experiment, except that in their experiment they exposed the insects to artificial diet (modified after Pleau et al. 2002) with and without Cry34/35Ab1 proteins. They concluded

that Cry34/35Ab1 was perceived as a poor host for WCR larvae. However, the factors responsible for host recognition require specific extraction techniques if they are to be separated from corn (Bernklau et al. 2009), and these factors are likely not present in artificial diet. In addition, Cry proteins are tied up in plant cells under normal circumstances and not directly available to searching larvae as was done by Binning et al.(2005).

Contact cues associated with the roots are the driving factor of host recognition (Branson and Ortman 1969, Strnad and Dunn 1990), and this study demonstrates that each corn type, Bt, RNAi, the vector stack, and isoline, contains sufficient contact cues to elicit a localized search response by both Cry3Bb1 resistant and susceptible larvae when the larvae are removed from the germinated corn roots. The toxins present in the Bt/RNAi roots did not significantly affect the host response despite what may have happened in other Bt assays such as in the study by Clark et al. (2006). Phenological differences in the corn may play a role in this discrepancy since corn seedlings of a slightly younger age were used in this study.

Overall the data between the two sets of assays were similar with the primary difference being the addition of a paired resistant colony. Although the susceptible colony had significantly less host recognition patterns than the resistant colony on RNAi, the susceptible colony never reached the level of ranging behaviors that was demonstrated by larvae exposed to the controls. The same is true for the resistant colony on the isoline corn roots compared to the susceptible. The differences observed in the response levels of resistant and susceptible neonates to RNAi but not RNAi + Bt suggest that the Bt provides more of a contact cue when coupled with RNAi than when RNAi is used alone. Alternatively, the RNAi may interfere with the contact cues needed to produce a stronger recognition response by the larvae when used alone.

Contact cues in the isoline root as perceived by the resistant larvae may not be as strongly perceived as the in the susceptible larvae or perhaps there is a slight difference in contact cue chemical composition. In the current set of assays, although differences were detected between colonies, overall all corn lines were recognized as suitable hosts. In RIB fields, the WCR would be attracted to these hybrids, presumably feed, then be exposed to the Bt toxins or RNA in the roots the same as the isoline plants. Neonates did not perceive isoline as a nonhost over Bt or RNAi hybrids therefore equal larval pressure could be expected on Bt, RNAi, and the isoline plants in a refuge in a bag field, which is positive for resistance management strategies as it helps hinder resistance development.

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