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Abstract

Non-target effects of Cry1Ab x CP4 EPSPS and Cry1Ab+Cry3Bb1 x CP4 EPSPS Bt transgenic new maize hybrids on insidious flower bugs [*Orius insidiosus* (Say)] was studied in Nebraska (Mead, Clay Center, and Concord) during 2007 and 2008. The Bt effect was compared to CP4 EPSPS maize (isoline), conventional maize, and insecticide applications of permethrin (Pounce® 1.5G) and bifenthrin (Capture® 2EC) to control first and second generations of *Ostrinia nubilalis* (Hübner), respectively. Yellow sticky cards, visual observations, and destructive samplings were used to evaluate *O. insidiosus* abundance. The yellow sticky card data in 2007 showed that *O. insidiosus* abundance was lower on Pounce® 1.5G treated non-Bt isoline maize plots compared to the BT transgenic hybrids at 60 and 90 days after planting (DAP). From visual observations, numbers of *O. insidiosus* were lower in Pounce® 1.5G treated plots and no adverse effects of the Bt hybrids was observed. In 2008, no significant differences were found among treatments in the sticky card data, but the *O. insidiosus* population significantly increased, with increasing DAP, where the lowest and highest numbers were recorded at 30 and 120 DAP, respectively. In the visual observation and destructive samplings, numbers of *O. insidiosus* were lower at Concord compared to other sites. Results from the visual observation data in 2008 also revealed that *O. insidiosus* abundance was lower on Pounce® 1.5G treated plots compared to other treatments. This study showed no adverse effects of the new BT transgenic hybrids that included stacked resistance genes on *O. insidiosus* compared to the non-Bt maize hybrids.

Keywords: *Orius insidiosus*; Non-target effects; BT transgenic maize

Introduction

Insect resistance based on *Bacillus thuringiensis* (Bt) (Berliner) endotoxins is the most widely used trait following herbicide tolerance in commercial transgenic crops [1]. The deployment of transgenic plants resistant to insects offered expectations as a means of pest control that led to a reduction in pesticide use in intensive cropping systems.

Although the increased global adoption of transgenic crops [2] shows usefulness for many growers and their acceptance in many markets, the imposition of moratoria in several countries reflects skepticism and public concern about a range of issues around transgenics including potential impacts on the environment. Potential adverse effects of transgenics on the environment include effects on non-target species, invasiveness, release or "escape" into the environment, and development of resistance to transgenic products [3]. To address these concerns, governments have authorized regulatory bodies like the U.S. Environmental Protection Agency to regulate the deployment of transgenics requiring environmental risk assessment data as part of the registration process [4].

Orius insidiosus (insidious flower bugs) (Hemiptera: Anthocoridae) are generalist predators which are frequently reported in ecological studies as important non-target organisms in transgenic maize [5-8]. In the Midwest, including Nebraska, *O. insidiosus* is a common predator in maize (Wright 2004) and soybean fields [9].

Orius spp. are important natural enemies of pest insects and mites in many cropping systems such as maize, soybeans, vegetables, and

fruit crops [10,11]. Nearly all *Orius* spp. are predaceous as nymphs and adults. The primary food of *Orius* spp. consists of small insects and insect eggs, plant pollen, and plant sap [12]. Nymphs and adults of *O. insidiosus* are commonly found on maize silks and serve as natural enemies of key maize pests such as of *Ostrinia nubilalis* (Hübner), *Helicoverpa zea* (Hübner) [13], *Spodoptera frugiperda* (J.E. Smith) [14], *Rhopalosiphum maidis* (Fitch) [9,15,16], *Frankliniella* spp. [12,17,18], spider mites, white flies (*Bemisia* spp.), and eggs of other insects in the field. *O. insidiosus* are commercially mass produced and sold as biocontrol agents against pests of glasshouse-grown vegetables and ornamental crops [19].

The potential non-target impact of transgenic maize was studied using *O. insidiosus* as a key non-target arthropod [5,8,20]. Effective and reliable sampling of *O. insidiosus* nymphs and adults is important

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in assessing the impact of transgenic corn on non-target organisms, particularly for environmental risk assessments. Previous ecological studies have assessed the non-target effects of transgenic maize by using visual observations, pitfall traps, sticky cards, sweep nets, and beat buckets [7,8,21,22].

Non-destructive (visual observations), yellow sticky card s, and destructive sampling techniques have been used to monitor *O. insidiosus* nymphs and adults together with above ground arthropods pests for the non-target impact of transgenic plants [5,6,8,22,23]. These techniques were used to approach the objective of this study, to evaluate the non-target effects of different Bt transgenic maize hybrids on *O. insidiosus* abundance compared to insecticide applications and non-transgenic maize.

Materials and Methods

Experimental sites and description

The experiments were conducted during 2007 and 2008 at three geographically different experimental research stations of University of Nebraska-Lincoln. The experimental fields were located at the Agricultural Research and Development Center, Mead, (N41°1.07' WO96°27.263' in 2007 and N41°11.09'WO96°27.411' in 2008), South Central Agricultural Laboratory, Clay Center, (N40 °34.216' WO98 °07.958' in 2007 and N40 °34.272' WO98° 07.822' in 2008), and the Northeast Research and Extension Center, Haskell Agricultural Laboratory, Concord, (N42°23.037' WO96°57.193' in 2007 and N42°23.149' WO96°57.331' in 2008) . Soil types were Sharpsburg silty clay loam, Kennebec silty clay loam, and Butler/Crete silt loam, respectively. The experimental fields at all locations were previously planted with soybeans in a no tillage system.

Agronomic practices

Plantings were done in a no-till corn system on 10, 11 and 15 May in 2007, and during 19, 20 and 21 May in 2008 at Mead, Clay Center, and Concord, respectively. Fertilizer management, irrigation, and herbicide application were made based on the normal agronomic recommendations of each specific site.

Experimental design and treatments

A randomized complete block design with four replications was used. The treatments were: a) a Cry1Ab X CP4 EPSPS maize, b) CP4 EPSPS maize (isoline), c) CP4 EPSPS maize (isoline) plus an insecticide application to control the first generation of *O. nubilalis*, d) Cry1Ab+Cry3Bb1X CP4 EPSPS maize, e) CP4 EPSPS maize (isoline) plus an insecticide application to control second generation of *O. nubilalis*, and f) a conventional maize without insecticide application. The Cry1Ab Bt transgenic maize is used to control lepidopteran pests while Cry3Bb1 is used against corn root worms (*Diabrotica spp.*). The CP4 EPSPS is a genetically engineered glyphosate tolerant maize variety which allows the use of glyphosate as a postemergence herbicide.

In the case of CP4 EPSPS maize plus an insecticide application to control the first generation of *O. nubilalis* both in 2007 and 2008, permethrin (Pounce® 1.5G) (FMC Corporation, PA) was applied at the recommended rate of 12 oz. /1000 row ft band using an improvised jar shaker applicator at whorl maize stage (V9-V12 growth stages). Bifenthrin (Capture® 2 EC) (Bayer, NJ) was sprayed at the rate of 6.66 ml/ 2 gallons of water using a carbon-gated sprayer for the control of second generation *O. nubilalis*. Individual plots were 60 square meters. There were 8 rows in each plot with ~400 plants per plot (~50 plants per

row). A 3 m spacing between treatments and blocks was planted with conventional corn hybrid.

Sampling methods

O. insidiosus nymphs and adults were monitored using visual observations, and adults with yellow sticky cards, in 2007 and 2008. A destructive sampling technique was added in 2008 to validate the actual nymph and adult counts. Visual observations were made on 20 randomly selected plants from rows 2 and 3 in each plot at reproductive stages, R1 (silking) and R2 (blister) i.e. 80 and 90 DAP, respectively. Nymphs and adults of *O. insidiosus* were observed on maize ears, and silks were tapped and *O. insidiosus* falling from the silk were collected with a clean sheet of bond paper underneath to quantify the number of nymphs and adults. The mean nymph plus adult counts per plant were used for the analysis.

Two yellow sticky cards (23 x 28 cm) per plot (sticky on one side only) (Pherocon® AM, Trécé Inc., Adair, OK) [7,8] were used. The traps were attached to wooden stakes (2.5 x 2.1 x 244 cm) that were placed between rows 5 and 6, 6 and 7 of each plot at the seedling stage (V3). The yellow stick cards were attached on the wooden stakes at 30, 60, 90, and 120 DAP. The cards were folded and clipped with 2 binder clips around the wooden stake facing the maize rows at the canopy level during the vegetative stage and parallel to the ears in the reproductive stages. After 7 days, the yellow sticky cards were collected, sealed in ziplock plastic bags, and brought to the laboratory for quantification. *O. insidiosus* adults were counted with the aid of a dissecting microscope. The adult counts from the 2 yellow sticky cards were pooled, and mean adult counts per card per day were used for the analysis.

Destructive sampling was done on five randomly selected maize ears from row 4 of each plot at R2. The randomly sampled maize ears were cut from the plant using a knife and kept in a ziplock plastic bag separately and brought to the laboratory for counting. Adults and nymphs of *O. insidiosus* were counted using a dissecting microscope. Mean number of nymphs and adults of the five ears per plot were pooled for the analysis. Voucher specimens of *O. insidiosus* were kept at University of Nebraska-Lincoln, Department of Entomology.

Data analysis

Analysis of variance (ANOVA) was performed using SAS's PROC GLM procedure (SAS, 2003) [24]. The level of significance was set at $P=0.05$. Whenever there was significant interaction among factors (treatment, sampling period, location, season), each factor was analyzed with respect to the levels of the other factor. In the absence of significant interaction, data were pooled. The treatment x site effects generally revealed no significant differences, and these were not presented in the results and discussion. For parameters that showed significant difference among treatments, individual means were separated using the Student's Newman Keuls test (SNK).

Results

There was a significant interaction in 2007 between treatments and sampling period for the yellow sticky card data ($F=2.29$, $P=0.0050$, $df=15,216$), so treatments were compared at a specific sampling period. Abundance of *O. insidiosus* also significantly varied among locations ($F=16.72$, $P<0.0001$, $df=2,216$). Significant differences among treatments were observed at 60 DAP ($F=3.48$, $P=0.0076$, $df=5, 64$) and 90 DAP ($F=4.26$, $P=0.0117$, $df=5, 64$); numbers of *O. insidiosus* were significantly lower on Pounce® 1.5G treated CP4 EPSPS maize (isoline) compared to the rest of the treatments including the transgenic hybrids

Treatment	Sampling periods (days after planting)			
	30 days	60 days	90 days	120 days
Cry1Ab	0.05 ± 0.02	0.88 ± 0.15a	0.81 ± 0.11a	1.14 ± 0.09
CP4 EPSPS maize	0.018 ± 0.01	0.77 ± 0.15ab	0.84 ± 0.12a	1.06 ± 0.16
Pounce® 1.5G	0.05 ± 0.02	0.46 ± 0.09b	0.45 ± 0.07b	1.57 ± 0.18
Cry1Ab x Cry3Bb1	0.018 ± 0.01	0.75 ± 0.13ab	0.77 ± 0.09a	1.28 ± 0.27
Capture® 2 EC	0.036 ± 0.01	0.93 ± 0.19a	0.82 ± 0.12a	1.17 ± 0.18
Conventional corn	0.01 ± 0.01	0.65 ± 0.12ab	0.85 ± 0.08a	1.19 ± 0.16

Means within a column followed by the same letter are not statistically different from each other (SNK, P = 0.05). Ns = not significant. Pounce® 1.5G and Capture® 2 EC was applied on CP4 EPSPS maize

Table 1: Mean number of *O. insidiosus* (± S E) in BT transgenic maize hybrids and non transgenic insecticide treated and non-treated hybrids during the 2007 cropping season.

Experimental Site	Season				
	2007		2008		
	Sticky card	Visual observation	Sticky card	Visual observation	Destructive sampling
ClayCenter	0.84 ± 0.07a	0.25 ± 0.03c	0.66 ± 0.08b	1.74 ± 0.73a	2.93 ± 0.20a
Concord	0.70 ± 0.05b	0.77 ± 0.05a	0.83 ± 0.13a	0.37 ± 0.03b	0.66 ± 0.11c
Mead	0.53 ± 0.05c	0.58 ± 0.04b	0.54 ± 0.06b	1.63 ± 0.16a	1.81 ± 0.13b

Means within a column followed by the different letter are significantly different from each other (SNK, P = 0.05)

Table 2: Mean number of *O. insidiosus* (± S E) in BT transgenic maize hybrids and non transgenic insecticide treated and non-treated hybrids at C lay Center, Concord, and Mead in Nebraska during the 2007 and 2008 cropping seasons.

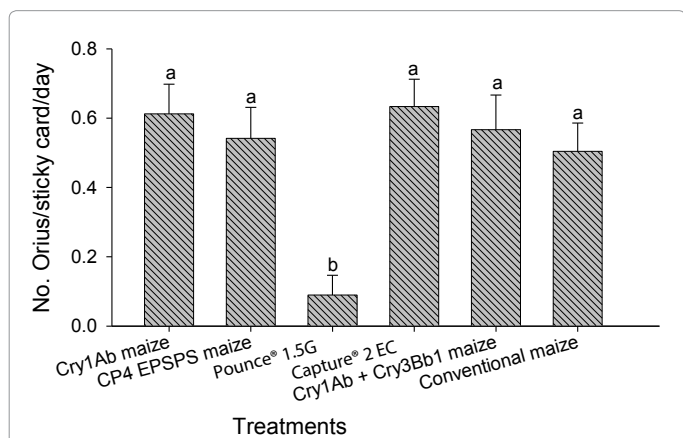


Figure 1: Mean number of *O. insidiosus* (± SE) in BT transgenic maize hybrids and non-transgenic insecticide treated and non-treated hybrids using a visual observation sampling technique during the 2007 cropping season in Nebraska. Bars followed by different letter are significantly different from each other (SNK, P=0.05). Pounce® 1.5G and Capture® 2 EC was applied on CP4 EPSPS maize.

Cry1Ab, and Cry1Ab+Cry3Bb1. Similarly at 90 DAP, lower *O. insidiosus* numbers were recorded from Pounce® 1.5G treated plots compared to other treatments; there were no significant differences in abundance of *O. insidiosus* among the rest of the treatments (Table 1). Although there were significant differences among locations, and sampling techniques in terms of *O. insidiosus* abundance, the results were not consistent in the different sampling techniques. In the season 2007, the sticky card sampling recorded higher numbers of *O. insidiosus* from Clay Center, followed by Concord, and Mead, respectively (Table 2). However, in the visual observation sampling technique, we found higher numbers of *O. insidiosus* at Concord followed by Mead and Clay Center, respectively (Table 2). The visual observation data in 2007 also showed significant

differences among treatments ($F=54.4, P<0.0001, df=5, 54$). Similar to the sticky card data, *O. insidiosus* populations were significantly lower on Pounce® 1.5G treated plots than the rest of the treatments (Figure 1). Moreover, data not shown adverse effect of the Bt transgenic hybrids compared to the isolate and conventional counterparts (Figure 1).

During the 2008 cropping season, the sticky card data showed no significant differences among treatments ($F=2.13, P=0.0624, df=5,274$). However, there was significant differences among sampling periods ($F=255.56, P<0.0001, df=3,216$) and locations ($F=9.17, P=0.0001, df=2,216$). *O. insidiosus* abundance significantly increased with DAP and the highest (1.95 *O. insidiosus* per sticky card per day) was recorded at 120 DAP and no *O. insidiosus* recorded at 30 DAP (Figure 2). When we compared the experimental sites sampled by sticky card, *O. insidiosus* populations were higher at Concord and there was no significant difference between Clay Center and Mead (Table 2). However, in the visual observation and destructive sampling techniques, lower numbers of *O. insidiosus* were recorded from Concord compared to the other sites.

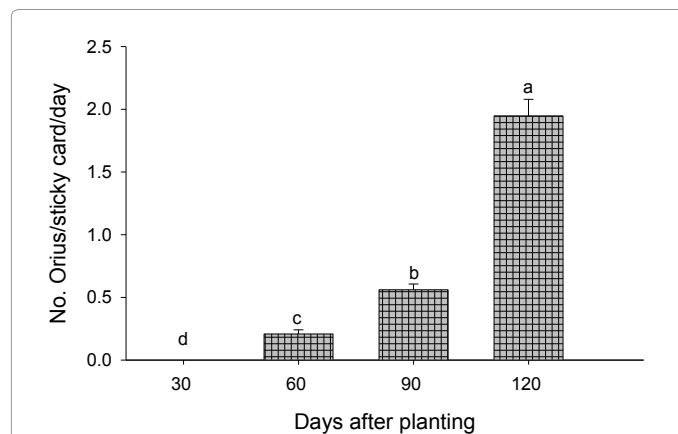


Figure 2: Mean number of *O. insidiosus* (± SE) in BT transgenic maize hybrids and non-transgenic insecticide treated and non-treated hybrids at different sampling periods using a yellow sticky card trapping method during the 2008 cropping season in Nebraska. Bars followed by different letter are significantly different from each other (SNK, P=0.05).

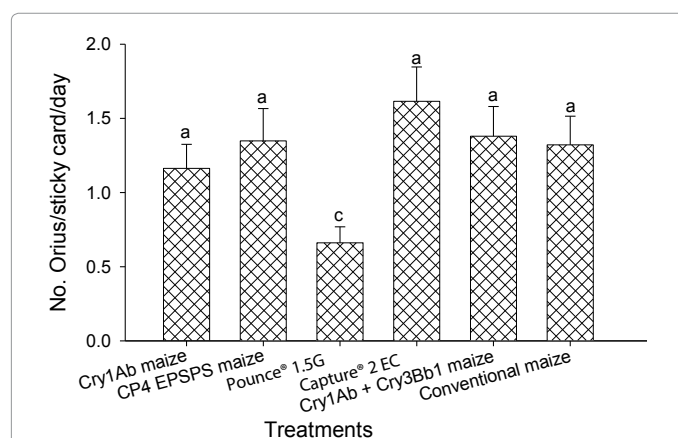


Figure 3: Abundance of *O. insidiosus* (mean ± SE) in BT transgenic maize hybrids and non-transgenic insecticide treated and non-treated hybrids during the 2008 cropping season in Nebraska using a visual observation sampling technique. Bars followed by the same letter are not statistically different from each other (SNK, P=0.05). Pounce® 1.5G and Capture® 2 EC was applied on CP4 EPSPS maize.

In the visual observations in 2008, there was a significant three-way interaction among sampling periods, locations (sites), and treatments ($F=18.23, P<0.0001, df=12,108$). Therefore, treatments were compared for each location separately at a specific sampling period. At Clay Center, significantly lower numbers of *O. insidiosus* were recorded from Pounce® 1.5G treated transgenic isolate maize hybrid (glyphosate resistant) both at 80 and 90 DAP compared to the other treatments (Table 3). Similarly, at Concord, abundance of *O. insidiosus* was significantly higher in Cry1Ab and Cry1Ab X Cry1Bb hybrid compared to the non-transgenic isolate treated with Pounce® 1.5G to control first generation of *O. nubilalis*. Moreover, at 90 DAP; a higher number of *O. insidiosus* was recorded from Capture® 2 EC treated plots compared to Pounce® 1.5G treated plots (Table 3). At Mead, *O. insidiosus* abundance was also significantly lower in Pounce® 1.5G treated plots than the other treatments at 80 DAP, and there were no significant differences among treatments at 90 DAP ($F=0.87, P=0.5263, df=5,15$). The overall treatment effect in the visual observations of 2008 season indicated that significantly lower numbers of *O. insidiosus* were recorded from Pounce® 1.5 G treated isolate than other treatments including the Bt transgenic hybrids (Figure 3). Moreover, *O. insidiosus* abundance showed a similar trend in the destructive sampling where *Orius* counts were significantly lower in Pounce® 1.5G treated plots than the Bt transgenic hybrids, the non-transgenic isolate, conventional maize, and Capture® 2 EC sprayed conventional maize (Figure 4).

Discussion

Visual observations, yellow sticky cards and destructive sampling techniques revealed the same trend of significantly fewer mean adult

counts of *O. insidiosus* on CP4 EPSPS maize plus Pounce® 1.5G for the control of first generation *O. nubilalis* at R2 (blister) stage. Neither Bt transgenic maize hybrids had observable effects on populations of *O. insidiosus* in all sampling techniques used in the study. *O. insidiosus* nymphs and adults were fewer on insecticide treated CP4 EPSPS maize. These findings support previous ecological studies on non-target predators that transgenic maize does not have a significant negative effect on the predator *O. insidiosus*, but our results differ with those previously reported because we obtained significant differences in the sampling techniques [7,8,20,22,25,26].

The results of our study suggested that visual observation, yellow sticky cards, and destructive sampling are effective in monitoring abundance of *O. insidiosus* in non-target studies. These results corroborate other ecological field studies on non-target arthropods of transgenic maize. Al-deeb et al. [5] used visual counts of *O. insidiosus* in Bt and non-Bt maize fields at three locations in Kansas to show that Bt maize does not have significant effects on *O. insidiosus*. Musser et al. [7] also recommended the use of field counts of immature and adults, because these counts are accurate, have no associated supply costs, and can be made quickly. In a similar study using yellow sticky cards, Pilcher et al. [8] showed that significantly higher numbers of adult *O. insidiosus* preferred the early planting date of Bt hybrids during the first *O. nubilalis* generation. The variation in *O. insidiosus* population abundance among the three sites may be due to slight variation in biotic and abiotic factors [22]. Moreover, development of *O. insidiosus* is very dependent on temperature [12], and availability of food supply [19,27].

In conclusion, our findings support non-target arthropod ecological

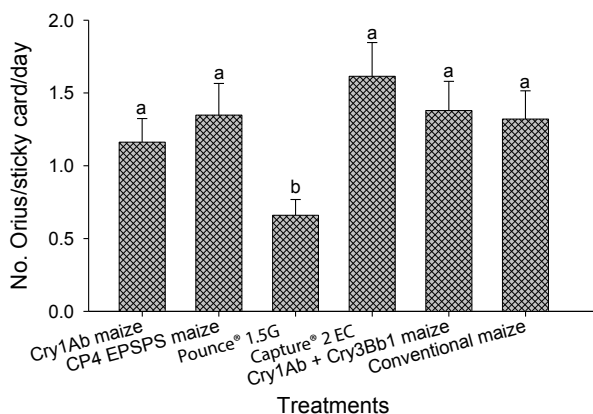


Figure 4: Abundance of *O. insidiosus* (mean ± SE) in BT transgenic maize hybrids and non-transgenic insecticide treated and non-treated hybrids during the 2008 cropping season in Nebraska using a destructive sampling technique. Bars followed by the same letter are not statistically different from each other (SNK, P=0.05). Pounce® 1.5G and Capture® 2 EC was applied on CP4 EPSPS maize.

Treatments	Clay Center		Concord		Mead	
	80 days	90 days	80 days	90 days	80 days	90 days
Cry1Ab	2.08 ± 0.19a	1.09 ± 0.16b	0.34 ± 0.03a	0.53 ± 0.13ab	2.18 ± 0.27a	0.78 ± 0.13
CP4 EPSPS Maize	2.00 ± 0.31a	1.88 ± 0.04a	0.19 ± 0.05ab	0.44 ± 0.14ab	2.85 ± 0.44a	0.75 ± 0.19
Pounce® 1.5G	0.85 ± 0.22b	1.00 ± 0.12b	0.09 ± 0.024b	0.23 ± 0.06b	1.29 ± 0.27c	0.51 ± 0.19
Capture® 2 EC	2.54 ± 0.36a	1.88 ± 0.08a	0.30 ± 0.07ab	0.74 ± 0.11a	3.33 ± 0.09a	0.91 ± 0.17
Cry1Ab x Cry3Bb1	2.30 ± 0.15a	1.71 ± 0.04a	0.34 ± 0.07a	0.49 ± 0.12ab	2.70 ± 0.38a	0.74 ± 0.04
Conventional Maize	1.94 ± 0.17a	1.63 ± 0.19a	0.29 ± 0.08ab	0.51 ± 0.04ab	2.84 ± 0.19a	0.73 ± 0.07

Means within a column followed by the same letter are not statistically different from each other (SNK, P = 0.05). Ns = not significant. Pounce® 1.5G and Capture® 2 EC was applied on CP4 EPSPS maize.

Table 3: Abundance of *O. insidiosus* (mean ± S E) in BT transgenic maize hybrids and non transgenic insecticide treated and non-treated hybrid s at C lay Center, Concord, and Mead in Nebraska during the 2008 cropping season using a visual observation sampling technique.

field studies that Cry1Ab, and Cry1Ab+Cry3Bb1 maize have no impact on *O. insidiosus* populations. However, the pyrethroid insecticide (Pounce® 1.5G) applications to control target pests significantly affected non-target natural enemies of the target pests.

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