Entomology/ Scientific Notes

Molecular identification for *Helicoverpa* spp. in Bt and non-Bt crops in central Argentina

Abstract – The objective of this work was to identify, by molecular analysis, the species of the genus *Helicoverpa* in corn, soybean, and lucerne Bt and non-Bt crops, in central Argentina. A total of 1,343 carterpillars were analyzed by PCR-RFLP. *Helicoverpa zea* was the only species detected on non-Bt and on most Bt corn hybrids. The species *H. gelotopoeon* predominated on non-Bt soybean and on lucerne, and *H. zea* larvae occurred in low proportion. Bt soybean did not show *Helicoverpa* spp. infestations. The exotic *H. armigera* was not found, though adult moths of this species were light-trapped in a complementary sampling.

Index terms: *Helicoverpa zea*, *Helicoverpa gelotopoeon*, *Helicoverpa armigera*, PCR-RFLP, Bt crops, *Helicoverpa* complex.

Identificação molecular de *Helicoverpa* spp. em culturas Bt e não Bt no centro da Argentina

Resumo – O objetivo deste trabalho foi identificar, por análise molecular, as espécies do gênero *Helicoverpa* nas lavouras − Bt e não Bt − de milho, soja e alfafa, no centro da Argentina. Um total de 1.343 lagartas foram analisadas por PCR-RFLP. A espécie *H. zea* foi a única detectada no milho não Bt e na maioria do Bt. A espécie *H. gelotopoeon* predominou na soja não Bt e na alfafa, mas *H. zea* ocorreu em baixa proporção. A soja Bt não apresentou infestações por *Helicoverpa* spp. A exótica *H. armigera* não foi encontrada, porém, mariposas adultas dessa espécie foram capturadas com armadilha luminosa em uma amostragem complementar.

Termos para indexação: *Helicoverpa zea*, *Helicoverpa gelotopoeon*, *Helicoverpa armigera*, PCR-RFLP, culturas Bt, complexo *Helicoverpa*.

Argentina ranks among the world's top exporters of soybean and corn, and it is also one of the largest lucerne producers. These crops are all affected by species of *Helicoverpa* (Lepidoptera: Noctuidae: Heliothinae). Two native bollworms, the polyphagous *H. zea* and *H. gelotopoeon*, are particularly frequent in damaged crops in the region. They have been traditionally controlled using chemical insecticides and, when available, transgenic cultivars expressing *Bacillus thuringiensis* (Bt) toxins. In recent years, the invasive "Old World bollworm", *H. armigera*, has entered the South American continent. It was first reported in Brazil in 2013, but its introduction in that country is believed to have occurred in 2008 or earlier (Sosa-Gómez et al., 2016). In Argentina, the presence of *H. armigera* has been recorded since 2013 (Murúa et al., 2016). The correct identification

Emilia Inés Balbi(1 ✉) (iD),
Fernando Miguel Flores(1) (iD),
Daniela Sandra Tosto(2) (iD) and
Joel Demián Arneodo(3) (iD)

(1) Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Marcos Juárez, Ruta 12, Km 3, CP 2580 Marcos Juárez, Argentina.
E-mail: balbi.emilia@inta.gob.ar, flores.fernando@inta.gob.ar

(2) Instituto Nacional de Tecnología Agropecuaria, Instituto de Agrobiotecnología y Biología Molecular (IABIMO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Nicolás Repetto y de los Reseros, CP 1686 Hurlingham, Argentina.
E-mail: tosto.daniela@inta.gob.ar

(3) Instituto Nacional de Tecnología Agropecuaria, Instituto de Microbiología y Zoología Agrícola, Nicolás Repetto y de los Reseros, CP 1686 Hurlingham, Argentina.
E-mail: arneodo.joel@inta.gob.ar

✉ Corresponding author

of *Helicoverpa* spp. is problematic because of their similar morphology and overlapping host range. Adult moths can be distinguished based on their genitalia; however, larvae are practically identical. Therefore, a PCR-RFLP method has been developed to identify the main *Helicoverpa* spp. in the country (Arneodo et al., 2015).

Routine monitoring and management of *Helicoverpa* caterpillars in Argentina usually do not imply species discrimination, which is costly and time-consuming. Thus, the identity of *Helicoverpa* spp. (including the exotic *H. armigera*) feeding on the different crops remains poorly documented.

The objective of this work was to identify, by molecular analysis, the species of the genus *Helicoverpa* in corn, soybean, and lucerne Bt and no Bt crops, in central Argentina.

The research was carried out in the agricultural core region of central temperate Argentina, where most (> 70%) of the country's soybean, corn, and lucerne are produced. Sampling areas included agricultural and mixed production systems (agriculture/livestock or agriculture/dairy farms) in five counties: Rafaela, Manfredi, Marcos Juárez, Laboulaye, and Pergamino (Figure 1). Plots of different Bt and non-Bt corn hybrids, Bt and non-Bt soybean, and non-Bt lucerne were sampled in each county, during February 2016 (Table 1), when *Helicoverpa* larval infestations became noticeable. Corn and lucerne plots were visited at the flowering stage; soybean plots were visited at the vegetative and flowering stages. In each plot, two transects were randomly sampled. If available, 50 *Helicoverpa* spp. larvae were collected and conserved at -20°C until processing. A total of 1,450 larvae were sampled in this survey. DNA was individually extracted from the last abdominal segment of worms using CTAB. Partial sequences of cytochrome oxydase I (*COI*) gene were amplified by PCR with primers H3Fw (5'-CGAGCAGAATTAGGTAAYCC-3') and H3Rv (5'-GCTGATGTRAAATAAGCTCGAG-3').
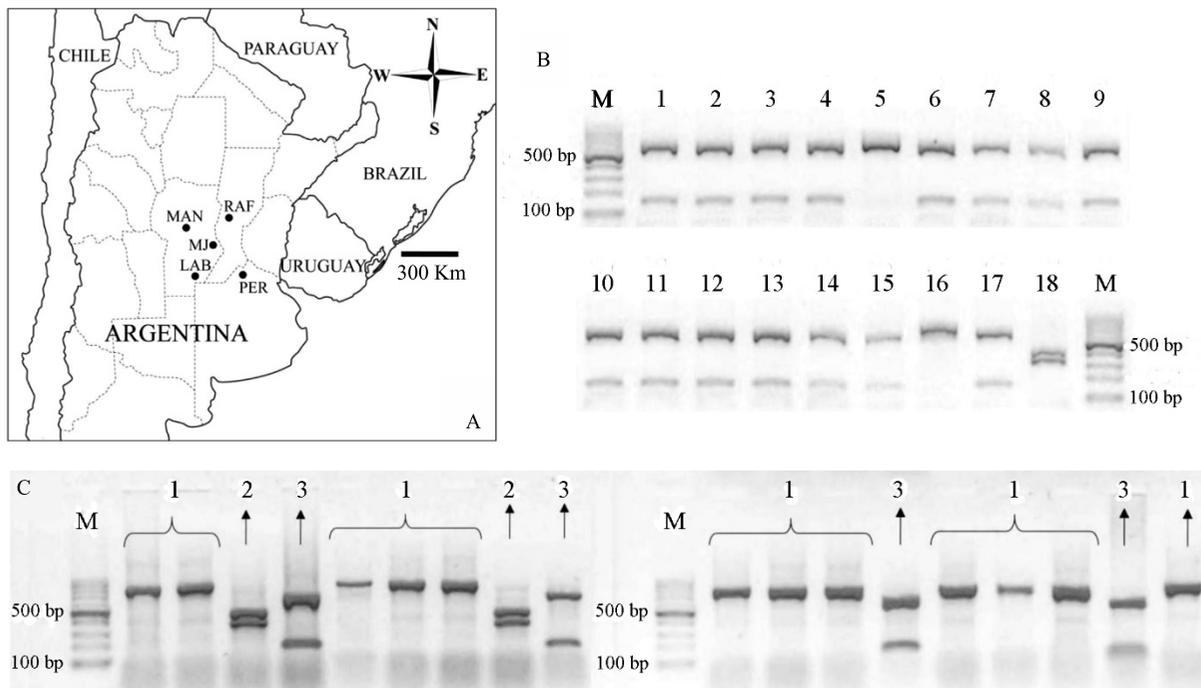

Figure 1. *Helicoverpa* spp. identified in central Argentina. (A) Sampling sites: RAF, Rafaela; MAN, Manfredi; MJ, Marcos Juárez; LAB, Laboulaye; PER, Pergamino. (B) PCR-RFLP of larvae collected on soybean, in Marcos Juárez county (lanes 1 – 15); lane 16, *H. zea* control.; lane 17, *H. gelotopoeon* control; lane 18, *H. armigera* control; M, molecular weight marker. (C) PCR-RFLP of adult moths collected in Marcos Juárez: 1, *H. zea*; 2, *H. armigera*; 3, *H. gelotopoeon*; M, molecular weight marker.

Table 1. Sampling data and species discrimination of *Helicoverpa* spp. larvae and moths collected in central Argentina.

	Larvae		
Location and sampling date	Crop (insecticide protein expressed; adoption year)	No. of larvae analyzed	Species identified (%)
Manfredi Feb 2016	Non-Bt soybean	50	*H. gelotopoeon* (100%)
Manfredi Feb 2016	Intacta RR2 soybean (Cry 1 Ac; 2012)	-	None
Manfredi Feb 2016	Non-Bt corn	39	*H. zea* (100%)
Manfredi Feb 2016	MG corn (Cry 1Ab; 2007)	50	*H. zea* (100%)
Manfredi Feb 2016	Power Core corn (Cry1A.105, Cry2Ab, Cry1Fa2; 2012)	44	*H. zea* (100%)
Manfredi Feb 2016	VT Triple Pro corn (Cry1A.105, Cry2Ab, Cry3Bb1; 2010)	45	*H. zea* (100%)
Manfredi Feb 2016	Viptera 3 corn (Vip3Aa20; 2011)	-	None
Manfredi Feb 2016	Non-Bt lucerne	50	*H. gelotopoeon* (100%)
Marcos Juárez Feb 2016	Non-Bt Soybean	50	*H. gelotopoeon* (96%), *H. zea* (4%)
Marcos Juárez Feb 2016	Intacta RR2 soybean (Cry 1 Ac; 2012)	-	None
Marcos Juárez Feb 2016	Non-Bt corn	42	*H. zea* (100%)
Marcos Juárez Feb 2016	MG corn (Cry 1Ab; 2007)	50	*H. zea* (100%)
Marcos Juárez Feb 2016	Power Core corn (Cry1A.105, Cry2Ab, Cry1Fa2; 2012)	50	*H. zea* (100%)
Marcos Juárez Feb 2016	VT Triple Pro corn (Cry1A.105, Cry2Ab, Cry3Bb1; 2010)	50	*H. zea* (100%)
Marcos Juárez Feb 2016	Viptera 3 corn (Vip3Aa20; 2011)	-	None
Marcos Juárez Feb 2016	Non-Bt lucerne	50	*H. gelotopoeon* (98%), *H. zea* (2%)
Laboulaye Feb 2016	Non-Bt soybean	50	*H. gelotopoeon* (100%)
Laboulaye Feb 2016	Non-Bt Corn	30	*H. zea* (100%)
Laboulaye Feb 2016	MG corn (Cry 1Ab; 2007)	40	*H. zea* (100%)
Laboulaye Feb 2016	Power Core corn (Cry1A.105, Cry2Ab, Cry1Fa2; 2012)	50	*H. zea* (100%)
Laboulaye Feb 2016	VT Triple Pro corn (Cry1A.105, Cry2Ab, Cry3Bb1; 2010)	50	*H. zea* (100%)
Laboulaye Feb 2016	Non-Bt lucerne	50	*H. gelotopoeon* (100%)
Rafaela Feb 2016	Non-Bt soybean	50	*H. gelotopoeon* (100%)
Rafaela Feb 2016	Intacta RR2 soybean (Cry 1 Ac; 2012)	-	None
Rafaela Feb 2016	Non-Bt corn	46	*H. zea* (100%)
Rafaela Feb 2016	MG corn (Cry 1Ab; 2007)	41	*H. zea* (100%)
Rafaela Feb 2016	Power Core corn (Cry1A.105, Cry2Ab, Cry1Fa2; 2012)	41	*H. zea* (100%)
Rafaela Feb 2016	VT Triple Pro corn (Cry1A.105, Cry2Ab, Cry3Bb1; 2010)	49	*H. zea* (100%)
Rafaela Feb 2016	Viptera 3 corn (Vip3Aa20; 2011)	-	None
Rafaela Feb 2016	Non-Bt lucerne	48	*H. gelotopoeon* (98%), *H. zea* (2%)
Pergamino Feb 2016	Non-Bt soybean	-	None
Pergamino Feb 2016	Intacta RR2 soybean (Cry 1 Ac; 2012)	-	None
Pergamino Feb 2016	Non-Bt corn	50	*H. zea* (100%)
Pergamino Feb 2016	MG corn (Cry 1Ab; 2007)	28	*H. zea* (100%)

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Pergamino Feb 2016	Power Core corn (Cry1A.105, Cry2Ab, Cry1Fa2; 2012)	50	*H. zea* (100%)
Pergamino Feb 2016	VT Triple Pro corn (Cry1A.105, Cry2Ab, Cry3Bb1; 2010)	50	*H. zea* (100%)
Pergamino Feb 2016	Viptera 3 corn (Vip3Aa20; 2011)	-	None
Pergamino Feb 2016	Non-Bt lucerne	50	*H. gelotopoeon* (98%), *H. zea* (2%)

Adult moths			
Location and sampling date	Predominant crops in the area	No. of adults analyzed	Species identified (%)
Manfredi Nov 2015 and Feb 2016	Soybean and corn	50	*H. zea* (76%), *H. gelotopoeon* (22%), *H. armigera* (2%)
Marcos Juárez Nov 2015 – Jan 2016	Soybean and corn	50	*H. zea* (66%), *H. gelotopoeon* (30%), *H. armigera* (4%)
Laboulaye Nov 2015 and Feb 2016	Soybean, corn, lucerne, and natural grasses	50	*H. zea* (86%), *H. gelotopoeon* (14%)
Rafaela Jan and Feb 2018	Lucerne, soybean, corn, and spontaneous vegetation	50	*H. zea* (92%), *H. gelotopoeon* (8%)
Pergamino Nov 2015 and Mar 2016	Soybean, corn, and lucerne	50	*H. zea* (96%), *H. gelotopoeon* (2%), *H. armigera* (2%)

Amplicons were digested with *Hinf*I endonuclease, the resulting fragments were separated by agarose gel electrophoresis (1%), and species were identified according to their differential RFLP patterns (Arneodo et al., 2015). Adults of *H. zea*, *H. gelotopoeon* and *H. armigera*, identified by microscopic examination of the genitalia, were included as controls in every batch of tests.

A log-linear maximum likelihood analysis of variance was performed with a model of the main effects for the variables location, crop and species. To confirm transgenic events, pieces of leaves of the different Bt corn hybrids (15 per plot) were analyzed at the Genetically Modified Organisms Detection Laboratory, Biotechnology Institute, CICVyA-National Institute of Agricultural and Livestock Technology, Argentina, ISO 17025. In pest-infested plots, GMO detection analyses were performed on the same leaves from which the larvae had been collected. The methods implemented were based on Sybr Green qPCR. In addition to the main survey (that is, *Helicoverpa* spp. larvae directly collected from the crops), a smaller sampling of adult moths was undertaken in the same counties. Fifty *Helicoverpa* spp. specimens per location (total=250) were captured using light-traps and identified by PCR-RFLP (Table 1). All soybean, corn and lucerne crops surveyed in central Argentina, in the 2016 summer, showed *Helicoverpa* spp. infestations,

except for Bt soybean ('Intacta RR2'), 'Viptera 3' Bt corn, and a non-Bt soybean plot in Pergamino (Table 1). *COI* fragments were amplified from most *Helicoverpa* specimens collected. Amplification failures were likely due to poor DNA quality. In total, 1343 *Helicoverpa* spp. larvae (out of the 1,450 collected ones) were successfully analyzed by the PCR-RFLP method for species discrimination (Figure 1).

A strong association between the pest and the host plant was observed, regardless of the sampling site. The variables insect species and crop had a significant effect on population distribution (Pr> ChiSq <0.00001). The species *H. zea* was the only one found on corn [non-Bt and transgenic 'MG' (Cry1Ab), 'VT Triple Pro' (Cry1A.105 + Cry2Ab + Cry3Bb1), and 'Power Core' (Cry1A.105 + Cry2Ab + Cry1Fa2) hybrids. In 'Viptera 3' (Vip3Aa20) corn hybrids, lepidopteran larvae were not detected. The transgenic condition of the Bt corn hybrids was confirmed in all cases. The analyzed leaf samples contained the expected Bt gene(s) related to the insecticidal characteristics of each corn hybrid. Resistance of *H. zea* to Cry 1Ab and Cry1A.105 + Cry2Ab toxins have been previously reported throughout the American continent (Dively et al., 2016). Recently, a reduced susceptibility to Vip3Aa toxin was reported in the USA (Yang et al., 2020). Concerning Fabaceae, *H. gelotopoeon* was largely the most abundant species of the genus in both

non-Bt soybean (96–100% of *Helicoverpa* specimens collected) and lucerne (98–100%). However, *H. zea* made up 4% of the sample in a non-Bt soybean plot in Marcos Juárez, and 2% in lucerne plots in Rafaela, Marcos Juárez and Pergamino. Similarly, Murúa et al. (2016) revealed low prevalence and incidence of *H. zea* (<4% of total Heliothinae sampled in a few plots) in soybean crops, in the NW of the country, where *H. gelotopoeon* predominated by far. In addition, Margheritis & Rizzo (1965), and Navarro et al. (2009) mentioned lucerne as an occasional host to *H. zea* in Argentina, albeit without providing quantitative data. Furthermore, *H. zea* is considered a sporadic pest of soybean and lucerne in the USA, where *H. gelotopoeon* is not present (Pearson et al., 1989; Swenson et al., 2013). Neither *Helicoverpa* spp. nor other lepidopteran pests were found on transgenic Bt soybean.

None of the 1343 sampled larvae from the different crops examined by PCR-RFLP corresponded to the introduced species *H. armigera*. Nevertheless, *H. armigera* represented a small share of the additional adult moth sampling in some locations (up to 4%), where *H. zea* constituted the vast majority of the specimens, followed by *H. gelotopoeon* (Table 1, Figure 1). These data suggest that *H. armigera* occurred in low abundance in central Argentina, during the evaluation period. In this line, a thorough study on the abundance of *H. armigera* and *H. zea* moths, caught in light traps in Distrito Federal (central Brazil), showed that *H. armigera* populations declined in the growing seasons following the big outbreak of 2012/2013. The authors found a significant negative correlation between the average maximum temperature and relative humidity (boosted by El Niño events) and the number of moths collected, with the highest Oceanic Niño Index (ONI) values registered in 2015/2016 (Specht et al., 2021). In addtion to the small population size, other factors could also explain the absence of *H. armigera* larvae in the samples. For instance, other plants might be serving as hosts for this insect. A survey conducted during 2015/2016 in corn, in the state of Rio Grande do Sul (southern Brazil) showed similar results. Even though *H. armigera* has been reported since 2011, in that region, no *Helicoverpa* larvae other than *H. zea* were recovered from infested corn ears. This was possibly due to the advantage of *H. zea* over *H. armigera* in intraguild interactions in corn, and to reduced *H. armigera* populations because

of the absence of cotton crops, which is a preferred host (Bentivenha et al., 2016). In northern Argentina, infestations with *H. armigera* larvae have already been recorded on chickpea, soybean, sunflower, and spiny plumeless thistle (Arneodo et al., 2015; Murúa et al., 2016). Given the damage potential of *H. armigera*, and because meteorological and agroecological conditions vary from year to year, the presence of this pest across the country should be periodically monitored. The economically important genus *Helicoverpa* contains species with worldwide (for instance, *H. armigera*), continental (*H. zea*), and regional (*H. gelotopoeon*) distribution. The presence of *Helicoverpa* bollworms in central Argentina is well known to farmers, but this is the first large-scale molecular survey to clearly identify, at the species level, larvae feeding on the main Bt and non-Bt crops in the region. The information provided here will be useful for analyzing possible changing scenarios for the *Helicoverpa* complex in the core agricultural lands of Argentina.

Acknowledgments

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