Appendix 7. Insect Resistance Monitoring in Iberian populations of *Sesamia nonagrioides*: 2011 Season

MINISTERIO
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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

INSECT RESISTANCE MONITORING ASSOCIATED WITH MON810 MAIZE CULTIVATION IN THE EU

Report: Season 2011

Within the frame of an Agreement established between Monsanto International Sarl and the Consejo Superior de Investigaciones Científicas (CSIC) for the prevention of pest' resistance to Bt plants, technical support work on the monitoring of resistance development of corn borers to Cry1Ab protein expressed in maize MON810 within Iberian populations of corn borers has been carried out in the Centro de Investigaciones Biológicas (CSIC).

CONTENTS

1. Aims

Baseline data on susceptibility of corn borers (*Sesamia nonagrioides* and *Ostrinia nubilalis*) to the Cry1Ab protein contained in MON810 maize were developed from different Iberian agroecological areas during the years 2004 and 2005. These data have provided insight into the natural variability of pest populations in the geographical range of adoption and they can be used to assess changes in susceptibility to Cry1Ab in the transgenic crop.

In accordance with the Plan for the Monitoring of Resistance to the Cry1Ab protein of MON810 corn within populations of corn borers, subsequent routine monitoring for each target pest should be carried out. The objective is to detect, in a timely manner, shifts relative to baseline susceptibility that could result in inadequate protection against the target species.

Different geographical areas where the commercial growing of MON810 varieties is considerable were selected. According to the Protocol, each target population is monitored every two years, what is assumed to be an acceptable interval for the early detection of resistance in a field population if it would occur. For practical reasons, the populations have been divided into two groups so that each year sampling is carried out in one of the groups.

The objectives of this study for the maize season 2011 were:

1) To detect shifts in susceptibility to MON810 maize of *S. nonagrioides* populations of one maize growing area of the Iberian Peninsula: Northeast Iberia.

2) To collect populations of *O. nubilalis* from Central and Northeast Iberia in order to send them to the BioOK laboratory in Germany for their analysis. This institute is carrying out the European resistance monitoring programme of *O. nubilalis* for MON810 maize, by an agreement with Monsanto International Sarl.

3) To analyze the susceptibility to Cry1Ab of a laboratory strain of *O. nubilalis* to verify the activity of the batch of toxin used in the bioassays with field populations.

2. Methodology

Last instar larvae of each population of the corn borers *O. nubilalis* and *S. nonagrioides* were collected from at least 3 locations in the different maize growing areas selected. The samples were taken during September and October of 2011 from refuge areas and fields of conventional maize adjacent to Bt maize **(Annex I)**.

Testing early generations is recommended in resistance monitoring plans (Sivasupramaniam 2007). Therefore, when possible, susceptibility to the protein Cry1Ab was carried out from January to March 2012 on F1 progeny.

The protocol followed in the bioassays is described in **Annex II**.

The toxin Cry1Ab used for all the bioassays performed in this season is a different batch from previous years. This lot was delivered in October 2011 by Monsanto. Stock solutions were prepared from the original and kept in the freezer at -20ºC and aliquots were thawed only when the bioassay was ready to be performed.

Laboratory populations of *S. nonagrioides* and *O. nubilalis* served as control using the same stock solution, comparing its susceptibility to Cry1Ab with those of field populations.

As it was concluded with the results obtained in the 2009 season, the susceptibility has been determined by MICs in *S. nonagrioides* populations and by LCs and MICs in a laboratory population of *O. nubilalis*.

3. Results

3.1. Collection of larvae

Numbers of larvae collected for the bioassays in Spain and for sending to BioOK in Germany are showed in **Annex I**.

A total of 564 larvae of *S. nonagrioides* were found in four of the eight fields inspected in Northeast Iberia. In the case of *O. nubilalis*, six fields in different locations of Central Iberia were examined, but larvae could only be collected in three of them. Similarly, in Northeast Iberia larvae were collected in six of the eight fields prospected, but only in two of them there was a high number (> 100).

3.2. Susceptibility to Cry1Ab in the 2011 campaign

To determine the susceptibility to Cry1Ab, larval mortality and larval molt inhibition data at the different concentrations of Cry1Ab tested were analyzed by probit analysis. Lethal concentrations at 50% (LC_{50}) and 90% (LC_{90}) were estimated for the laboratory population of *O. nubilalis*, and moulting inhibition concentrations at 50% (MIC_{50}) and 90% (MIC_{90}) for populations of both *O. nubilalis* and *S. nonagrioides* **(Table 1).** The significance of differences in susceptibility between the laboratory strain and the field population of *S. nonagrioides* was tested by determining the 95%

fiducial limits of molt inhibition concentration ratios (MICR) at the MIC_{50} (Robertson *et al.,* 2007). Fitted curves of susceptibility to the toxin Cry1Ab of laboratory and field populations of the two species were generated taking into account the molting inhibition of neonate larvae after seven days feeding on treated diet **(Figure 1)**.

3.2.1. S. nonagrioides

The bioassay to evaluate the susceptibility of the Northeast population of *S. nonagrioides* to Cry1Ab was performed with neonates of the F1 generation of the field-collected larvae after the winter diapause period. As it was established by the results of the campaign of 2009, only values of MIC have been used to assess the susceptibility of this species to Cry1Ab.

This is the first time to use this new batch of the Cry1Ab protein. However, the results of MIC_{50} and MIC_{90} are in the range of those obtained in previous years. Susceptibility of the laboratory strain (MIC_{50} = 9 ng Cry1Ab/cm^2) was higher than that of the population coming from Northeast Iberia (MIC_{50} = 20 ng Cry1Ab/cm^2) (**Table 1A**). When both strains were compared by the MICR, it could be seen that the one from Northeast Iberia was twice more tolerant to Cry1Ab than laboratory colony at MIC_{50} and MIC_{90} levels. Similar differences between laboratory and field colonies have been observed historically, as well as variations in susceptibility of a population in different years (**Table 3)**. Nevertheless, information from the last seasons suggests that these differences and oscillations in susceptibility values to the toxin Cry1Ab are common in *S. nonagrioides* and they are not an evidence of resistance acquisition of the tested population (Farinós *et al.* 2004).

3.2.2. O. nubilalis

In the case of the laboratory strain of *O. nubilali*s, susceptibility to the new batch of Cry1Ab toxin was analyzed by LCs and MICs. This population displayed LC_{50} and MIC_{50} values of 4 and 2.8 ng Cry1Ab/cm^2, respectively. The LC values are slightly lower than those reported in 2011 and 2010, and in the range of values obtained between 2004 and 2008 for the same population (**Table 4**). Likewise, MIC_{50} and MIC_{90} are comprised between the MIC values of previous years. The fiducial limits for the MIC values could not be calculated because of a poor fit of the by the probit analysis model (**Table 1B**; **Figure 1-B2**), although the MIC_{50} value is comprised within the range of concentrations tested.

Table 1. Susceptibility to Cry1Ab toxin of a laboratory population and a field population of *S.nonagrioides* (A) and a laboratory population of *O. nubilalis* (B) during the 2011 campaign.

A) *Sesamia nonagrioides*

Population	Year	n[a]	Slope ± SE	χ^2	d.f.	MIC_{50}[b] (FL 95%)	MICR (MIC_{50})[c] (FL 95%)	MIC_{90}[b] (FL 95%)	MICR (MIC_{90})[c] (FL 95%)
Laboratory	2011	863	1.5 ± 0.1	75.7	25	9 (6-13)	1	68 (45-127)	1
Northeast Iberia	2011	864	1.5 ± 0.1	70.1	25	20 (14-27)	2.2 (1.6-3.0)	135 (91-232)	2.0 (1.3-2.9)

B) *Ostrinia nubilalis*

Population	Year	n[a]	Slope ± SE	χ^2	d.f.	LC_{50}[b] (FL 95%)	LC_{90}[b] (FL 95%)
Laboratory	2012	796	1.8 ± 0.1	34.8	28	4 (3-5)	20 (16-28)
						MIC_{50}[b] (FL 95%)	MIC_{90}[b] (FL 95%)
Laboratory	2012	560	5.0 ± 0.6	472	19	2.8[d]	5.0[d]

[a] n does not include controls

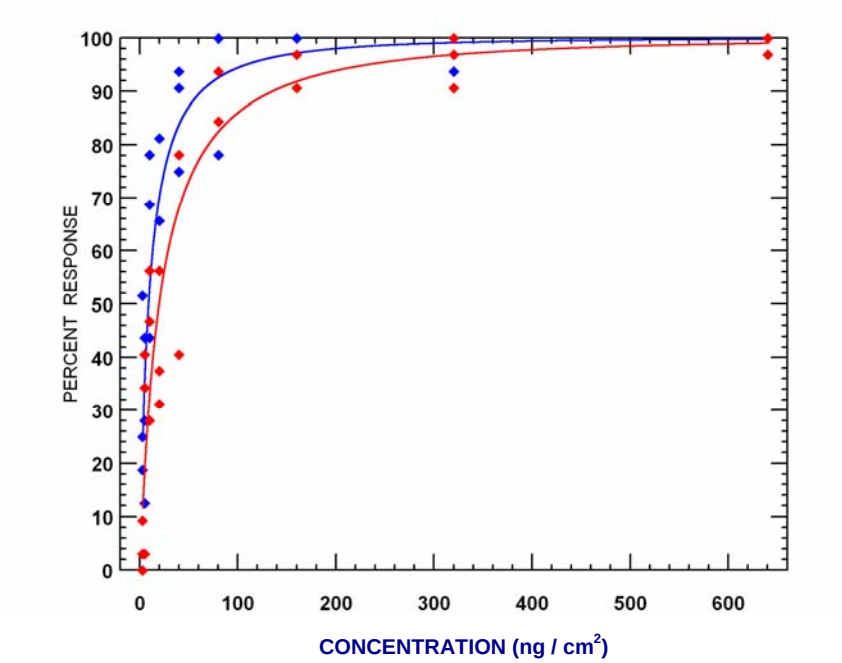
[b] 50% and 90% lethal concentrations (LC_{50} and LC_{90}) or moulting inhibition concentrations (MIC_{50} and MIC_{90}) and their 95% fiducial limits (FL95%) are expressed in ng Cry1Ab/cm^2.

[c] Lethal concentrations significantly different ($P < 0.05$) if the LCR 95% confidence interval does not include 1.

[d] FL 95% could not be estimated because the coefficient g was >0.5 at the 95% probability level.

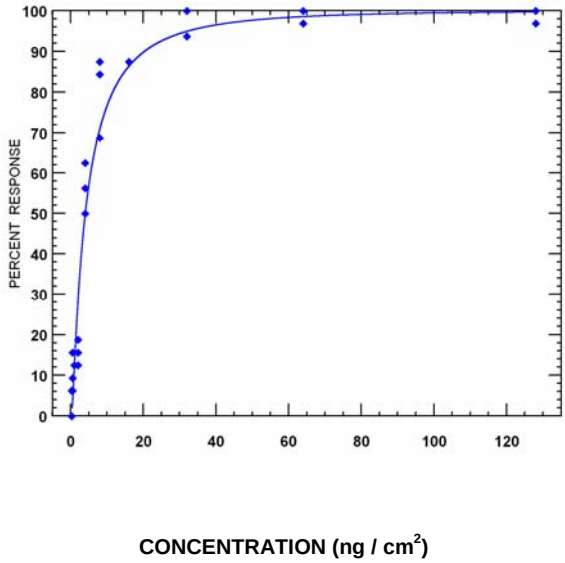
Figure 1. Fitted curves of susceptibility to the toxin Cry1Ab (PoloPlus, LeOra Software, 2002-2009).
A: Laboratory colony (blue) and a field population from Northeast Iberia (red) of *Sesamia nonagrioides* (slopes of individual population lines were constrained to be parallel). Response is molt inhibition after seven days feeding on treated diet.
B: Laboratory colony of *Ostrinia nubilalis*. Response is mortality (B1) or molt inhibition (B2) after seven days feeding on treated diet.

A) *Sesamia nonagrioides*

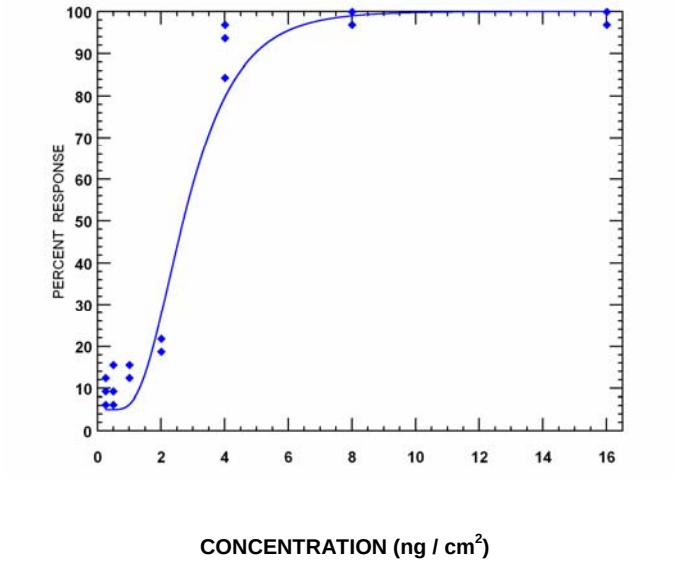


B) *Ostrinia nubilalis*

B1) Lethal Concentration (LC) values



B2) Molt Inhibition Concentration (MIC) values



3.3. Survival of larvae recovered from bioassays on MON810 leaves

All larvae of *S. nonagrioides* from the field population of Northeast Iberia which survived the bioassay with different concentrations of Cry1Ab were taken apart (243, 245 and 233 larvae from the 1st, 2nd and 3rd replicate, respectively). They were transferred to plastic boxes in groups of ≈ 50 larvae, provided with newly detached MON810 maize leaves without the central nerve, and fed *ad libitum* on them, to record any survivors. No surviving larvae were reported after 10 days from any of the boxes.

3.4. Historical susceptibility of corn borers to Cry1Ab

3.4.1. S. nonagrioides

Bioassays of susceptibility performed in the laboratory with the progenies of the field populations of *S. nonagrioides* since 2004 have yielded low variability in MIC_{50} and MIC_{90} values. MIC_{50}s ranged between 7 and 28 ng Cry1Ab/cm^2, recorded in populations from Central Iberia that were collected in 2006 and 2008, respectively (**Table 3**). These results evidenced a magnitude variation of 4.0-fold. Likewise, values of MIC_{50} of laboratory strains were also very uniform, ranging between 8 and 19 ng Cry1Ab/cm^2, which means a magnitude variation of 2.4-fold.

Following this progression, MIC_{50} values obtained during this campaign for the field populations of Northeast Iberia (20 ng Cry1Ab/cm^2) and for the laboratory strain (9 ng Cry1Ab/cm^2) are within the range of values got in the past years (**Table 3**).

3.4.2. O. nubilalis

LC and MIC values of the control laboratory strain were very consistent in the interval of years examined (2004-2012), being the maximum magnitude of variation about 5-fold (for both LC_{50} and LC_{90}values) and 6- and 8-fold (for MIC_{50} and MIC_{90} values, respectively) (**Table 4**).

Taking into consideration MIC_{50} values obtained for both corn borers, larvae of *O. nubilalis* in most cases showed higher susceptibility to the Cry1Ab toxin than *S. nonagrioides*.

Table 3. Susceptibility to Cry1Ab toxin of laboratory populations and Iberian field populations of *S. nonagrioides* collected in refuge areas of MON810 between 2004 and 2011. Bioassays performed during this campaign are shaded.

Population[a]	Year	n[b]	Slope ± SE	χ^2	d.f.	MIC_{50}[c] (FL 95%)	MIC_{90}[c] (FL 95%)
Laboratory	2004	575	1.7 ± 0.2	32.6	13	18 (11-25)	99 (66-208)
Laboratory	2007	669	1.7 ± 0.2	23.2	16	16 (11-22)	94 (69-147)
Laboratory	2009	671	1.6 ± 0.2	65.0	19	19 (10-30)	120 (76-255)
Laboratory	2010	859	1.3 ± 0.1	40.7	25	8 (5-11)	74 (51-117)
Laboratory [e]	2012	863	1.5 ± 0.1	75.7	25	9 (6-13)	68 (45-127)
Southwest Iberia (Spain) [a]	2005	192	4.6 ± 0.7	125.2	10	16[d]	30[d]
Southwest Iberia (Portugal) [a]	2005	660	1.0 ± 0.1	26.2	19	8 (3-16)	152 (94-309)
Southwest Iberia (Spain) [a]	2007	670	1.1 ± 0.1	17.7	16	17 (10-25)	226 (153-385)
Southwest Iberia F2	2010	768	1.7 ± 0.1	48.8	22	16 (11-21)	86 (60-141)
Central Iberia	2004	672	1.0 ± 0.1	30.5	19	12 (5-22)	248 (143-588)
Central Iberia	2006	672	0.8 ± 0.1	42.5	19	7 (1-17)	321 (157-1360)
Central Iberia	2008	672	1.6 ± 0.2	22.3	19	28 (18-38)	170 (124-259)
Central Iberia	2010	800	1.2 ± 0.1	63.2	37	10 (6-14)	119 (81-200)
Northeast Iberia	2005	560	1.4 ± 0.2	20.8	19	9 (3-15)	76 (54-117)
Northeast Iberia	2007	671	1.5 ± 0.2	23.9	19	14 (8-20)	99 (71-158)
Northeast Iberia	2009	863	1.4 ± 0.1	28.2	25	22 (16-28)	188 (138-277)
Northeast Iberia [e]	2011	864	1.5 ± 0.1	70.1	25	20 (14-27)	135 (91-232)

[a] Since 2008 the population called *Southwest Iberia* includes sampling areas from Spain and Portugal. Previously the populations were separated by country.

[b] n does not include controls

[c] 50% and 90% lethal concentrations (LC_{50} and LC_{90}) or moulting inhibition concentration (MIC_{50} and MIC_{90}) and their 95% fiducial limits (FL95%) are expressed in ng Cry1Ab/cm^2 .

[d] FL 95% could not be estimated because the coefficient g was >0.5 at the 95% probability level.

[e] From 2012 bioassays of susceptibility are performed with a new batch of the Cry1Ab protein.

Table 4. Susceptibility to Cry1Ab toxin of Iberian field populations of *O.nubilalis* collected in refuge areas of MON810 between 2004 and 2008, and of laboratory strains between 2004 and 2012. Bioassays performed during this campaign are shaded.

Population	Year	n[a]	Slope ± SE	χ^2	d.f.	LC_{50}[b] (FL 95%)	LCL_{90}[b] (FL 95%)	Slope ± SE	χ^2	d.f.	MIC_{50}[b] (FL 95%)	MIC_{90}[b] (FL 95%)
Laboratory	2004	480	2.0 ± 0.2	63.3	13	4 (2-7)	19 (12-54)	2.0 ± 0.3	101.1	10	2.1[c]	9.0[c]
Laboratory	2007	479	1.5 ± 0.2	37.4	19	2 (1-4)	17 (11-31)	2.3 ± 0.8	7.6	19	0.6 (0.03-1.2)	2.3 (1.2-3.2)
Laboratory	2008	832	1.4 ± 0.1	62.7	25	2 (2-3)	20 (13-33)	2.2 ± 0.2	40.1	25	0.8 (0.6-1.0)	2.9 (2.3-4.1)
Laboratory	2010	768	3.1 ± 0.3	52.3	22	9 (7-11)	26 (19-44)	1.7 ± 0.2	6.5	18	3.4 (1.6-5.6)	19.0 (10.0-107.3)
Laboratory	2011	672	1.3 ± 0.1	28.1	19	10 (8-13)	90 (53-194)	2.4 ± 0.2	32.7	16	2.0 (1.5-2.5)	6.7 (5.1-10.1)
Laboratory[e]	2012	796	1.8 ± 0.1	34.8	28	4 (3-5)	20 (16-28)	5.0 ± 0.6	472	19	2.8[c]	5.0[c]
Southwest Iberia (Spain)	2004	670	1.6 ± 0.1	59.1	19	6 (4-9)	41 (27-77)	2.3 ± 0.2	36.9	19	5.4 (4.0-6.9)	19.8 (15.1-29.1)
Southwest Iberia (Portugal)	2005	672	2.6 ± 0.2	66.4	19	14 (11-17)	43 (31-67)	7.8 ± 1.0	16.1	19	9.4 (8.7-10.1)	13.7 (12.4-15.9)
Southwest Iberia (Spain)	2006	576	1.8 ± 0.2	35.0	16	6 (4-8)	32(23-54)	4.2 ± 0.6	22.8	19	1.9 (1.5-2.2)	3.8 (3.3-4.9)
Southwest Iberia	2008	672	1.7 ± 0.1	19.2	19	5 (4-7)	32 (24-44)	2.6 ± 0.3	27.6	19	1.3 (1.0-1.6)	4.0 (1.0-1.6)
Central Iberia	2005	672	1.9 ± 0.1	46.7	19	12 (9-15)	57 (41-92)	3.3 ± 0.3	103.8	19	4.8 (2.9-6.6)	11.8 (8.4- 23.9)
Central Iberia	2006	665	1.1 ± 0.1	35.4	19	2 (1-4)	33 (21-68)	1.9 ± 0.3	135.8	19	1.1[c]	5.2[c]
Central Iberia	2008	576	2.1 ± 0.2	29.0	16	3 (2-3)	10 (8-15)	2.8 ± 0.3	41.3	19	1.3 (0.9-1.6)	3.7 (2.9-5.5)
Northeast Iberia	2004	575	2.0 ± 0.1	66.4	16	6 (4-8)	27 (18-56)	2.2 ± 0.2	180.5	19	2.8 (0.8-4.5)	10.5 (6.2-51.4)
Northeast Iberia	2006	663	1.1 ± 0.1	64.6	19	3 (1-5)	42 (22-138)	1.5 ± 0.3	23.1	19	0.5 (0.05-1.0)	3.2 (1.8-4.8)
Northeast Iberia	2008	672	1.6 ± 0.1	45.0	19	9 (7-12)	58 (38-108)	3.0 ± 0.3	37.8	19	1.6 (1.3-1.9)	4.2 (3.4-5.9)

[a] n does not include controls

[b] 50% and 90% lethal concentrations (LC_{50} and LC_{90}) or moulting inhibition concentration (MIC_{50} and MIC_{90}) and their 95% fiducial limits (FL95%) are expressed in ng Cry1Ab/cm^2 .

[c] FL 95% could not be estimated because the coefficient g was >0.5 at the 95% probability level.

[e] From 2012 bioassays of susceptibility are performed with a new batch of the Cry1Ab protein.

4. Conclusions

1. Shifts in the susceptibility to the toxin Cry1Ab expressed in the MON810 maize have been evaluated by means of molt inhibition concentration (MIC) values in two populations of *S. nonagrioides* (a field population from Northeast Iberia and a laboratory strain) and by means of lethal concentrations (LC) and MIC in a laboratory strain of *O. nubilalis*.

2. A new batch of toxin, different from that used in previous years, has been used in all cases. Levels of susceptibility (LCs and MICs) to Cry1Ab of this batch showed by laboratory strains of both species of corn borers, *S. nonagrioides* and *O. nubilalis*, are similar to levels of susceptibility obtained in previous campaigns. Thus, it can be assumed that batches' performance is comparable. Consequently, results obtained this season with the new lot could be compared with historical data obtained since 2004.

3. The susceptibility of the field population of *S. nonagrioides* from Northeast Iberia was determined in the first generation (F1) after the winter diapause. Susceptibility to the Cry1Ab toxin of this population has been assessed for fourth time since 2005. Levels of larval molt inhibition (MIC_{50} and MIC_{90}) are within the range of variability in susceptibility to the toxin expected for field populations of this corn borer. Larvae of this field population resulted about 2-fold less susceptible than those of the laboratory strain.

4. The laboratory strain of *O. nubilalis* showed susceptibility levels to the Cry1Ab toxin comparable with those obtained for laboratory strains in previous years. Both LC and MIC values evidenced consistency through time, showing 5- and 6-fold variation in both LC_{50} and MIC_{50} values, respectively.

5. The analysis of the historical series of data of susceptibility of *S. nonagrioides* to Cry1Ab did not reveal signs of resistance to this toxin in field populations of *S. nonagrioides* from Northeast Iberia.

Madrid, 19th July 2012

ANNEX I

LOCATIONS AND NUMBERS OF CORN BORERS COLLECTED IN 2011

Species	Area	Country	Fields (Province)[1]	Postal Code	Date	No of larvae collected	No of larvae for BioOK Laboratory
Sesamia nonagrioides	Northeast Iberia	Spain	Alfamén (Z)	50461	26-29/09/2011	64	-
			Agramonte (Z)	50461	26-29/09/2011	103	-
			Ejea de los Caballeros (Z)	50600	26-29/09/2011	0	-
			Sariñena 1 (HU)	22200	26-29/09/2011	0	-
			Sariñena 2 (HU)	22200	26-29/09/2011	0	-
			Valtierra (NA)	31514	26-29/09/2011	229	-
			Alagón (Z)	50630	26-29/09/2011	168	-
			Gallur (Z)	50650	26-29/09/2011	0	-
						Total: 564	
Ostrinia nubilalis	Central Iberia	Spain	Motilleja 1 (AB)	02220	4-6/10/2011	-	173
			Motilleja 2 (AB)	02220	4-6/10/2011	-	0
			La Herrera (AB)	02162	4-6/10/2011	-	0
			Barrax (AB)	02639	4-6/10/2011	-	123
			La Gineta (AB)	02110	4-6/10/2011	-	0
			El Salobral (AB)	02140	4-6/10/2011	-	108
							Total: 404
	Northeast Iberia	Spain	Alfamén (Z)	50461	26-29/09/2011	-	31
			Agramonte (Z)	50461	26-29/09/2011	-	30
			Ejea de los Caballeros (Z)	50600	26-29/09/2011	-	0
			Sariñena 1 (HU)	22200	26-29/09/2011	-	0
			Sariñena 2 (HU)	22200	26-29/09/2011	-	167
			Valtierra (NA)	31514	26-29/09/2011	-	20
			Alagón (Z)	50630	26-29/09/2011	-	117
			Gallur (Z)	50650	26-29/09/2011	-	17
							Total: 382

[1] Spanish provinces: AB = Albacete; HU = Huesca; NA = Navarra; Z = Zaragoza

ANNEX II

Methodology applied in the Plan for the Monitoring of Resistance to the Cry1Ab protein within populations of corn borers.

Sampling and rearing of insects

Larvae of *Sesamia nonagrioides* and *Ostrinia nubilalis* were collected from refuge areas and fields of conventional maize adjacent to Bt maize fields. Sampling was carried during September and October of 2011, by cutting the stalk of the maize plants and avoiding collection of more than one larvae of each species per plant.

In the laboratory the larvae were dipped in a solution containing 1% bleach, to avoid contamination by pathogens, and placed in 21 x 16 x 4 cm plastic boxes (50 larvae of *S. nonagrioides* or 100 larvae of *O. nubilalis*) and were fed on an artificial diet. Immediately after asepsis, larvae of *O. nubilalis* were sent to the BioOK laboratory (Germany), in accordance with the agreement established with Monsanto International Sarl.

Most of the larvae of *S. nonagrioides* collected from the field were in diapause or started diapause when placed on the rearing chamber at 15 ± 1ºC, 70 ± 5% relative humidity and a photoperiod of 12:12 hours (light: dark), after reaching the last larval instar. The larvae were kept in diapause during different time periods, to allow spacing out of the bioassays across several months. To interrupt diapause the larvae were placed under conditions 28 ± 1ºC, 70 ± 5% relative humidity and continuous light. Once the diapause was interrupted, the larvae pupated and the process continued in an insectarium at temperature of 25 ± 3ºC, 70 ± 10% relative humidity and a photoperiod of 16:8 hours (light: dark). Pupae of *S. nonagrioides* were sexed and 7 to 10 couples were placed in oviposition cages consisting of ventilated metacrylate cylinders, 30 cm high and 12 cm diameter that covered a pot with 5-6 maize plantlets, where the females laid the eggs. After 7 days the eggs were collected and placed into ventilated plastic boxes containing wet filter paper. The eggs were incubated under the same conditions and neonate larvae (< 1 day old) were selected for the bioassays.

Rearing diet

The diet for both species was established from that described by Poitout and Buès (1970) with the following modifications:

S. nonagrioides

Components	Amount	Provider
Distilled H_2O	1 l	
Agar	26 g	Panreac
Maize flour	160 g	Santiveri
Wheat germ	40 g	Santiveri
Yeast	43 g	Santiveri
Ascorbic acid	6 g	Panreac
Benzoic acid	1,25 g	Fluka
Nipagin (Methyl p-hidroxibenzoato)	1 g	Fluka
Wesson's salts mixture	1,55 g	Sigma

O. nubilalis

Components	Amount	Provider
Distilled H_2O	1 l	
Agar	24 g	Panreac
Maize flour	168 g	Santiveri
Wheat germ	42 g	Santiveri
Yeast	45 g	Santiveri
Ascorbic acid	9 g	Panreac
Benzoic acid	3 g	Fluka
Nipagin (Methyl p-hydroxybenzoate)	1.5 g	Fluka
Sorbic acid	1.2 g	Panreac

Bioassays

The bioassays were carried out in accordance with the methods described by Farinós et al. (2004). Monsanto provided 10 ml of a solution of Cry1Ab toxin diluted in a pH 10.5 sodium carbonate buffer, at a toxin concentration of 2.03 mg/ml and 95% purity. Serial dilutions were prepared in a pH 10.5 sodium carbonate buffer.

All assays were performed in "Bio-Ba-128" plastic trays (Color-Dec Italy, Capezzano Pianore, Italy). Each tray contains 128 wells, where 0.5 ml of rearing diet is placed and flattened, corresponding to a surface of 1.77 cm^2 and a height of about 10 mm. Once solidified, 50 µl of a solution containing different concentrations of toxin were added to the surface of the diet. The controls consisted of the carbonate buffer solution used to dilute the toxin. One neonate larva was placed in each well using a fine paintbrush and it was covered with a breathing

adhesive cover “Bio-Cv-16” (Color-Dec Italy, Capezzano Pianore, Italy). The trays were incubated in rearing chambers at 25 ± 1ºC, 70 ± 5% relative humidity and total darkness. Measured endpoints of the tests are mortality and moulting inhibition relative to the negative control after 7 days of exposure, where mortality equals larvae not showing any reaction when prodded and molting inhibition larvae that have either died or not molted to the 2nd instar after the 7 days.

The concentration ranges used were comprised between 2.5 and 640 ng Cry1Ab/cm^2 for the populations of *S. nonagrioides*, and between 0.25 and 128 ng Cry1Ab/cm^2 for the populations of *O. nubilalis*. These concentrations were established according to values of mortality and growth inhibition obtained in the laboratory of the CIB in previous years.

In order to determine the susceptibility of each population, 7 to 10 different concentrations resulting in mortality higher than 0% and below 100% were used. Three replicates were prepared for each concentration, including the control. Each replicate consisted of 32 larvae per concentration (64 larvae in the case of controls), giving a total of 96 larvae for each concentration tested (192 for controls). For each replicate neonate larvae from different oviposition cages were used.

Statistical analysis

The results obtained for mortality or growth inhibition at different concentrations of Cry1Ab were adjusted by probit weighted regression lines, and the lethal concentrations (LCs) and moulting inhibition concentrations (MICs) for 50% (LC_{50}, MIC_{50}) and 90% (LC_{90}, MIC_{90}) of each population were estimated together with their 95% confidence limits using the POLO-PC programme (LeOra Software, 1987). Mortality of the control must be below 25% for *S. nonagrioides* and 20% for *O. nubilalis*, so that the replicate is included in the statistical analysis.

The bioassay is considered valid if the average response of 50% obtained is comprised between at least 2 concentrations above it and 2 concentrations below it, from all the concentrations tested.

The significance of changes in susceptibility was tested by the 95% confidence limits of lethal concentration ratios (LCR) at the LC_{50} (Robertson et al 2007) or moult inhibition concentration ratios (MICR) at the MIC_{50}. Plots showing the percent response to the different concentrations of the Cry1Ab protein were performed with the program Polo Plus 1.0 (LeOra Software, 2002-2012).

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