APPENDIX 5: INSECT RESISTANCE MONITORING REPORT: 2009 SEASON

(------------------- , G. 2010, Insect resistance monitoring associated with MON 810 maize cultivation in the EU. Report: season 2009., *Ministerio de Educacion y Ciencia*)

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INSECT RESISTANCE MONITORING ASSOCIATED WITH MON810 MAIZE CULTIVATION IN THE EU

Report: Season 2009

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 Department of Environmental Biology of the Centro de Investigaciones Biológicas (Consejo Superior de Investigaciones Científicas, CSIC), within the frame of a Multiyear Agreement established between Monsanto International Sarl and CSIC for the prevention of resistance of corn borers to Bt plants.

CONTENTS

1. Aims

Susceptibility baselines to MON810 in corn borers (*Sesamia nonagrioides* and *Ostrinia nubilalis*) of Iberian and French populations were established during the years 2004 and 2005. Once this objective was accomplished, the second objective of the Resistance Monitoring Programme is to detect changes relative to baseline susceptibility that could result in inadequate protection against these 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.

Thus, the objectives of this study for the maize season 2009 were:

1) To detect changes in susceptibility to MON810 maize of *S. nonagrioides* populations of two maize growing areas of the Iberian Peninsula: Northeast Iberia (Ebro Valley) and Southwest Iberia (Extremadura in Spain and South of Portugal).

2) To collect populations of *O. nubilalis* from two Iberian areas: Central Iberia (Albacete) and Northeast Iberia (Ebro Valley) to be sent to the BioOK laboratory in Germany, for them to detect changes in susceptibility to MON810 maize of these populations following a similar methodology.

3) To compare data of susceptibility to the protein Cry1Ab obtained by mortality (lethal concentrations, LC) or by growth inhibition (molting inhibition concentrations, MIC) in the laboratory and field populations tested.

4) To analyze susceptibility of corn borers to Cry1Ab observed since 2004 by means of growth inhibition data to obtain values of MIC, and compare them among populations and with the values of LC of the same populations obtained in the past.

2. Methodology

A minimum of 300 larvae of each population of both *O. nubilalis* and *S. nonagrioides*, collected from 2-3 locations in the different maize growing areas selected, were used for the bioassays to detect changes in susceptibility to Cry1Ab toxin. The samples were taken during September and October of 2009 from refuge areas and fields of conventional maize adjacent to Bt maize (Annex I).

The susceptibility to the protein Cry1Ab was carried out on F1 progeny from January to March 2010. At the same time, the susceptibility of a laboratory population of each species was also tested to assess differences with field populations. The methodology applied in the bioassays is described in Annex II. For these assays there were stock solutions available, prepared from the Cry1Ab toxin delivered in 2003 by Monsanto and kept in the freezer at -20ºC. At the same time, 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.

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.

As it happened in 2008, the minimum number of larvae of *S. nonagrioides* required for the bioassay was not found in Southwest Iberia: in Spain seven fields were prospected, but only 3 larvae were found; in Portugal only 56 larvae were collected in the four maize fields examined. However, in Northeast Iberia last instar larvae were found in three of the four fields inspected (Annex I).

The pressure of *O. nubilalis* in Central Iberia was low. From the four locations inspected, larvae were collected only in two. Both sampling locations were separated by less than 25 km, but it has to be considered that area planted with MON810 varieties in Central Spain has been decreasing since 2005 (3128 ha in 2009) and it is concentrated now in a small region. In Northeast Iberia levels of attack of this species were higher and larvae were taken from three of the four fields explored (Annex I).

3.2. Susceptibility to Cry1Ab in the 2009 campaign

The raw results corresponding to mortality and growth inhibition at the different concentrations of Cry1Ab are showed in Annex III. These results were analysed by probit analysis, with an estimation of the lethal concentration at 50% (LC_{50}) and 90% (LC_{90}), or the moulting inhibition concentration at 50% (MIC_{50}) and 90% (MIC_{90}) of each population (Table 1). Fitted curves of susceptibility to the toxin Cry1Ab of laboratory and field populations of both corn borers species were generated taking into account the mortality or moulting inhibition of neonate larvae after seven days feeding on treated diet (Figure 1).

3.2.1. S. nonagrioides

The concentration needed to kill the 50% of the laboratory population (LC_{50} = 69 ng Cry1Ab/cm^2) notably differed from that needed for the population collected in Northeast Iberia (LC_{50} = 482 ng Cry1Ab/cm^2) (Table 1A). Data of the LCR would suggest that the population of Northeast was about 7 times less susceptible to the toxin Cry1Ab than the laboratory strain. However, it has been demonstrated that variability in susceptibility to the toxin Cry1Ab between populations collected in different years is common in *S. nonagrioides* and variations in this species are not an evidence of resistance acquisition of the tested population (Farinós *et al.* 2004). Furthermore, all bioassayed larvae that were found alive at the highest dose died when they were fed on Bt maize plants.

Thus, it was necessary to find a parameter that reflected the actual situation of susceptibility of corn borers to the toxin Cry1Ab. When susceptibility was measured by moulting inhibition, values of MIC_{50} were very similar in both populations (MIC_{50} = 19 and 22 ng Cry1Ab/cm^2 for the laboratory and the field population, respectively; Table 1A). In this case the MICR showed no significant differences in values of MIC_{50} between both strains. As a consequence, MIC values in *S. nonagrioides* were more precise to reflect the susceptibility of this species to the toxin Cry1Ab than LC values. Fitted curves of susceptibility also reveal that the response to increasing concentrations of the toxin is contradictory when data were analyzed by mortality of neonate larvae (Figure 1A, blue line), but very consistent when data were analyzed by moulting inhibition (Figure 1A, red line).

3.2.2. O. nubilalis

The susceptibility of the laboratory strain of *O. nubilalis* analyzed showed that the concentration needed to kill the 50% of the population (LC_{50} = 8.8 ng Cry1Ab/cm^2) was lower than for *S. nonagrioides*. Susceptibility was also measured by moulting inhibition giving a value of MIC_{50} of 3.4 ng Cry1Ab/cm^2 (Table 1B). Even so, these values were slightly higher than those obtained in previous years with the same population, revealing the variability in susceptibility to Cry1Ab.

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

A) *Sesamia nonagrioides*

Population	Year	n[a]	Slope ± SE	χ^2	d.f.	LC_{50}[b] (FL 95%)	LC_{90}[b] (FL 95%)	LCR (LC_{50})[c] (FL 95%)
Laboratory	2009	671	1.4 ± 0.1	34.8	19	69 (48-96)	565 (358-1127)	1
Northeast Iberia	2009	863	0.9 ± 0.1	23.4	25	482 (330-802)	12614 (5028-58507)	6.9 (4.1-11.7)
						MIC_{50}[b] (FL 95%)	**MIC_{90}[b] (FL 95%)**	**MICR (MIC_{50})[c] (FL 95%)**
Laboratory	2009	671	1.6 ± 0.2	65.0	19	19 (10-30)	120 (76-255)	1
Northeast Iberia	2009	863	1.4 ± 0.1	28.2	25	22 (16-28)	188 (138-277)	1.1 (0.8-1.7)

B) *Ostrinia nubilalis*

Population	Year	n[a]	Slope ± SE	χ^2	d.f.	LC_{50}[b] (FL 95%)	LC_{90}[b] (FL 95%)
Laboratory	2010	768	3.1 ± 0.3	52.3	22	8.8 (6.7-11.0)	26.2 (19.4-43.8)
						MIC_{50}[b] (FL 95%)	**MIC_{90}[b] (FL 95%)**
Laboratory	2010	768	1.7 ± 0.2	6.5	18	3.4 (1.6-5.6)	19.0 (10.0-107.3)

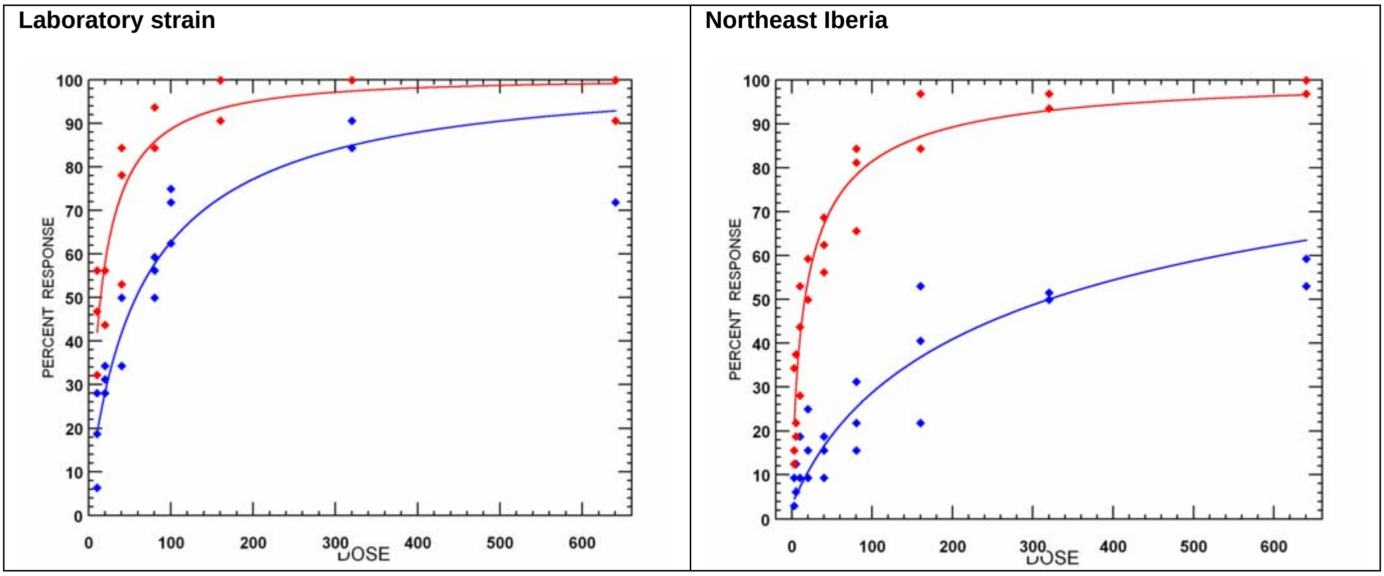
[a] n does not include controls

[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.

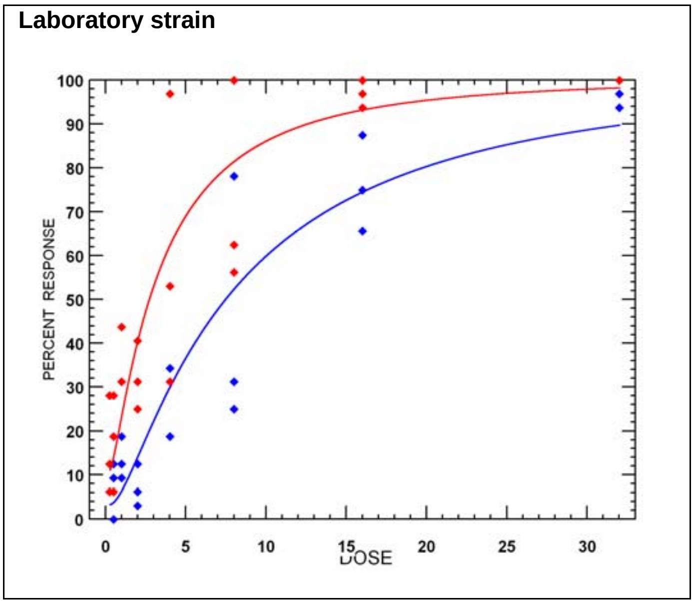
[c] Lethal concentrations significantly different ($P < 0.05$) if the LCR 95% confidence interval does not include 1.

Figure 1. Fitted curves of susceptibility to the toxin Cry1Ab of laboratory and field populations of *Sesamia nonagrioides* (A) and *Ostrinia nubilalis* (B) when slopes of individual population lines were constrained to be parallel (PoloPlus, LeOra Software, 2002-2009). Response is the mortality of neonate larvae (blue line) or moulting inhibition (red line) after seven days feeding on treated diet. Dose is expressed in ng Cry1Ab/cm^2.

A) ***Sesamia nonagrioides***



B) ***Ostrinia nubilalis***



3.3. Historical susceptibility of corn borers to Cry1Ab

3.3.1. S. nonagrioides

Bioassays performed with *S. nonagrioides* since 2004 have yielded highly variable values of LC_{50} and LC_{90} of the laboratory strains and the field populations collected in the three different Iberian areas (Table 3). The lowest value of LC_{50} was recorded in 2005 for the population of Southwest Iberia (12 ng Cry1Ab/cm^2), and the highest was recorded in 2009 for the population collected in Northeast Iberia (482 ng Cry1Ab/cm^2), being in this case the magnitude of variation around 40-fold. When LC_{90} values were compared differences were even higher, with a maximum magnitude of variation over 200-fold. However, this variability was not a sign of development of resistance of larvae to Cry1Ab.

All the data of susceptibility of *S. nonagrioides* to Cry1Ab recorded since 2004 were reanalyzed by means of moulting inhibition data, using the MIC_{50} and MIC_{90} as comparators among populations. In this case the lowest value of MIC_{50} was recorded in 2006 for the population of Central Iberia (7 ng Cry1Ab/cm^2), and the highest was recorded in 2008 for the population collected in Central Iberia (28 ng Cry1Ab/cm^2), giving as a result a magnitude variation of around 4-fold. Values of MIC_{90} were also very homogeneous, being the maximum difference between populations of around 4-fold. Values of MIC_{50} of laboratory strains were almost identical, ranging from 16 to 19 ng Cry1Ab/cm^2. Thus, the evaluation of MIC_{50} and MIC_{90} values seems to be a better reflect of actual changes of susceptibility of *S. nonagrioides* to the Cry1Ab toxin than LC_{50} and LC_{90} values.

3.3.2. O. nubilalis

Larvae of *O. nubilalis* in general showed higher susceptibility to the toxin Cry1Ab (Table 4) than *S. nonagrioides*. The degree of variability in LC_{50} and LC_{90} values in this species was much lower than in *S. nonagrioides*. LC_{50} and LC_{90} values were more consistent in all regions and years examined, being the maximum magnitude of variation about 7-fold (for LC_{50} values) and 6-fold (for LC_{90} values).
Likewise, the data of susceptibility of *O. nubilalis* to Cry1Ab recorded since 2004 were reanalyzed by moulting inhibition, using the MIC_{50} and MIC_{90} as comparators among populations. Values of MIC_{50} of laboratory strains ranged from 0.6 to 3.4 ng Cry1Ab/cm^2. The lowest value of MIC_{50} was registered in 2006 for the population of Northeast Iberia (0.5 ng Cry1Ab/cm^2), and the highest was recorded in 2005 for the population collected in Southwest Iberia (9.4 ng Cry1Ab/cm^2), giving as a result a

magnitude variation of around 19-fold. Values of MIC_{90} were more homogeneous, being the maximum difference between populations of around 9-fold. Since the concentrations used in the past were fixed according to the LC_{50} values, there is a possibility that the use of lower and more suitable concentrations to estimate MIC_{50} will contribute to obtain better-adjusted results in the future also for *O. nubilalis*. Thus, for this species both LC and MIC values could be used as comparators among populations to evaluate changes in susceptibility to the Cry1Ab toxin.

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 2009. Bioassays performed during this campaign are shaded.

Population[a]	Year	n[b]	Slope ± SE	χ^2	d.f.	LC_{50}[c] (FL 95%)	LC_{90}[c] (FL 95%)	Slope ± SE	χ^2	d.f.	MIC_{50}[c] (FL 95%)	MIC_{90}[c] (FL 95%)
Laboratory	2004	575	1.4 ± 0.1	42.1	16	23 (14-32)	179 (113-385)	1.7 ± 0.2	32.6	13	18 (11-25)	99 (66-208)
Laboratory	2007	669	1.1 ± 0.1	46.1	19	39 (20-62)	541 (297-1467)	1.7 ± 0.2	23.2	16	16 (11-22)	94 (69-147)
Laboratory	2009	671	1.4 ± 0.1	34.8	19	69 (48-96)	565 (358-1127)	1.6 ± 0.2	65.0	19	19 (10-30)	120 (76-255)
Southwest Iberia (Spain) [a]	2005	192	4.0 ± 0.5	41.3	19	12 (9-15)	57 (41-92)	4.6 ± 0.7	125.2	10	16[d]	30[d]
Southwest Iberia (Portugal) [a]	2005	660	0.9 ± 0.1	25.3	18	18 (7-31)	566 (293-1779)	1.0 ± 0.1	26.2	19	8 (3-16)	152 (94-309)
Southwest Iberia (Spain) [a]	2007	670	0.8 ± 0.1	15.1	19	54 (35-78)	1796 (956-4659)	1.1 ± 0.1	17.7	16	17 (10-25)	226 (153-385)
Central Iberia	2004	672	1.1 ± 0.1	36.2	19	32 (18-48)	523 (299-1278)	1.0 ± 0.1	30.5	19	12 (5-22)	248 (143-588)
Central Iberia	2006	672	0.1 ± 0.1	34.8	19	29 (14-49)	1137 (513-4934)	0.8 ± 0.1	42.5	19	7 (1-17)	321 (157-1360)
Central Iberia	2008	672	1.1 ± 0.1	18.9	19	74 (43-108)	1066 (668-2134)	1.6 ± 0.2	22.3	19	28 (18-38)	170 (124-259)
Northeast Iberia	2005	560	1.1 ± 0.1	21.9	19	23 (13-36)	362 (242-649)	1.4 ± 0.2	20.8	19	9 (3-15)	76 (54-117)
Northeast Iberia	2007	671	1.0 ± 0.1	20.8	19	60 (36-89)	1317 (704-3597)	1.5 ± 0.2	23.9	19	14 (8-20)	99 (71-158)
Northeast Iberia	2009	863	0.9 ± 0.12	23.4	25	482 (330-802)	12614 (5028-58507)	1.4 ± 0.1	28.2	25	22 (16-28)	188 (138-277)

[a] Since 2008 the population called *Southwest Iberia* includes 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.

Table 4. Susceptibility to Cry1Ab toxin of laboratory and Iberian field populations of *O.nubilalis* collected in refuge areas of MON810 between 2004 and 2009. 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)
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.

4. Conclusions

1. Changes in the susceptibility to the toxin Cry1Ab expressed in the MON810 maize have been evaluated by means of mortality and moulting inhibition data in two populations of *S. nonagrioides* (one from Northeast Iberia and one laboratory strain) and one laboratory population of *O. nubilalis*. The susceptibility of the population of *S. nonagrioides* from Southwest Iberia could not be evaluated due to the low levels of the pest found in this area during the 2009 campaign.

2. Susceptibility to the Cry1Ab toxin of the population of *S. nonagrioides* from Northeast Iberia has been assessed for third time since 2004. Differences found in the susceptibility to the toxin are within the range of variability expected for this corn borer. Values of MIC_{50} and MIC_{90} were comparable with those showed by the laboratory strain, and with the range obtained with populations of this species collected from the same geographical area in previous years. Susceptibility measured by larval mortality (LC values) did not show such consistency through time.

3. The susceptibility to the Cry1Ab toxin of the laboratory strain of *O. nubilalis* was comparable with values of susceptibility obtained for laboratory strains in previous years. LC_{50} and LC_{90} values evidenced consistency through time and they were comprised within a narrow range. MIC_{50} and MIC_{90} values were also consistent in this species.

4. The analyses of historical series of data of susceptibility to the Cry1Ab toxin have revealed that in *S. nonagrioides* MIC values were more precise to reflect changes in the susceptibility to the toxin Cry1Ab than LC values with the bioassay utilized. In the case of *O. nubilalis*, both LC and MIC values could be used as comparators among populations to evaluate changes in susceptibility to the Cry1Ab toxin. In view of the results, concentrations of the toxin used in the bioassays of susceptibility in future campaigns will be chosen according to MIC values.

5. No indications of resistance to the Cry1Ab protein have been found in the field populations of *S. nonagrioides* from the sampling area considered.

[redacted]

Madrid, 4th June 2010

ANNEX I

LOCATIONS AND NUMBERS OF CORN BORERS COLLECTED IN 2009

Species	Population	Country	Fields (Province)[1]	Postal Code	Date dd/mm/yyyy	No of larvae collected	No of larvae sent to Germany
Sesamia nonagrioides	Southwest Iberia	Spain	Don Benito (BA)	06400	09/09/2009	1	-
			Madrigalejo (CC) (3 fields)	10110	09-10/09/2009	1	-
			Vegas Altas (BA)	06731	09/09/2009	0	-
			Guadiana del Caudillo (BA)	06186	10/09/2009	1	-
			Lobón-Arroyo (BA)	06498	11/09/2009	0	-
		Portugal	Coruehe	2100	23/10/2009[2]	15	-
			Avis	7480-148	23/10/2009	10	-
			Arraiolos	7480-909	23/10/2009	14	-
			Avis	7480-999	23/10/2009	17	-
						Total: 59	
	Northeast Iberia	Spain	Épila (ZA)	50290	13/10/2009	191	-
			Ejea de los Caballeros (ZA)	50600	13-14/10/2009	130	-
			Candasnos (HU)	22591	15/10/2009	168	-
			Bujaraloz (ZA)	50177	15/10/2009	0	-
						Total: 489	
Ostrinia nubilalis	Central Iberia[3]	Spain	Motilleja (AB)	02220	22/09/2009	-	0
			El Salobral (AB)	02140	22-23/09/2009	-	168
			Aguas Nuevas (AB)	02049	23/09/2009	-	228
			La Gineta (AB)	02110	23/09/2009	-	0
							Total: 396
	Northeast Iberia[3]	Spain	Épila (ZA)	50290	13/10/2009	-	0
			Ejea de los Caballeros (ZA)	50600	13-14/10/2009	-	195
			Candasnos (HU)	22591	15/10/2009	-	214
			Bujaraloz (ZA)	50177	15/10/2009	-	100
							Total: 509

[1] Spanish provinces: AB = Albacete; BA = Badajoz; CC = Cáceres; LE = Lleida; HU = Huesca; ZA = Zaragoza.
[2] Date of the shipment from Portugal
[3] Pairs of fields El Salobral / Aguas Nuevas and Candasnos / Bujaraloz were separated by less than 25 Km.

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 out from mid September to mid November of 2008, 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 with a diet treated with antibiotics. Most of the larvae 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 stage. 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 *O. nubilalis* were sexed and 50 to 70 couples were placed in oviposition cages, where the adults emerged. The oviposition cages consisted of wire cylinders, 16 cm high and 15 cm diameter. The bottom of the cage was covered with filter paper, the top was covered with a plastic where the females laid the eggs, and cotton soaked in a solution of 10% honey was placed on the sides. 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. Every two or three 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

In case the diet was used to rear larvae collected from the field, one bactericidal antibiotic and one amoebicidal antibiotic were added to the diet:

Component	Proportion	Provider
Aureomycine (chlortetracycline chlorhydrate 5,5%)	0,6 g	Cyanamid Ibérica S.A.

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 of the 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 10mm. 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, 6 to 9 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 & Preisler, 1992) or moult inhibition concentration ratios (MICR) at the MIC_{50}.

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ANNEX III

RAW DATA OF MORTALITY AND MOULTING INHIBITION OF THE BIOASSAYS PERFORMED WITH THE DIFFERENT POPULATIONS

a) ***Sesamia nonagrioides***

Table 1. *S. nonagrioides LABORATORY STRAIN*

	Replicate 1			**Replicate 2**			**Replicate 3**		
Concentration (ng Cry1Ab/cm^2)	No of tested larvae	No of dead larvae (D)	No of larvae that did not moult (D+L1)	No of tested larvae	No of dead larvae (D)	No of larvae that did not moult (D+L1)	No of tested larvae	No of dead larvae (D)	No of larvae that did not moult (D+L1)
0	64	6	6	64	8	15	64	5	5
10	32	6	15	32	9	18	31	2	10
20	32	9	18	32	10	18	32	11	14
40	32	11	25	32	16	27	32	11	17
80	32	16	27	32	18	30	32	19	27
160	32	23	32	32	24	32	32	20	29
320	32	29	32	32	27	32	32	29	32
640	32	32	32	32	29	32	32	23	29

Table 2. *S. nonagrioides NORTHEAST IBERIA*

	Replicate 1			Replicate 2			Replicate 3		
Concentration (ng Cry1Ab/cm^2)	No of tested larvae	No of dead larvae (D)	No of larvae that did not moult (D+L1)	No of tested larvae	No of dead larvae (D)	No of larvae that did not moult (D+L1)	No of tested larvae	No of dead larvae (D)	No of larvae that did not moult (D+L1)
0	64	3	3	64	2	6	64	3	11
2.5	32	1	4	32	1	5	32	3	11
5	32	4	7	32	2	6	32	4	12
10	32	3	9	32	6	14	32	3	17
20	32	8	19	32	3	19	32	5	16
40	32	5	18	32	6	22	32	3	20
80	32	10	27	32	7	26	32	5	21
160	32	17	31	32	7	31	32	13	27
320	32	16	31	32	16	31	31	16	29
640	32	17	31	32	19	32	32	17	31

Table 3. *O. nubilalis LABORATORY STRAIN*

	Replicate 1			Replicate 2			Replicate 3		
Concentration (ng Cry1Ab/cm^2)	No of tested larvae	No of dead larvae (D)	No of larvae that did not moult (D+L1)	No of tested larvae	No of dead larvae (D)	No of larvae that did not moult (D+L1)	No of tested larvae	No of dead larvae (D)	No of larvae that did not moult (D+L1)
0	64	3	5	64	1	5	64	2	9
0.25	32	2	2	32	4	9	32	2	4
0.5	32	0	2	32	4	6	32	3	9
1	32	4	10	32	6	14	32	3	10
2	32	2	8	32	4	13	32	1	10
4	32	11	31	32	10	17	32	6	10
8	32	25	32	32	10	20	32	8	18
16	32	28	32	32	24	31	32	21	30
32	32	37	32	32	31	32	32	30	32
64	32	32	32	32	32	32	32	32	32
128	32	32	32	32	32	32	32	32	32