Appendix 7. Insect Resistance Monitoring in Iberian populations of *Sesamia nonagrioides*: 2010 Season

MINISTERIO
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INSECT RESISTANCE MONITORING ASSOCIATED WITH MON810 MAIZE CULTIVATION IN THE EU

Report: Season 2010

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 (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

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 2010 were:

1) To detect shifts in susceptibility to MON810 maize of *S. nonagrioides* populations of two maize growing areas of the Iberian Peninsula: Central Iberia and Southwest Iberia (including areas from Spain and Portugal).

2) To collect populations of *O. nubilalis* from Southwest Iberia (including areas from Spain and Portugal) to be sent to the BioOK laboratory in Germany. 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 of a laboratory strain of *O. nubilalis* to Cry1Ab.

2. Methodology

Last instar larvae of each population of the corn borers *O. nubilalis* and *S. nonagrioides* were collected from 2-3 locations in the different maize growing areas selected. The samples were taken during September and October of 2010 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 2011 on F1 progeny.

The protocol followed 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.

In the last campaign (2009) the susceptibility to the Bt toxin was measured by lethal concentration (LC) and molting inhibition concentration (MIC) values in the two target species of corn borers. The study concluded that in *S. nonagrioides* MIC values were more precise to reflect changes in susceptibility to Cry1Ab than LC values; on the other hand, in *O. nubilalis* both LC and MIC values could be used as comparators among populations to evaluate shifts in susceptibility to the Bt toxin. Taking into account these results, the susceptibility was 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 small number of larvae of *S. nonagrioides* was found in Southwest Iberia: in Spain only 41 larvae were collected after prospects in four different locations, and no larvae were found in three of them; in Portugal, only 123 larvae were collected from one location examined. This situation is similar to that of campaigns of 2008 and 2009. In Central Iberia enough last instar larvae (570) were found in three of the four fields inspected (Annex I).

More than five hundred larvae of *O. nubilalis* were collected in three locations of Southwest Iberia: two in Spain (from the four locations inspected) and one in Portugal.

3.2. Susceptibility to Cry1Ab in the 2010 campaign

Data corresponding to mortality and growth inhibition at the different concentrations of Cry1Ab tested were analysed 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). Fitted curves of susceptibility to the toxin Cry1Ab of laboratory and field populations of the two corn borers were generated taking into account the moulting inhibition of neonate larvae after seven days feeding on treated diet (Figure 1).

3.2.1. S. nonagrioides

The larvae collected in Southwest Iberia (a total of 164) did not reach the minimum number required for the bioassay. Thus, the bioassay of the Southwest population was made with the F2 generation from the field collected larvae. Some studies have shown for the Cry1Ab protein that there was no significant difference between the results of bioassays using advanced generations compared to those using earlier generations (Marçon *et al.* 1999; Saeglitz *et al.* 2006).

Due to the results of last campaign (2009), from this season onwards, only values of MIC will be used to assess the susceptibility of this species to Cry1Ab. Susceptibility of the field populations of *S. nonagrioides* were very similar (MIC_{50} = 16 and 10 ng Cry1Ab/cm^2 in the populations from Southwest and Central Iberia, respectively), being slightly lower in the laboratory colony (MIC_{50} = 8 ng Cry1Ab/cm^2) (Table 1A). All bioassayed larvae that were found alive at the highest dose died when they were fed on Bt maize plants. When the three populations were compared by the MICR, only the one from Southwest Iberia was twice more tolerant to Cry1Ab than laboratory colony at MIC_{50} level, but they were not significantly different at the MIC_{90} level. It has been previously showed that oscillations in susceptibility values to the toxin Cry1Ab in different years is common in *S. nonagrioides* and these variations are not an evidence of resistance acquisition of the tested population (Farinós *et al.* 2004).

Fitted curves of susceptibility to Cry1Ab revealed the similarity of values found in the three populations tested (Figure 1A).

3.2.2. O. nubilalis

Susceptibility of the laboratory strain of *O. nubilalis* to the Cry1Ab toxin was analyzed by LC and MIC. This population displayed LC_{50} and MIC_{50} values of 10 and 2 ng Cry1Ab/cm^2, respectively. These values very similar to those obtained for the same population in the last season (LC_{50} = 9 ng Cry1Ab/cm^2 and MIC_{50} = 3 ng Cry1Ab/cm^2).

Table 1. Susceptibility to Cry1Ab toxin of one laboratory population and two field population of *S.nonagrioides* (A) and a laboratory population of *O. nubilalis* (B) during this 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	2010	859	1.3 ± 0.1	40.7	25	8 (5-11)	1	74 (51-117)	1
Southwest Iberia F2	2010	768	1.7 ± 0.1	48.8	22	16 (11-21)	2.0 (1.4-2.9)*	86 (60-141)	1.2 (0.8-1.8)
Central Iberia	2010	800	1.2 ± 0.1	63.2	37	10 (6-14)	1.3 (0.9-1.9)	119 (81-200)	1.6 (1.0-2.6)

B) *Ostrinia nubilalis*

Population	Year	n[a]	Slope ± SE	χ^2	d.f.	LC_{50}[b] (FL 95%)	LC_{90}[b] (FL 95%)
Laboratory	2011	672	1.3 ± 0.1	28.1	19	9.7 (7.5-13.1)	90 (53-194)
						MIC_{50}[b] (FL 95%)	**MIC_{90}[b] (FL 95%)**
Laboratory	2011	576	2.4 ± 0.2	32.7	16	2.0 (1.5-2.5)	6.7 (5.1-10.1)

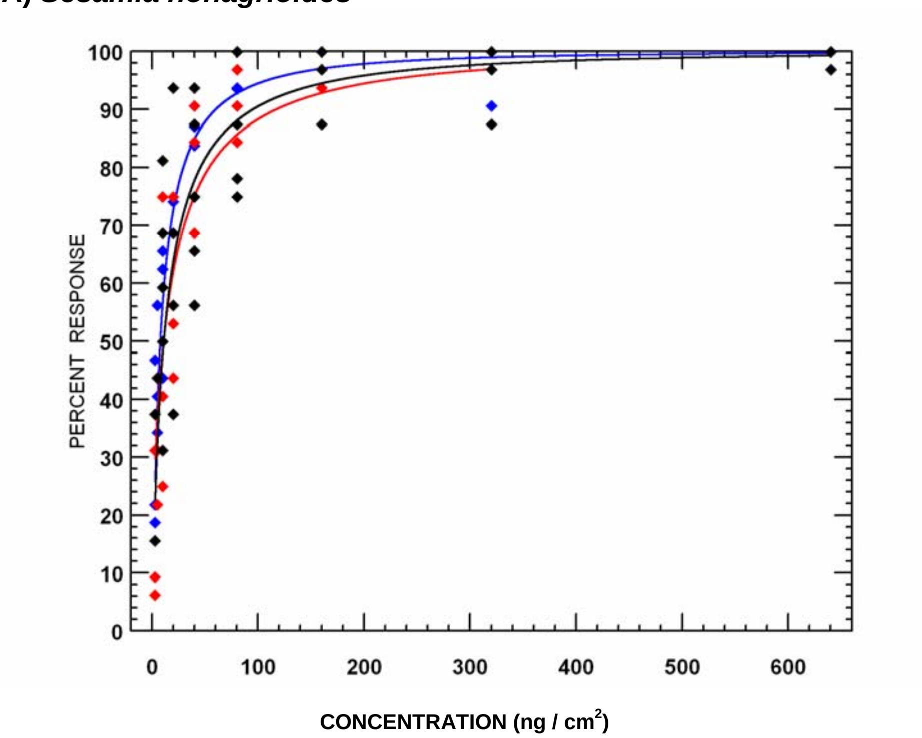
[a] n does not include controls

[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.

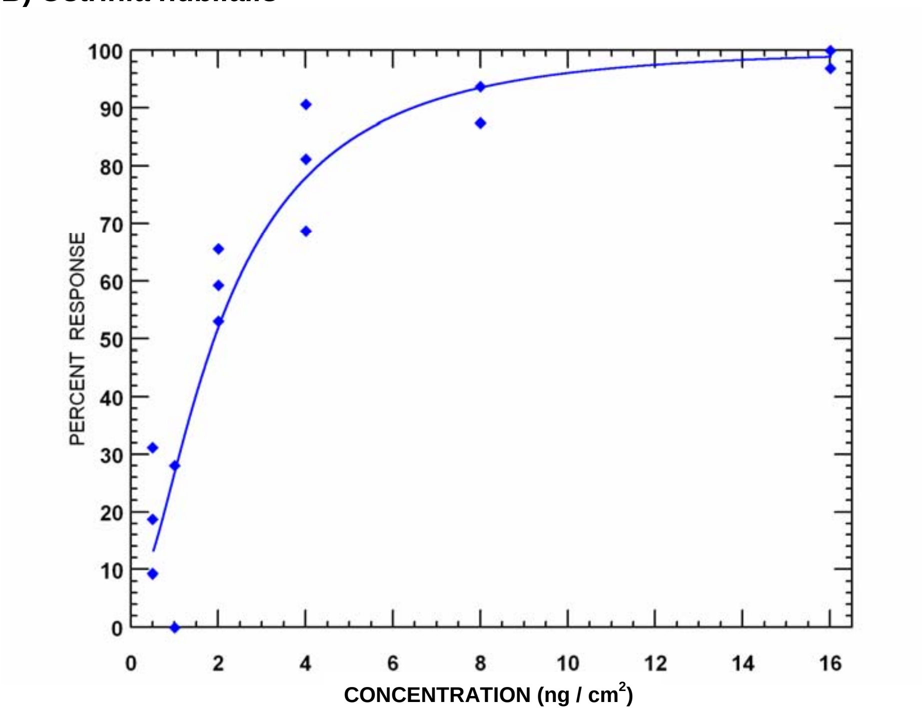
[c] Lethal concentrations significantly different ($P < 0.05$) if the LCR 95% confidence interval does not include 1.

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

A) ***Sesamia nonagrioides***



B) ***Ostrinia nubilalis***



3.3. Historical susceptibility of corn borers to Cry1Ab

3.3.1. S. nonagrioides

Bioassays performed with 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 in 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.

Every year, the larvae that survived the diet overlay bioassays with purified toxin were placed on Bt maize to check for resistance, and all of them died in the following three days.

3.3.2. O. nubilalis

Larvae of *O. nubilalis* in general showed higher susceptibility to the toxin Cry1Ab than *S. nonagrioides* (Table 4). LC_{50} and LC_{90} values of field collected larvae were consistent in all regions and years examined, being the maximum magnitude of variation about 7-fold (for LC_{50} values) and 9-fold (for LC_{90} values). The lowest value of MIC_{50} in field populations 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). Values of MIC_{90} were more homogeneous, being the maximum difference between populations of around 9-fold. This result can be explained because the concentrations used in the past (before the season 2010) were fixed according to the LC_{50} values. Starting from this season (2010) lower and more suitable concentrations to assess MIC_{50} have been used to obtain better-adjusted results of this estimate.

When only the control laboratory strain was considered, values of MIC_{50} were comprised between 0.6 and 3.4 ng Cry1Ab/cm^2. The variation was 5- and 6-fold for LC_{50} and MIC_{50} values, respectively.

In view of the results, both LC and MIC values could be used as comparators among populations of *O. nubilalis* 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 2010. 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)
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)

[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 2010. 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)
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. Shifts in the susceptibility to the toxin Cry1Ab expressed in the MON810 maize have been evaluated by means of MIC values in three populations of *S. nonagrioides* (two field populations, from Southwest and Central Iberia, and one laboratory strain) and by means of LC and MIC in one laboratory population of *O. nubilalis*. The susceptibility of the population of *S. nonagrioides* from Southwest Iberia was determined in the second generation (F2) due to the low numbers of last instar larvae collected in this area during the 2010 campaign.

2. Susceptibility to the Cry1Ab toxin of the populations of *S. nonagrioides* from Southwest and Central Iberia has been assessed for fourth time since 2004, but it has been the first time that larvae collected from Spain and Portugal have been analyzed together. In both cases, differences found in the susceptibility to the toxin are within the range of variability expected for field populations of this corn borer. Values of MIC_{50} and MIC_{90} were also comparable with those showed by the laboratory strain.

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. Both LC and MIC values evidenced consistency through time, showing around 5-fold variation in both LC_{50} and MIC_{50} values.

4. The analyses of historical series of data of susceptibility of *S. nonagrioides* to Cry1Ab did not reveal signs of resistance to this toxin in field populations from the sampling areas considered.

Madrid, 23th May 2011

ANNEX I

LOCATIONS AND NUMBERS OF CORN BORERS COLLECTED IN 2010

Species	Population	Country	Fields (Province)[1]	Postal Code	Date	No of larvae collected	No of larvae sent to Germany
Sesamia nonagrioides	Southwest Iberia	Spain	Don Benito (BA)	06400	6-8/09/2010	0	-
			Navalvillar de Pela (BA)	06760	7/09/2010	0	-
			Madrigalejo (CC)	10110	7/09/2010	0	-
			Porzuna (CR)	13120	15-16/09/2010	41	-
		Portugal	Avis	7480	8/10/2010[2]	123	-
						Total: 164	
	Central Iberia	Spain	Motilleja (AB)	02220	21/09/2010	195	-
			La Herrera (AB)	02162	21/09/2010	251	-
			La Gineta (AB)	02110	22/09/2010	0	-
			Aguas Nuevas (AB)	02049	22/09/2010	124	-
						Total: 570	
Ostrinia nubilalis	Southwest Iberia	Spain	Don Benito (BA)	06400	6-8/09/2010	193	193
			Navalvillar de Pela (BA)	06760	7/09/2010	0	0
			Madrigalejo (CC)	10110	7/09/2010	0	0
			Porzuna (CR)	13120	15-16/09/2010	188	188
		Portugal	Santiago - do - Cacém	7565-100	12-13/10/2010	167	167
						Total: 548	**Total: 548**

[1] Spanish provinces: AB = Albacete; BA = Badajoz; CC = Cáceres; CR = Ciudad Real.
[2] Date of the shipment from Portugal

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 2010, 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. 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 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 *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

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 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, 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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