Appendix 3. Insect Resistance Monitoring in Iberian collections of *Sesamia nonagrioides*: 2013 Season

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INSECT RESISTANCE MONITORING REPORT FOR *Sesamia nonagrioides* ASSOCIATED WITH MON 810 MAIZE CULTIVATION IN THE EU

Season 2013

CONTENTS

1. Introduction

Maize containing event MON 810 is transgenic improved maize expressing the Cry1Ab protein derived from *Bacillus thuringiensis* subsp. *kurstaki*, and conferring protection against certain lepidopteran insect pests such as *Ostrinia nubilalis* and *Sesamia nonagrioides*. Resistance development in targeted lepidopteran pests is a potential concern arising from the widespread cultivation of MON 810 maize varieties. In order to maintain the benefits obtained from growing MON 810 maize varieties, Monsanto, following directions described in the industry IRM (Insect Resistance Management) working group guidelines proposed to the competent authority (EU Commission), available since 2003 but published in 2007 (Alcalde et al., 2007) and subsequently updated as the EuropaBio Harmonised IRM plan (EuropaBio, 2012), established an insect resistance monitoring program across Europe and in particular in areas where commercial activity of MON 810 genetically improved maize is occurring or planned for the European targeted pests *O. nubilalis* and *S. nonagrioides*. This report focuses on the monitoring plan for *S. nonagrioides*.

The Mediterranean corn borer, *Sesamia nonagrioides* Lefèbvre (Lepidoptera: Noctuidae), is one of the most damaging pests of maize in Spain and the circum-Mediterranean countries (Castañera, 1986; Farinós et al., 2012). This species completes a variable number of generations per year depending on latitude, ranging from two in southern France to up to four in Morocco (Anglade 1972, Eizaguirre and Fantinou, 2012). Larvae of the first generation are particularly destructive because they tunnel throughout the maize stem during the whole larval stage, causing great damage to maize seedlings and making their control particularly difficult.

In accordance with the EuropaBio Harmonised IRM plan (EuropaBio, 2012) the baseline susceptibility of *S. nonagrioides* to the Bt Cry1Ab protein needs to be established after which subsequent routine monitoring for changes in susceptibility 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. This program will enable early detection of potential development of resistance in *S. nonagrioides* if it occurs.

Previous baseline susceptibility to Cry1Ab protein has been established for *S. nonagrioides* populations collected in different maize areas in Spain (González-Núñez et al., 2000, Farinós et al., 2004). 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.

This report focuses monitoring resistance of *S. nonagrioides* to Cry1Ab in the main Iberian areas of adoption of MON 810, mostly located in Spain. Baseline was gathered for other areas in Europe but since adoption in those areas is less than 20%, monitoring resistance is not necessary according to the EuropaBio Harmonized IRM plan (EuropaBio, 2012) and therefore is not reported. In Iberia, each target field population is monitored every two years, but for practical reasons they have been divided into two groups so that each year sampling is carried out in one of the groups.

The objectives of the 2013 maize growing season are:

1) To determine the susceptibility of a *S. nonagrioides* population of Northeast Iberia to the Cry1Ab protein expressed in MON 810 maize varieties by means of moulting inhibition concentration (MIC) values and a diagnostic dose.

2) To collect larvae of *O. nubilalis* from Central and Northeast Iberia to send them to the laboratory BTL GmbH Sagerheide (Germany) for testing their susceptibility to the Cry1Ab protein. This laboratory is carrying out the European resistance monitoring programme of *O. nubilalis* for MON 810 maize.

3) To analyze the susceptibility to Cry1Ab of laboratory strains of *S. nonagrioides* and *O. nubilalis* to verify the activity of the batch of protein used in the bioassays with field populations.

2. Materials and Methods

2.1. Insect collection

Three areas have been identified in Iberia where the penetration of MON 810 has been significant: Iberia Northeast (the Ebro valley), Central Iberia (particularly the province of Albacete) and Iberia Southwest (comprising Extremadura and Western Andalucía in Spain and Southern Portugal). Susceptibility of filed populations of *S. nonagrioides* and *O. nubilalis* to Cry1Ab in these areas has been assessed since 2004 every two years. For this season, larvae of *S. nonagrioides* have been collected in Northeast Iberia and larvae of *O. nubilalis* in Northeast and Central Iberia.

Last instar larvae of both corn borers were collected before harvesting in naturally infested fields or refuges to MON 810 maize varieties fields following standard operative procedures (SOP) of each species (EuropaBio, 2012). For each region that was sampled, it was tried to choose three sampling sites separated by at least 50 km.

The samples were collected during September and October of 2013 from refuges and fields of conventional maize adjacent to MON 810 maize by cutting the stalk of the maize plants and taking only one larvae of each species per plant to avoid collecting siblings. Testing early generations is recommended in resistance monitoring plans (Sivasupramaniam, 2007). Therefore, susceptibility to the protein Cry1Ab was carried out on F1 progeny.

This insect collection and area setting scheme is in compliance with the EuropaBio Harmonized IRM plan (EuropaBio, 2012).

2.2. Insect culture

In the laboratory the field collected 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).* Both species were fed on an artificial diet established from that described by Poitout and Buès (1970) with some modifications **(Tables 1 and 2).**

Immediately after asepsis, larvae of *O. nubilalis* from Central and Northeast Iberia were sent to BTL GmbH Sagerheide (Germany) to be analyzed there.

Larvae of *S. nonagrioides* from Almudévar (Northeast Iberia), collected in October, were in diapause, so they were placed on a rearing chamber at 15 ± 1ºC, 70 ± 5% relative humidity and a photoperiod of 12:12 hours (light: dark). They were kept in diapause during 2 months. To interrupt diapause, larvae were placed under conditions 28 ± 1ºC, 70 ± 5% relative humidity and continuous light. Once the diapause was interrupted, larvae pupated and the process continued in a growth chamber (Sanyo MLR-350 H, Sanyo, Osaka, Japan) 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 confined in ventilated plastic cylinders (12 cm diameter x 30 cm high) containing 5-7 maize seedlings for oviposition. 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.

2.3. Quality of the laboratory strain

To preserve the quality of the laboratory strain of *S. nonagrioides,* this population is refreshed every year with new healthy individuals collected in the field, to ensure that the population does not collapse. To that end, the progenies of the same populations collected in the field for the monitoring are used. Before introducing new individuals they are maintained separately for at least two generations in the laboratory after checking, by the susceptibility bioassay, that LC_{50} values are not significantly different to that of the laboratory strain. It is also verified that the new population is free of pathogens (namely *Nosema* sp.) by inspecting a number of larvae in slides under the microscope.

2.4. Bioassays

2.4.1. Susceptibility to Cry1Ab in dose-response bioassays

Two batches of Cry1Ab protein had been used since the start of the MON 810 monitoring plan (2004) to the last season (2012). The first batch (B1) was provided by Monsanto in 2003 (concentration 2.03 mg/ml in sodium bicarbonate buffer, pH 10.5; purity 95%). To prepare the test concentrations, a bicarbonate buffer pH 10.5 was used. The second batch (B2) was provided by Monsanto in October 2011 (concentration 1.8 mg/ml in 50 mM sodium bicarbonate buffer, pH 10.25; purity 91%). In February 2014 Monsanto provided a third batch (B3) of Cry1Ab with the same characteristics (concentration, purity and buffer) than the previous one. Stock solutions were prepared from the original and kept in the freezer at -80ºC until used. Aliquots were thawed only when the bioassay was ready to be performed. To prepare the test concentrations, a sodium bicarbonate buffer (50 mmol/l) with pH 10.25 was used. The bioassays performed in this season have been performed using the batches B2 and B3 of Cry1Ab.

The bioassays were carried out in accordance with the methods described by Farinós et al. (2004).

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 Cry1Ab were added to the surface of the diet. The controls consisted of the sodium bicarbonate buffer solution used to dilute the toxin. After drying the wells under a laminar flow hood, 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 (lethal concentration, LC) and moulting inhibition (moulting inhibition concentration, MIC) relative to the negative control after 7 days of exposure, where mortality equals larvae not showing any reaction when prodded and moulting inhibition larvae that have either died or not molted to the 2nd instar after the 7 days.

The concentration ranges used were comprised between 1.25 and 160 ng Cry1Ab/cm^2 for the populations of *S. nonagrioides*, and between 0.25 and 32 ng Cry1Ab/cm^2 for the laboratory population of *O. nubilalis* tested. These concentrations have been established according to values of moult inhibition obtained in the laboratory in previous years with the batch B2. In order to determine the susceptibility of each population, 7 to 10 different concentrations resulting in mortality or molt inhibition higher than 0% and below 100% were used. At least three replicates were prepared for each concentration, including the control. Each replicate consisted of 32 larvae per concentration (64 for 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. 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.

The susceptibility has been determined by MICs in *S. nonagrioides* populations because our previous results have proved that it is a robust parameter for this species; and by LCs and MICs in a laboratory population of *O. nubilalis*.

2.4.2. Diagnostic dose

Another approach to the dose-mortality testing for monitoring Bt maize resistance would be the use of diagnostic doses (Sims et al., 1997; Marçon et al., 2000). The diagnostic dose (DD) is here defined to cause 99% of moulting inhibition to first instar larvae (MIC_{99}). An important advantage of this technique is that it is much less time-consuming because fewer individuals must be tested and more populations can be tested (Roush & Miller, 1986; Halliday & Burnham, 1990).

A diagnostic dose (MIC_{99}) of 726 ng Cry1Ab/cm^2, according to the one obtained last season with data from larvae collected in different locations of Southwest, Central and Northeast from 2008 to 2012 (see report 2012 for details), was used for the population of *S. nonagriodes* collected in Northeast of Spain in 2013.

2.4.3. Larval survival on MON 810 tissue

MON 810 maize was grown in the greenhouse and leaf material from plant growth stages V5-V8

harvested for their use in a confirmatory experiment. The confirmatory experiment consisted in exposing surviving larvae from the protein bioassays and left-over larvae generated from field collections (which were not used in bioassays) to MON 810 leaves for a period of 10 days and observing survival. Larvae were transferred to plastic boxes in groups of ≈ 50 larvae, provided with newly detached MON 810 maize leaves without the central nerve, and were allowed to feed ad libitum.

2.5. Statistical analysis

The results obtained for mortality or growth inhibition at different concentrations of Cry1Ab (dose-response bioassays) were adjusted by probit weighted regression lines. 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 PoloPlus 1.0 (LeOra Software, 2002-2014).

3. Results and Discussion

3.1. Collection of larvae

A total of 742 larvae of *S. nonagrioides* were collected in the 6 fields of the provinces of Navarra and Huesca inspected in Northeast Iberia, although only in three of them (Tafalla, Candasnos 1 and Almudévar) larvae were assembled in sufficient number (**Table 3**). The larvae from Almudévar were in diapause, whereas the rest pupated soon after in the laboratory. Locations where the larvae of *S. nonagrioides* were collected are displayed in a map in **Annex II.**

In Northeast Iberia, larvae of *O. nubilalis* were principally collected from three fields: Ribaforada, Candasnos 1 and Almudévar (**Table 3**), being larvae from Almudévar in diapause. Most of the larvae (142, 145 and 165 from each field, respectively) were sent to the laboratory BTL GmbH Sagerheide (Germany), for testing their susceptibility to the Cry1Ab protein.In Central Iberia a total of 9 fields, all located in the province of Albacete, were inspected, although larvae of *O. nubilalis* were only found in two of them (La Herrera and Barrax) in sufficient number. These two locations are separated by less than 50 km but it was not possible to find other fields fulfilling this requirement, since maize in the province of Albacete is very concentrated in a relatively small area. Larvae (207 and 225 from each field, respectively) were sent to Germany just after asepsis

3.2. Susceptibility to Cry1Ab in the 2013 campaign

To determine the susceptibility to Cry1Ab, larval mortality and larval molt inhibition records 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 *S. nonagrioides* and *O. nubilalis* **(Table 4).** The significance of differences in susceptibility between the laboratory strain and the field population of *S. nonagrioides* was tested by determining the 95% confidence intervals of molt inhibition concentration ratios (MICR) at the MIC_{50} (Robertson *et al.,* 2007). Since a new batch of toxin (B3) was received from Monsanto in February of 2014, this was also tested on the laboratory population of *S. nonagrioides* using the same concentrations used in the bioassay with the toxin batch B2, and their MIC_{50}s were compared by MICR **(Table 4)**. Fitted curves of susceptibility to the Cry1Ab protein 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

For this season, the values of MIC_{50} obtained for the laboratory population with the protein batches B2 and B3 were similar (7 and 5 ng Cry1Ab/cm^2, respectively), not presenting significant differences (MICR=0.7 (0.5-1.1)) **(Table 4A)**.

As it was informed in the "*Insect resistance monitoring report for Sesamia nonagrioides associated with MON 810 maize cultivation in the EU. Season 2009"*, data of MIC were calculated for first time for *S. nonagrioides* in 2009 and 2010 for field and laboratory populations, respectively, using adequate concentrations for this parameter. Previously, bioassays included concentrations effective to calculate LC values. After that Report, it was decided to use MIC_{50} instead LC_{50} from that point on, because MIC values are more consistent for this bioassay and species than LCs. To assess the quality of the LC and MIC values of the laboratory population, historical LC (from 2004 to 2011) and MIC (from 2010 to 2013) values of this strain have been compared with respect to LC and MIC values measured for first time in the same population (**Table 5).** LC values were significantly higher in 2007 and 2008 than in 2004 (**Table 5, Figure 2**), but they decreased again in 2010 and 2011 to reach values similar to the baseline. In the case of MIC values, they did not present significant differences compared to the baseline, regardless the toxin batch used (**Table 5, Figure 3**).

With respect to the field population assessed, the bioassay to evaluate the susceptibility of the population from the Northeast Iberia of *S. nonagrioides* to Cry1Ab was performed with neonates of the F1 generation of the field-collected larvae in two steps: three replicates were made in October 2013 using the F1 generation from last instar larvae that were not collected in diapause, and a fourth replicate (with larvae from Almudévar, Huesca) was performed two months later, after a diapause period. Then, all the replicates were analyzed together giving a total of 128 larvae tested per concentration (256 for the controls). The susceptibility to the Cry1Ab protein showed by the Northeast Iberia population (MIC_{50} = 19 ng Cry1Ab/cm^2) was lower than that of the control

laboratory population tested with the same toxin batch, B2 (MICR = 2.6 (2.0-3.4)) **Table 4A,** but both MIC_{50} and MIC_{90} values were at the same level as those obtained in previous years **(Table 6).** Differences between laboratory and field colonies have been observed historically, as well as changes in susceptibility to the toxin Cry1Ab of a population in different years (**Table 6**), suggesting that it could be due to common natural variations already reported in *S. nonagrioides* (González-Núñez et al. 2000; Farinós *et al.* 2004).

3.2.2. O. nubilalis

As in the case of *S. nonagrioides*, the quality of the laboratory strain of *O. nubilalis* is maintained by refreshing it every year with new healthy individuals collected in the field, following the same methodology explained above.

The susceptibility to Cry1Ab toxin of the laboratory strain of *O. nubilali*s was assessed by LCs and MICs. The results among the different replicates were quite variable **(Figure 1B)**. Values of LC_{50} and LC_{90} were 1.7 and 9.3 ng Cry1Ab/cm^2, respectively **(Table 4B)**, slightly lower than values obtained historically for the same population **(Table 7)**. The estimated MIC values for this season (MIC_{50} and MIC_{90} values were 1.1 and 2.5 ng Cry1Ab/cm^2, respectively) were also lower that those obtained previously **(Table 7)**.

3.3. Diagnostic dose

A diagnostic dose of 726 ng Cry1Ab/cm^2, according to the MIC_{99} value obtained last season with data from larvae collected in different locations of Southwest, Central and Northeast Iberia between 2008 and 2012 (see report 2012 for details), was used for the population of *S. nonagriodes* collected in Northeast of Spain in 2013. The molt inhibition got by neonates with this concentration was 97 ± 2 % (mean ± standard error).

3.4. Survival of larvae recovered from bioassays on MON 810 leaves

None of the larvae of *S. nonagrioides* from the Northeast Iberia population which were not killed by the treatment with Cry1Ab in the dose-response bioassay (1003 larvae) could survive after 10 days feeding ad libitum on MON 810 tissue. Additionally, there was no survivor among the neonate larvae that were not used in these bioassays and that were exposed to MON 810 leaves.

3.5. Historical susceptibility of corn borers to Cry1Ab

3.5.1. 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 ng Cry1Ab/cm^2 (Central Iberia in 2006) and 29 ng Cry1Ab/cm^2 (Southwest Iberia in 2012) (**Table 6**), evidencing a magnitude variation of 4.1-fold.

Likewise, values of MIC_{50} of laboratory strains were very uniform. Three toxin batches (B1, B2 and B3) have been used to date to assess their susceptibility to Cry1Ab. All together, MIC_{50} values are comprised between 5 and 19 ng Cry1Ab/cm^2 **(Table 6)**, which means a magnitude variation of 3.8-fold and indicates a variability similar to that found within field populations.

In the light of these results, MIC_{50} values obtained during this campaign for the field collected population and for the laboratory strain of this corn borer are within the range of values got in the past years.

3.5.2. O. nubilalis

LC and MIC values of the control laboratory strain were very consistent in the interval of years examined (2004-2013), being the maximum magnitude of variation 6-fold for both LC_{50} and MIC_{50} values (**Table 7**). 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*. Historical values of LC_{50} for *O. nubilalis* are showed in **Figure 3.**

4. Conclusions

1. It is the fifth time to determine the susceptibility to the Cry1Ab toxin of the field population of *S. nonagrioides* from Northeast Iberia since 2005, and in this period the MIC_{50} values have ranged between 9 and 22 ng Cry1Ab/cm^2. The MIC_{50} for this season was 19 ng Cry1Ab/cm^2, resulting 2.6-fold less susceptible than those of the laboratory strain tested with the same toxin batch.

2. A moult inhibition of 97 ± 2 % was observed in the Northeast Iberian population of *S. nonagrioides* when a diagnostic dose (MIC_{99}) of 726 ng Cry1Ab/cm^2 was used.

3. No survivors have been reported among larvae of the F1 generation exposed to MON 810 leaves.

4. The laboratory strain of *O. nubilalis* showed susceptibility levels to the Cry1Ab toxin comparable with those obtained for laboratory strains in previous years.

5. The analysis of the historical series of data of susceptibility to Cry1Ab of Northeast Iberian populations of *S. nonagrioides* has not evidenced resistance development to this toxin.

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Madrid, 18th July 2014

ANNEX I. TABLES AND FIGURES

Table 1. Artificial diet used for *S. nonagrioides*.

Components	Amount	Provider
Distilled H_2O	1 l	
Agar	26 g	Conda Pronadisa
Maize flour	160 g	Santiveri
Wheat germ	40 g	Santiveri
Yeast	43 g	Santiveri
Ascorbic acid	6 g	Panreac
Benzoic acid	1.25 g	Merck Millipore
Nipagin (Methyl p-hidroxibenzoato)	1 g	Sigma-Aldrich
Wesson's salts mixture	1.55 g	Sigma

Table 2. Artificial diet used for *O. nubilalis*.

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

Table 3. *Sesamia nonagrioides* and *Ostrinia nubilalis* larvae collection details for the 2013 season

Area	Country	Fields (Province)[a]	Postal Code	Date	Surface (Ha) [b]	Distance (m) to the nearest MON 810 field [c]	*S. nonagrioides* No of larvae collected	*O. nubilalis* No of larvae collected	*O. nubilalis* No of larvae sent to BTL (Germany)
Northeast Iberia	Spain	Ribaforada (NA)	31550	10/09/2013	3	0	76	205	142
		Valtierra (NA)	31514	09/09/2013	50	0	0	0	-
		Tafalla (NA)	31300	09/09/2013	8	0	258	0	-
		Candasnos 1 (HU)	22591	11/09/2013	70	3000	212	155	145
		Candasnos 2 (HU)	22591	11/09/2013	10,5	0	25	15	-
		Almudévar (HU)	22270	14/10/2013	5	0	171 [d]	227 [d]	165
Central Iberia	Spain	La Herrera (AB)	02162	24/09/2013	60	300	-	234	207
		Motilleja (AB)	02220	24/09/2013	45	0	-	0	-
		La Gineta 1 (AB)	02110	25/09/2013	2	< 300	-	1	-
		La Gineta 2 (AB)	02110	25/09/2013	1	0	-	2	-
		Barrax (AB)	02639	25/09/2013	2	0	-	285	225
		Aguas Nuevas (AB)	02049	25/09/2013	3	0	-	0	-
		El Salobral (AB)	02140	25/09/2013	3	0	-	3	-
		La Felipa 1 (AB)	02156	25/09/2013	40	>1000	-	0	-
		La Felipa 2 (AB)	02156	25/09/2013	60	>2000	-	15	-

[a] Spanish provinces: AB = Albacete; HU = Huesca; NA = Navarra

[b] Data are approximate.

[c] There could be other nearer fields that are not known by the technician and/or the farmer. "0" means that it is adjacent to a MON 810 field.

[d] Diapausing larvae

Table 4. 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 2013 campaign.

A) *Sesamia nonagrioides*

Population	Year	Toxin batch	n	Slope ± SE	χ^2	d.f.	MIC_{50}[a] (CI 95%)	MICR (MIC_{50})[b] (CI 95%)	MIC_{90}[a] (CI 95%)	MICR (MIC_{90})[b] (CI 95%)
Laboratory [c]	2013	B2	953	1.6 ± 0.1	65.9	22	7 (5-10)	1	48 (31-88)	1
Laboratory [c]	2014	B3	960	1.6 ± 0.1	65.6	22	5 (3-9)	0.7 (0.5-1.1)	42 (26-87)	0.9 (0.6-1.4)
Ebro	2013	B2	1274	1.4 ± 0.1	67.5	30	19 (14-25)	2.6 (2.0-3.4)*	163 (108-287)	3.4 (2.2-5.2)*

B) *Ostrinia nubilalis*

Population	Year	n	Slope ± SE	χ^2	d.f.	LC_{50}[a] (CI 95%)	LC_{90}[a] (CI 95%)
Laboratory	2014	698	1.8 ± 0.16	58.8	19	1.7 (1.1-2.6)	9.3 (5.8-20.3)
						MIC_{50}[a] (CI 95%)	MIC_{90}[a] (CI 95%)
Laboratory	2014	621	2.5 ± 0.4	43.8	16	0.8 (0.5-1.2)	2.8 (1.9-5.0)

[a] 50% and 90% lethal concentrations (LC_{50} and LC_{90}) or moulting inhibition concentrations (MIC_{50} and MIC_{90}) and their 95% confidence intervals (CI95%) are expressed in ng Cry1Ab/cm^2.
[b] Molt inhibition concentrations significantly different (*) ($P < 0.05$) with respect to the laboratory strain if the MICR 95% confidence interval does not include 1.
[c] The laboratory population of *S. nonagrioides* was tested in two different generations with the two toxin batches of Cry1Ab (B2 and B3, see text), to compare the effectiveness of both batches against this species.

Table 5. Lethal concentration ratio (LCR) and molt inhibition concentration ratio (MICR) at LC_{50} and MIC_{50} level, respectively, and their 95% confidence limits of a laboratory population of *S.nonagrioides* through time, with respect to LC and MIC values measured for first time in the same population (baseline, shaded values).

Report (season)	Batch of toxin	Measured endpoint	LCR (LC_{50})[a] (CI 95%)	MICR (MIC_{50})[a] (CI 95%)
2004	B1	LC	1	-
2007	B1	LC	1.7 (1.1-2.6)*	-
2008,2009	B1	LC	3.1 (2.2-4.4)*	-
2010	B1	LC-MIC	0.8 (0.5-1.1)	1
2011	B2	LC-MIC	1.1 (0.8-1.6)	1.2 (0.8-1.7)
2012	B2	MIC	-	0.7 (0.5-1.1)
2013	B2	MIC	-	1.0 (0.7-1.3)
2013	B3	MIC	-	0.7 (0.5-1.1)

[a] Lethal concentrations or molt inhibition concentrations significantly different (*) ($P < 0.05$) with respect to baseline if the 95% confidence intervals of LCR or MICR do not include 1. Values have been calculated according to Robertson et al. (2007).

Table 6. Susceptibility to Cry1Ab toxin of laboratory populations and Iberian field populations of *S. nonagrioides* collected in refuge areas of MON 810 between 2004 and 2013. Bioassays performed during this campaign are shaded.

Population[a]	Season	Batch of toxin	MIC_{50}[a] (CI 95%)	MIC_{90}[a] (CI 95%)
Laboratory	2004	B1	18 (11-25)	99 (66-208)
Laboratory	2007	B1	16 (11-22)	94 (69-147)
Laboratory	2008-9	B1	19 (10-30)	120 (76-255)
Laboratory	2010	B1	8 (5-11)	74 (51-117)
Laboratory	2011	B2	9 (6-13)	68 (45-127)
Laboratory	2012	B2	7 (5-10)	62 (41-107)
Laboratory	2013	B2	7 (5-10)	48 (31-88)
Laboratory	2013	B3	5 (3-9)	42 (26-87)
Southwest Iberia (Spain)	2005	B1	16[b]	30[b]
Southwest Iberia (Portugal)	2005	B1	8 (3-16)	152 (94-309)
Southwest Iberia (Spain)	2007	B1	17 (10-25)	226 (153-385)
Southwest Iberia F2 (Spain and Portugal)	2010	B1	16 (11-21)	86 (60-141)
Southwest Iberia (Spain)	2012	B2	29 (19-41)	158 (101-339)
Central Iberia	2004	B1	12 (5-22)	248 (143-588)
Central Iberia	2006	B1	7 (1-17)	321 (157-1360)
Central Iberia	2008	B1	28 (18-38)	170 (124-259)
Central Iberia	2010	B1	10 (6-14)	119 (81-200)
Central Iberia	2012	B2	15 (8-25)	160 (79-608)
Northeast Iberia	2005	B1	9 (3-15)	76 (54-117)
Northeast Iberia	2007	B1	14 (8-20)	99 (71-158)
Northeast Iberia	2009	B1	22 (16-28)	188 (138-277)
Northeast Iberia	2011	B2	20 (14-27)	135 (91-232)
Northeast Iberia	2013	B2	19 (14-25)	163 (108-287)

[a] 50% and 90% lethal concentrations (LC_{50} and LC_{90}) or moulting inhibition concentration (MIC_{50} and MIC_{90}) and their 95% confidence intervals (CI95%) are expressed in ng Cry1Ab/cm^2 .

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

Table 7. Susceptibility to Cry1Ab toxin and a laboratory strain of *O. nubilalis* between the season 2004 and 2013. The bioassays performed during this campaign is shaded.

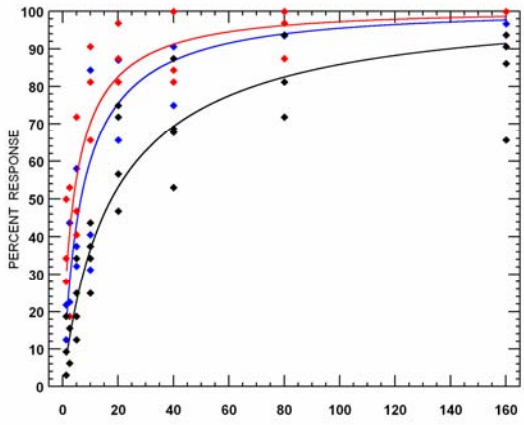
Population	Season	Batch of toxin	LC_{50}[a] (CI 95%)	LC_{90}[a] (CI 95%)	MIC_{50}[a] (CI 95%)	MIC_{90}[a] (CI 95%)
Laboratory	2004-2005	B1	4 (2-7)	19 (12-54)	2.1[b]	9.0[b]
Laboratory	2007	B1	2 (1-4)	17 (11-31)	0.6 (0.03-1.2)	2.3 (1.2-3.2)
Laboratory	2008	B1	2 (2-3)	20 (13-33)	0.8 (0.6-1.0)	2.9 (2.3-4.1)
Laboratory	2009	B1	9 (7-11)	26 (19-44)	3.4 (1.6-5.6)	19.0 (10.0-107.3)
Laboratory	2010	B2	10 (8-13)	90 (53-194)	2.0 (1.5-2.5)	6.7 (5.1-10.1)
Laboratory	2011	B2	4 (3-5)	20 (16-28)	2.8[b]	5.0[b]
Laboratory	2012	B2	2.5 (2.0-3.2)	16 (12-25)	1.1 (0.8-1.4)	2.5 (2.0-3.8)
Laboratory	2013	B3	1.7 (1.1-2.6)	9 (6-20)	0.8 (0.5-1.2)	2.8 (1.9-5.0)

[a] 50% and 90% lethal concentrations (LC_{50} and LC_{90}) or moulting inhibition concentration (MIC_{50} and MIC_{90}) and their 95% confidence intervals (CI 95%) are expressed in ng Cry1Ab/cm^2.

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

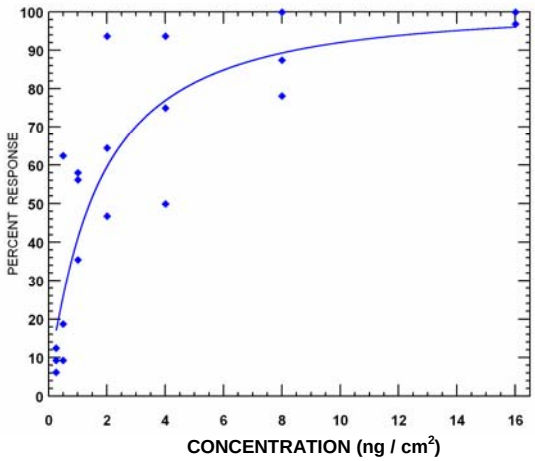
Figure 1. Fitted curves of susceptibility to the toxin Cry1Ab (PoloPlus, LeOra Software, 2002-2014).
A: Laboratory colonies tested with the toxin batches B2 (blue) and B3 (red), respectively, and a field population from Northeast Iberia (black) of *Sesamia nonagrioides* (slopes of individual population 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*

Lethal Concentration



Molt Inhibition Concentration

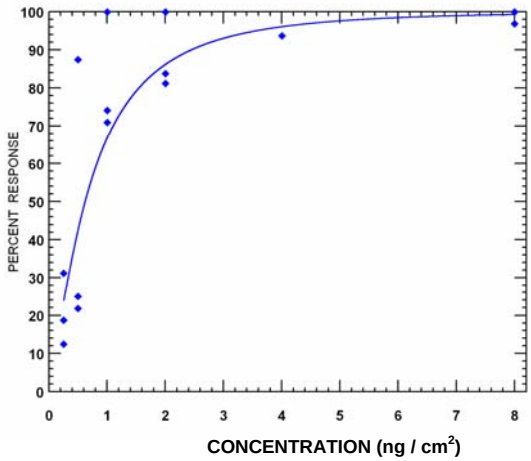


Figure 2. Susceptibility to Cry1Ab toxin, measured by LC_{50} and MIC_{50} values, of a laboratory population of *S.nonagrioides*. Colors indicate the B1 (blue), B2 (red) and B3 (green) toxin batches.

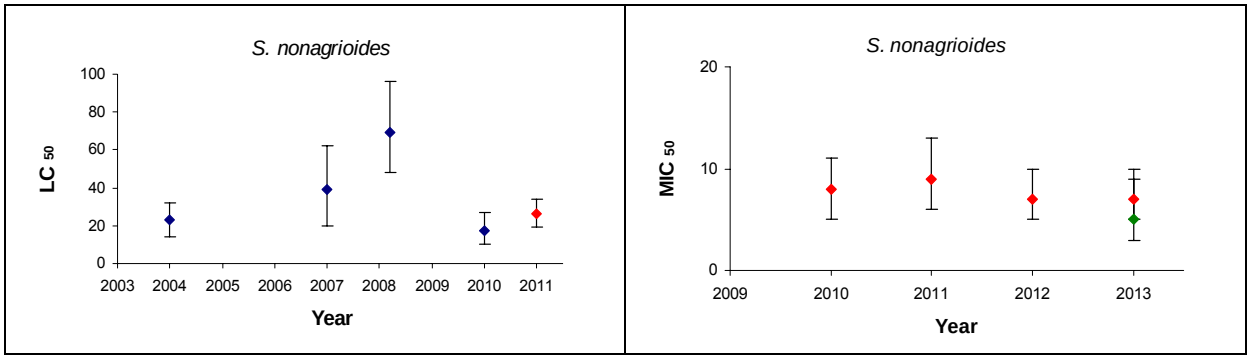
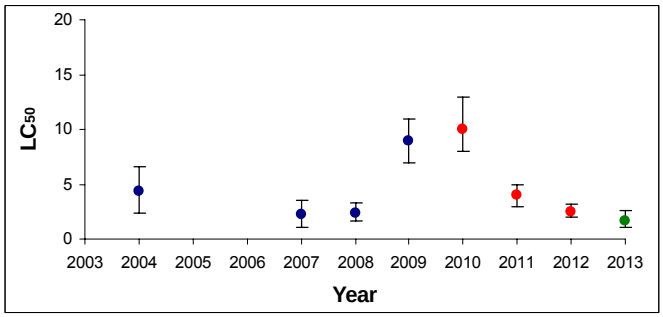


Figure 3. Susceptibility to Cry1Ab toxin, measured by LC_{50} values, of a laboratory population of *O. nubilalis.* Colors indicate the B1 (blue), B2 (red) and B3 (green) toxin batches.



ANNEX II: Sampling locations of *Sesamia nonagrioides* in 2013