Appendix 7. Insect Resistance Monitoring in Iberian collections of *Sesamia nonagrioides*: 2014 Season

INSECT RESISTANCE MONITORING REPORT FOR *Sesamia nonagrioides*

ASSOCIATED WITH MON 810 MAIZE CULTIVATION IN THE EU

Season 2014

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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 2014 maize growing season are:

1) To determine the susceptibility of a *S. nonagrioides* population of Southwest and Central 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 Southwest Iberia to send them to the laboratory BTL GmbH Sagerheide (Germany) for testing their susceptibility to the Cry1Ab protein expressed in MON 810 varieties. This laboratory is carrying out the European resistance monitoring programme of *O. nubilalis* for MON 810 maize.

3) To analyze the susceptibility to Cry1Ab protein 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 field 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 Southwest and Central Iberia and larvae of *O. nubilalis* in Southwest 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, the objective was to choose three sampling sites separated by at least 50 km.

The samples were collected during August and September 2014 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 Cry1Ab protein 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, 2**). Immediately

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

All the larvae of *S. nonagrioides* were collected in September 2014, and none of them had entered diapause, so they were reared in the laboratory following the standard methods, 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), until pupation. Pupae 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 strains

To preserve the quality of the laboratory strains of *S. nonagrioides* and *O. nubilalis,* this population is reinvigorated 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

Three batches of Cry1Ab protein have been used since the start of the MON 810 monitoring plan (2004) to the last season (2014). The first batch (B1) was provided by Monsanto in 2003 (concentration 2.03 mg/ml in sodium bicarbonate buffer, pH 10.5; purity 95%); the second batch (B2) was sent in October 2011 (concentration 1.8 mg/ml in 50 mM sodium bicarbonate buffer, pH 10.25; purity 91%); and the third batch (B3) was provided in February 2014 with the same characteristics (concentration, purity and buffer) than B2. 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 batch 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 moulted to the 2nd instar after the 7 days.

The concentration ranges used were comprised between 0.75 and 96 ng Cry1Ab/cm^2 for the populations of *S. nonagrioides*, and between 0.5 and 64 ng Cry1Ab/cm^2 for the laboratory population of *O. nubilalis* tested. These concentrations have been established according to values of mortality and moult inhibition obtained in the laboratory in the last season with the batches B2 and B3. In order to determine the susceptibility of each population, 7 to 10 different concentrations resulting in mortality or moult 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 is 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, since fewer individuals must be tested and more populations can be examined (Roush & Miller, 1986; Halliday & Burnham, 1990).

A diagnostic dose (MIC_{99}) of 726 ng Cry1Ab/cm^2 was estimated in 2013 with data from larvae collected in different locations of Southwest, Central and Northeast from 2008 to 2012 (see report 2012 for details). This value has been used for the populations of *S. nonagriodes* collected in Southwest and Central Spain in 2014.

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 about 10 days and observing survival. Larvae were transferred to plastic boxes in groups of ≈ 50 larvae, provided

with new 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 644 last instar larvae of *S. nonagrioides* were collected in Southwest Iberia after inspecting ten different fields of Spain and Portugal. The majority of the collected larvae came from three fields of two Spanish provinces: Sanlúcar de Barrameda (Cádiz) and Miajadas and El Batán (Cáceres) (**Table 3**).

In Central Spain *S. nonagrioides* larvae were searched in nine fields of the province of Albacete. Only in two of them (Motilleja and La Gineta) a sufficient number of larvae (> 100) were found (202 and 277 larvae, respectively). 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.

No larvae of *S. nonagrioides*, either from Southwest or Central Iberia, were in diapause. Locations where the larvae of *S. nonagrioides* were collected are displayed in a map in **Annex II.**

Larvae of *O. nubilalis* were collected in sufficient number in Southwest Iberia in two out of ten fields inspected: Mérida (Badajoz, Spain) and Elvas (Portugal) (**Table 3**). All the larvae (246 and 107 from each field, respectively) were in diapause and they were sent to the laboratory BTL GmbH Sagerheide (Germany), for testing their susceptibility to the Cry1Ab protein.

3.2. Susceptibility to Cry1Ab in the 2014 campaign

To determine the susceptibility to Cry1Ab, larval mortality and larval moult 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 moult inhibition concentration ratios (MICR) at the MIC_{50} (Robertson *et al.,* 2007). Fitted curves of susceptibility to the Cry1Ab protein of laboratory and field populations of the two species were generated taking into account the moulting inhibition of neonate larvae after seven days feeding on treated diet (**Figure 1**).

3.2.1. Sesamia nonagrioides

As it was informed in the "*Insect resistance monitoring report for Sesamia nonagrioides associated with MON 810 maize cultivation in the EU. Season 2009"*, MIC values were calculated for the first time for field and laboratory populations of *S. nonagrioides* in 2009 and 2010, 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. Thus, MIC values for the laboratory population are compared every year with respect to the MIC value measured for first time (2010) in the same population (**Table 5**). MIC values did not present significant differences from 2011 to 2013 compared to the baseline, regardless the toxin batch used. In this campaign the MIC_{50} value for the laboratory strain was 17 ng Cry1Ab/cm^2 (**Table 4A**), showing a small increase with respect to that of 2010 (**Table 5, Figure 2**).

The bioassays to evaluate the susceptibility of the two field populations (Southwest and Central Iberia) to Cry1Ab were performed with neonates of the F1 generation in September and November of 2014, respectively, since no larvae were collected in diapause. MIC_{50} values resulted in 31 and 15 ng Cry1Ab/cm^2 for Southwest and Central Iberia, respectively (**Table 4A**). The MIC_{50} value of the laboratory population did not present significant differences with that of the population of Central Spain (MICR=0.8 (0.6-1.2)), whereas MIC_{50} of Southwest was a little higher (MICR=1.8 (1.3-2.5)).

Both MIC_{50} and MIC_{90} values of the two populations tested (Southwest and Central Iberia) were at the same level as those obtained in previous years (**Table 6**). It is important to highlight that 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 these differences and oscillations in susceptibility values to the Cry1Ab protein can be attributed to common natural variations already reported in *S. nonagrioides* (González-Núñez et al. 2000; Farinós *et al.* 2004).

3.2.2. Ostrinia nubilalis

The susceptibility to Cry1Ab toxin of the laboratory strain of *O. nubilalis* was assessed by determination of LCs and MICs. Results among the different replicates were very variable (**Figure 1B**). The LC_{50} was 11.7 ng Cry1Ab/cm^2 (**Table 4B**). This value is between 3-7-fold higher than those obtained in the three previous years, although it is at the same level as values found in 2009

and 2010 (**Table 7**). Fewer differences were observed MIC values (MIC_{50} was 3.2 ng Cry1Ab/cm^2) with respect to former seasons (**Table 7, Figure 3**).

3.3. Diagnostic dose

A diagnostic dose of 726 ng Cry1Ab/cm^2, according to the MIC_{99} value agreed from data of 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. nonagrioides* collected in Southwest and Central Iberia in 2014. The moult inhibition got by neonates with this concentration was 96 ± 2 and 96 ± 1 % (mean ± standard error) for Southwest and Central Iberia, respectively.

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

None of the larvae of *S. nonagrioides* from the Southwest and Central Iberia population which were not killed by the treatment with Cry1Ab in the dose-response bioassay and in the DD bioassay (847 and 803 larvae, respectively) could survive after 12 days feeding ad libitum on MON 810 tissue. Additionally, there was no survivor among the neonate larvae (about 3000) 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. Sesamia nonagrioides

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 historically since 2004.

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} values. MIC_{50}s ranged between 7 ng Cry1Ab/cm^2 (Central Iberia in 2006) and 31 ng Cry1Ab/cm^2 (Southwest Iberia in 2014) (**Table 6**), resulting in a magnitude variation of 4.4-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.

3.5.2. Ostrinia nubilalis

LC and MIC values of the control laboratory strain have also been consistent in the interval of years examined (2004-2014), being the maximum magnitude of variation 6.8- and 5.7-fold for LC_{50} and MIC_{50} values, respectively (**Table 7**).

4. Conclusions

1. The susceptibility to the Cry1Ab toxin of the field population of *S. nonagrioides* from **Southwest Iberia** has been determined for the sixth time since 2005. The MIC_{50} obtained for this season was 31 ng Cry1Ab/cm^2. During the period 2005-2014 MIC_{50} values have ranged between 8 and 31 ng Cry1Ab/cm^2, therefore the maximum magnitude of variation was 3.9-fold. When the field and the laboratory populations were tested with the same toxin batch, the former resulted 1.8-fold less susceptible.

2. The susceptibility to the Cry1Ab toxin of the field population of *S. nonagrioides* from **Central Iberia** has been determined for the sixth time since 2004. The MIC_{50} obtained for this season was 15 ng Cry1Ab/cm^2. During the period 2004-2014 MIC_{50} values have fluctuated between 7 and 28 ng Cry1Ab/cm^2, so the greatest magnitude of variation was 4-fold. Central Iberian larvae collected in 2014 were not less susceptible than those of the laboratory strain.

3. A total of 353 larvae of *O. nubilalis* have been collected from two fields of Southwest Iberia and sent to the laboratory BTL GmbH Sagerheide (Germany) for testing their susceptibility to the Cry1Ab protein expressed in MON 810 varieties.

4. The use of a diagnostic dose (MIC_{99}) of 726 ng Cry1Ab/cm^2 revealed an average moult inhibition of 96 ± 1 % in the Southwest and Central Iberian population of *S. nonagrioides*.

5. No survivors have been reported from the more of 4500 larvae of the F1 generation from both field populations exposed to MON 810 leaves.

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

In short, the analysis of the historical series of data of susceptibility to Cry1Ab of the populations of *S. nonagrioides* from Southwest and Central Iberia has not evidenced resistance development to this toxin.

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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 2014 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 for BTL (Germany)
Southwest Iberia	Spain	Carmona (SE)	04104	18/08/2014	2.5	4	0	-
	Spain	Sanlúcar de Barrameda (CA)	11540	19/08/2014	24	0	216	-
	Spain	Logrosán (CC)	10120	01/09/2014	70	15	24	-
	Spain	Miajadas 1 (CC)	10100	02/09/2014	1.6	0	0	-
	Spain	Miajadas 2 (CC)	10100	02/09/2014	21	400	149	-
	Spain	El Batán (CC)	10816	04/09/2014	7	0	248	29 [d]
	Spain	Montehermoso (CC)	10810	04/09/2014	1.5	0	0	-
	Spain	Mérida (BA)	06800	02/09/2014	10	0	7	246 [d]
	Spain	Lácara (BA)	06487	03/09/2014	52	0	0	-
	Portugal	Elvas	7350	03/09/2014	35	0	0	107 [d]
Central Iberia	Spain	La Herrera (AB)	02162	22/09/2014	60	50	0	-
	Spain	Aguas Nuevas 1 (AB)	02049	22/09/2014	4	0	0	-
	Spain	Aguas Nuevas 2 (AB)	02049	23/09/2014	5	300	0	-
	Spain	Motilleja (AB)	02220	22/09/2014	200	0	202	-
	Spain	La Gineta (AB)	02110	23/09/2014	1	100	277	-
	Spain	Barrax (AB)	02639	23/09/2014	3.5	1000	0	-
	Spain	Santa Ana 1 (AB)	02328	23/09/2014	4	300	0	-
	Spain	Santa Ana 2 (AB)	02328	23/09/2014	1.5	600	0	-
	Spain	El Salobral (AB)	02140	23/09/2014	2	300	0	-

[a] Spanish provinces: AB = Albacete; BA = Badajoz; CA = Cádiz; CC = Cáceres; SE = Sevilla

[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 two field populations of *S.nonagrioides* (A) and a laboratory population of *O. nubilalis* (B) during the 2014 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	2014	B3	956	1.8 ± 0.2	74.1	22	17 (11-25)	1	91 (57-209)	1
Southwest	2014	B3	957	1.5 ± 0.2	36.8	22	31 (23-43)	1.8 (1.3-2.5) *	236 (140-569)	2.6 (1.4-4.9)*
Central	2014	B3	943	1.3 ± 0.1	44.4	22	15 (9-21)	0.8 (0.6-1.2)	138 (81-329)	1.5 (0.8-2.7)

B) *Ostrinia nubilalis*

Population	Year	Toxin batch	n	Slope ± SE	χ^2	d.f.	LC_{50}[a] (CI 95%)	LC_{90}[a] (CI 95%)
Laboratory	2015	B3	854	1.6 ± 0.2	56.9	19	11.7 (7.2-17.2)	72 (40-267)
							MIC_{50}[a] (CI 95%)	MIC_{90}[a] (CI 95%)
Laboratory	2015	B3	726	2.0 ± 0.2	133	15	3.2 (1.1-5.9)	13.6 (7-156)

[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.

Table 5. Lethal concentration ratio (LCR) and moult 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)
2014	B3	MIC	-	2.2 (1.6-3.1)*

[a] Lethal concentrations or moult 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 2014. 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)
Laboratory	2014	B3	17 (11-25)	91 (57-209)
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)
Southwest Iberia (Spain)	2014	B3	31 (23-43)	236 (140-569)
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)
Central Iberia	2014	B3	15 (9-21)	138 (81-329)
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 2014. The bioassay performed during this campaign is shaded.

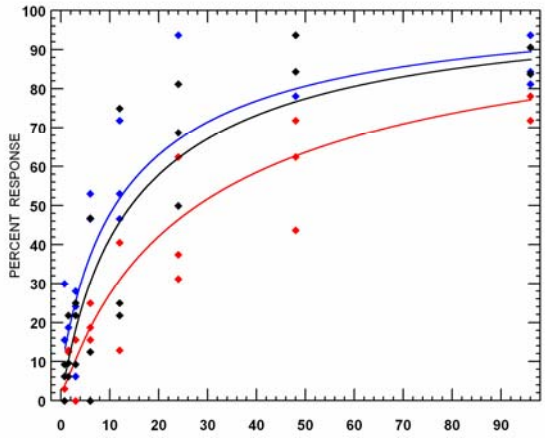
Population	Season	Batch of toxin	LC_{50}[a] (CI 95%)	LC_{90}[a] (CI 95%)	MIC_{50}[a] (CI 95%)	MIC_{90}[a] (CI 95%)
Laboratory	2004-2005	B1	4 (2-7)	19 (12-54)	2.1[b]	9.0[b]
Laboratory	2007	B1	2 (1-4)	17 (11-31)	0.6 (0.03-1.2)	2.3 (1.2-3.2)
Laboratory	2008	B1	2 (2-3)	20 (13-33)	0.8 (0.6-1.0)	2.9 (2.3-4.1)
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)
Laboratory	2014	B3	11.7 (7.2-17.2)	72 (40-267)	3.2 (1.1-5.9)	13.6 (7.0-156.1)

[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.

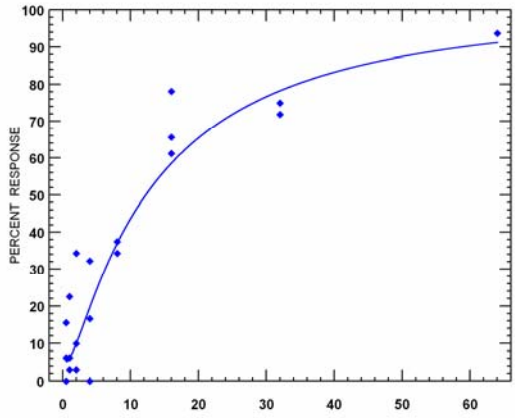
Figure 1. Fitted curves of susceptibility to the toxin Cry1Ab (PoloPlus, LeOra Software, 2002-2015).
A: Laboratory colony (black) and two field population from Central Iberia (blue) and Southwest Iberia (red) of *Sesamia nonagrioides*. Slopes of individual population lines were constrained to be parallel. Response is moult inhibition after seven days feeding on treated diet.
B: Laboratory colony of *Ostrinia nubilalis*. Response is mortality (B1) or moult inhibition (B2) after seven days feeding on treated diet.

A) *Sesamia nonagrioides*



B) *Ostrinia nubilalis*

B1. Lethal Concentration



B2. Moult Inhibition Concentration

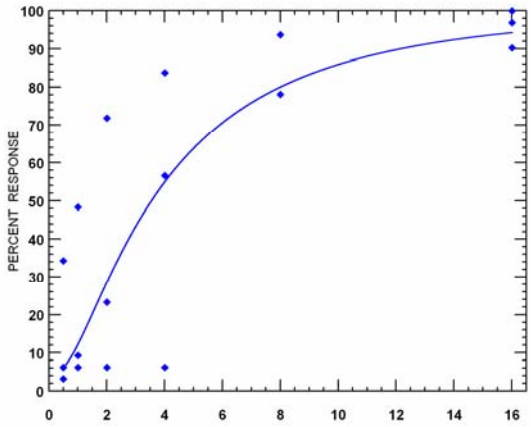


Figure 2. Susceptibility to Cry1Ab toxin, measured by MIC_{50} values, of a laboratory population of *S.nonagrioides*. Colors indicate the B2 (red) and B3 (green) toxin batches.

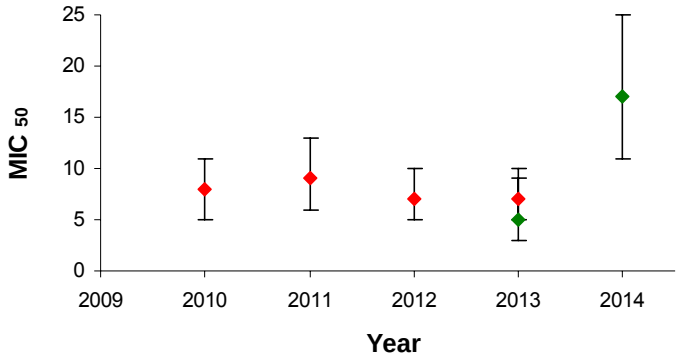
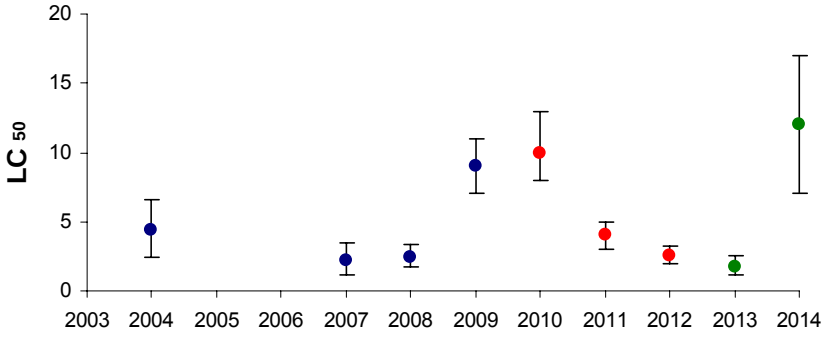
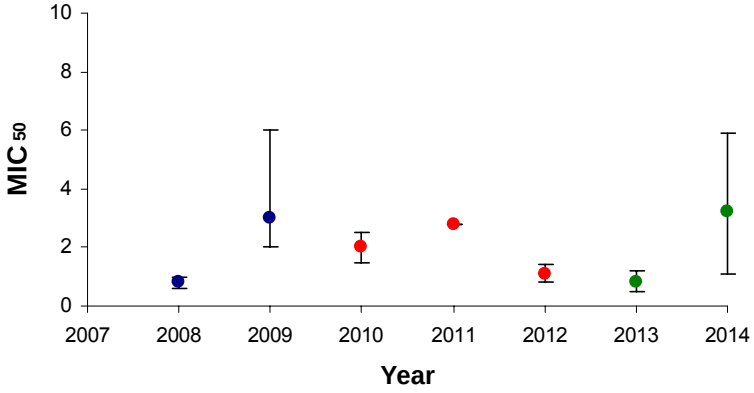


Figure 3. Susceptibility to Cry1Ab toxin, measured by LC_{50} and MIC_{50} values, of a laboratory population of *O. nubilalis.* Colors indicate the B1 (blue), B2 (red) and B3 (green) toxin batches.





ANNEX II: SAMPLING LOCATIONS FOR *S. nonagrioides* IN 2014