Appendix 7. Insect Resistance Monitoring in Iberian collections of *Sesamia nonagrioides*: 2012 Season

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

Season 2012

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. nonagriodes* if it occurs.

Previous baseline susceptibility to Cry1Ab protein has been established for *S. nonagrioides* populations collected in different maize areas in Spain (Gónzález-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 2012 maize growing season are:

1) To determine the susceptibility of *S. nonagrioides* populations to the Cry1Ab protein expressed in MON 810 maize varieties in Southwest and Central Iberia.

2) To collect larvae of *O. nubilalis* from Southwest Iberia to be sent to the laboratory BTL GmbH Sagerheide (Germany) for their analysis. 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 toxin used in the bioassays with field populations.

4) To explore the feasibility of monitoring resistance of *S. nonagrioides* to Cry1Ab using a diagnostic dose.

2. Materials and Methods

2.1. Insect collection

The three areas identified in Iberia where MON 810 adoption has been significant are the Ebro valley or Iberia Northeast, Central Iberia (particularly the province of Albacete) and Iberia Southwest. For these areas data on susceptibility of *S. nonagrioides* and *O. nubilalis* to Cry1Ab have been collected since 2004. For this season (2012) larvae of *S. nonagrioides* were 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 sampled that comprises the baseline, up to three sampling sites separated by at least 50 km were chosen. The samples were collected during September and October of 2012 from refuges and fields of conventional maize adjacent to Bt 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 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* were sent to BTL GmbH Sagerheide (Germany) to be analyzed there.

Most of the larvae of *S. nonagrioides* collected from the field were in diapause or started diapause when placed on the rearing chamber at 15 ± 1ºC, 70 ± 5% relative humidity and a photoperiod of 12:12 hours (light: dark), after reaching the last larval instar. These 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 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.

Table 1. Artificial diet used for *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

Table 2. Artificial diet used for *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

2.3. Bioassays

2.3.1. Susceptibility to Cry1Ab in dose-response bioassays

Two batches of Cry1Ab toxin have been used since the start of the MON 810 monitoring plan. The first batch 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 was provided by Monsanto in October 2011 (concentration 1.8 mg/ml in 50 mM sodium bicarbonate buffer, pH 10.25;

purity 91%). 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. The toxin Cry1Ab used for all the bioassays performed in this season (2012) belong to this second batch. To prepare the test concentrations, a sodium bicarbonate buffer (50 mmol/l) with pH 10.25 was used.

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 toxin 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 were established according to values of moult inhibition obtained in the laboratory in previous years. In order to determine the susceptibility of each population, 7 to 10 different concentrations resulting in mortality higher than 0% and below 100% were used. 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.

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

2.3.2. Diagnostic dose

Another approach to the dose-mortality testing for monitoring Bt maize resistance would be the use of diagnostic doses (Sims, 1997; Marçon et al., 2000). 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).

In order to conclude on the dose that will be used in the following growing seasons, the MIC_{99} was determined for field populations of *S. nonagrioides* from the past growing seasons. This will help to diminish the variation expected. Thus, the diagnostic dose (DD) was defined to cause 99% of moulting inhibition to first instar larvae (MIC_{99}) and determined for the field populations of *S. nonagrioides* collected in Iberia from 2008 to 2012.

2.3.3. Larval survival on MON 810 tissue

MON 810 containing 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.

2.4. 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,

2002-2012).

3. Results and Discussion

3.1. Collection of larvae

Details of larvae of *S. nonagrioides* collected for the bioassays in Iberia are shown in **Table 3**. This Table also includes information about larvae of *O. nubilalis* sent to the laboratory BTL GmbH Sagerheide (Germany). The position of the locations of collection within each area is displayed in a map in **Annex I.**

In Southwest Iberia a total of 19 different fields belonging to five provinces were inspected. Only 243 larvae of *S. nonagrioides*, dissimilar in size and larval instar, could be collected. From them, 98 (those from El Torno) were in diapause, whereas the rest started to pupate soon after in the laboratory. In the case of *O. nubilalis* 378 larvae were collected from two Southwest Iberian fields and all of them were in diapause. From them 260 were sent to Germany. This population should have been completed with larvae from the projected third Iberian point, located in Portugal; however, logistical complications prevented adequate collections for testing from the third Iberian site.

In Central Iberia 9 fields in different locations of the province of Albacete were examined. Only in three of them the number of larvae was sufficient (>100), giving a total of 544 larvae, and all of the larvae were in diapause. Here, two locations (La Herrera and Santa Ana) were not separated by 50 km and it was not possible to find other fields that fulfill this requirement. The main reason is that maize in the province of Albacete is very concentrated in a relatively small area.

Table 3. *Sesamia nonagrioides* and *Ostrinia nubilalis* larvae collection details for the 2012 season

Area	Country	Fields (Province)[a]	Postal Code	Date	Surface (Ha) [b]	Distance (m) to the nearest MON810 field [c]	*S. nonagrioides* No of larvae collected	*O. nubilalis* No of larvae collected	*O. nubilalis* No of larvae to BTL (Germany)
Southwest Iberia	Spain	Torrecera (CA)	11595	4/9/2012	12	0	0	0	-
		La Ina 1 (CA)	11595	4/9/2012	30	50	0	0	-
		La Ina 2 (CA)	11595	4/9/2012	15	200	0	0	-
		La Ina 3 (CA)	11595	4/9/2012	2	0	0	0	-
		Torre Melgarejo 1 (CA)	11592	4/9/2012	nd	nd	0	0	-
		Torre Melgarejo 2 (CA)	11592	4/9/2012	nd	nd	0	0	-
		Torre Melgarejo 3 (CA)	11592	4/9/2012	10	50	0	0	-
		El Trobal (SE)	41727	5/9/2012	6	2500	15	0	-
		Posadas 1 (CO)	14730	5/9/2012	12	0	0	0	-
		Posadas 2 (CO)	14730	5/9/2012	10	0	96	0	-
		Guadiana del Caudillo (BA)	06186	17/9/2012	14	0	0	0	-
		Valdebotoa 1 (BA)	06194	17/9/2012	40	10000	0	0	-
		Valdebotoa 2 (BA)	06194	17/9/2012	4	100	0	11	-
		Valdebotoa 3 (BA)	06194	17/9/2012	nd	0	0	0	-
		Navalvillar de Pela 1 (BA)	06760	18/9/2012	nd	0	0	0	-
		Navalvillar de Pela 2 (BA)	06760	18/9/2012	11	500	30	204 [d]	130[d]
		Villar de Rena (BA)	06716	19/9/2012	5	0	4	174 [d]	130[d]
		Obando (BA)	06730	19/9/2012	8	0	0	12 [d]	0
		El Torno (CR)	13194	1/10/2012	nd	0	98 [d]	-	-
Central Iberia	Spain	La Gineta 1 (AB)	02110	2/10/2012	2	0	0	-	-
		La Gineta 2 (AB)	02110	2/10/2012	3	0	2 [d]	-	-
		Barrax (AB)	02639	2/10/2012	nd	0	12 [d]	-	-
		Santa Ana 1 (AB)	02328	2/10/2012	10	300	0	-	-
		Santa Ana 2 (AB)	02328	2/10/2012	2	0	165 [d]	-	-
		Aguas Nuevas (AB)	02049	2/10/2012	3	0	0	-	-
		El Salobral (AB)	02140	2/10/2012	12	20	0	-	-
		La Herrera (AB)	02162	3/10/2012	60	0	198 [d]	-	-
		Motilleja (AB)	02220	3/10/2012	45	0	181 [d]	-	-

[a] Spanish provinces: AB = Albacete; BA = Badajoz; CA = Cádiz; CR = Ciudad Real; CO = Córdoba; SE = Sevilla

[b] Data are approximate.

[c] There could be other nearer fields that are not known by the technician and/or the farmer.

[d] Diapausing larvae

nd = no data

3.2. Susceptibility to Cry1Ab in the 2012 campaign

To determine the susceptibility to Cry1Ab, larval mortality and larval molt inhibition data at the different concentrations of Cry1Ab tested were analyzed by probit analysis. Lethal concentrations at 50% (LC_{50}) and 90% (LC_{90}) were estimated for the laboratory population of *O. nubilalis*, and moulting inhibition concentrations at 50% (MIC_{50}) and 90% (MIC_{90}) for populations of both *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). Fitted curves of susceptibility to the toxin Cry1Ab of laboratory and field populations of the two species were generated taking into account the molting inhibition of neonate larvae after seven days feeding on treated diet **(Figure 1)**.

3.2.1. S. nonagrioides

The bioassays to evaluate the susceptibility of the population from the Southwest and Central Iberia of *S. nonagrioides* to Cry1Ab were performed with neonates of the F1 generation of the field-collected larvae. The bioassay with the population of Central Iberia was performed after the winter diapause period, as all of the larvae collected were in diapause. However, the bioassay with the population of Southwest Iberia was performed in two steps: one replicate (with larvae from Posadas, Córdoba) was made in October 2012, since last instar larvae collected were not in diapause, and other four replicates (with larvae from El Torno, Ciudad Real) were performed in February 2013 after the winter diapause period. Then, all the replicates were analyzed together. In this case the total number of larvae tested per concentration was 80 (160 for the controls).

As it was established by the results of the 2009 season, only values of MIC have been used to assess the susceptibility of this species to Cry1Ab. This is the second maize season using the new batch of the Cry1Ab protein, delivered by Monsanto in October 2011. The results of MIC_{50} and MIC_{90} are in the range of those obtained in previous years. Susceptibility of the laboratory strain (MIC_{50} = 7 ng Cry1Ab/cm^2) was higher than that of the population coming from Southwest and Central Iberia (MIC_{50} = 29 and 15 ng Cry1Ab/cm^2, respectively) (**Table 4A** and **Figure 1A**). Similar sizes of differences between laboratory and field colonies have been observed historically, as well as variations in susceptibility of a population in different years (**Table 5**). Information from the last seasons suggests that these differences and oscillations in susceptibility values to the toxin Cry1Ab can be attributed to common natural variations in *S. nonagrioides* previously reported (Farinós *et al.* 2004).

3.2.2. O. nubilalis

In the case of the laboratory strain of *O. nubilali*s, susceptibility to Cry1Ab toxin was analyzed by LCs and MICs, displaying LC_{50} and LC_{90} values of 2.5 and 16 ng Cry1Ab/cm^2, respectively (**Table 4B**; **Figure 1B**), which is in the range of values obtained historically for the same population (**Table 6**). The estimated MIC values this year (MIC_{50} and MIC_{90} values were 1.1 and 2.5 ng Cry1Ab/cm^2, respectively) were even lower that those obtained for previous seasons (**Table 6**).

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 2012 campaign.

A) *Sesamia nonagrioides*

Population	Year	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	2012	879	1.4 ± 0.1	33.9	22	7 (5-10)	1	62 (41-107)	1
Southwest Iberia [c]	2012	798	1.7 ± 0.2	86.3	37	29 (19-41)	4.1 (2.9-5.9)*	158 (101-339)	2.6 (1.5-4.4)*
Central Iberia	2012	954	1.2 ± 0.1	106.4	22	15 (8-25)	2.1 (1.4-3.1)*	160 (79-608)	2.6 (1.4-4.6)*

B) *Ostrinia nubilalis*

Population	Year	n	Slope ± SE	χ^2	d.f.	LC_{50}[a] (CI 95%)	LC_{90}[a] (CI 95%)
Laboratory	2013	960	1.6 ± 0.1	36.9	22	2.5 (2.0-3.2)	16 (12-25)
						MIC_{50}[a] (CI 95%)	MIC_{90}[a] (CI 95%)
Laboratory	2013	672	3.9 ± 0.5	30.0	13	1.1 (0.8-1.4)	2.5 (2.0-3.8)

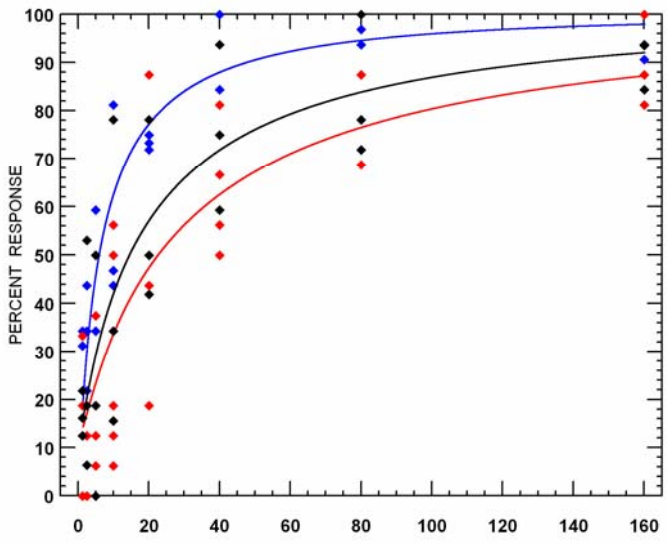
[a] 50% and 90% lethal concentrations (LC_{50} and LC_{90}) or moulting inhibition concentrations (MIC_{50} and MIC_{90}) and their 95% confidence intervals (CI95%) are expressed in ng Cry1Ab/cm^2.

[b] Lethal concentrations significantly different ($P < 0.05$) with respect to the laboratory strain if the MICR 95% confidence interval does not include 1.

[c] This bioassay was performed in two steps: One replicate was made in October 2012 with non-diapausing field larvae and other three replicates were performed in February 2013 after the winter diapause period. All the replicates were analyzed together.

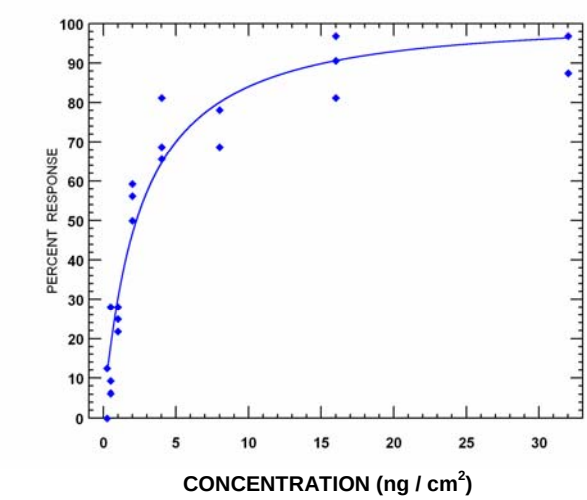
Figure 1. Fitted curves of susceptibility to the toxin Cry1Ab (PoloPlus, LeOra Software, 2002-2009).
A: Laboratory colony (blue) and two field populations from Soutwest Iberia (red) and Central Iberia (black) of *Sesamia nonagrioides* (slopes of individual population lines were constrained to be parallel). 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*

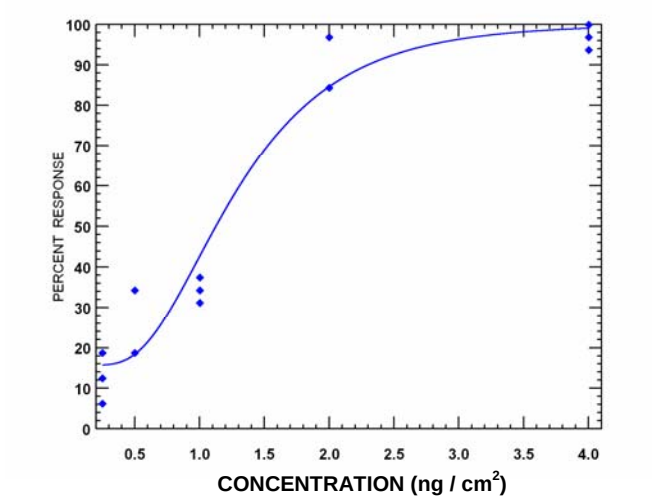


B) *Ostrinia nubilalis*

B1. Lethal Concentration (LC) values



B2. Molt Inhibition Concentration (MIC) values



3.3. Diagnostic dose

The diagnostic dose (MIC_{99}) obtained for the populations of *S. nonagriodes* collected in Iberian maize fields from 2008 to 2012 was 726 ng Cry1Ab/cm^2. This value represents the response of 6,646 neonates derived from larvae collected in different locations of Southwest (2010 and 2012), Central (2008, 2010 and 2012) and Northeast (2009 and 2011) Iberia.

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

None of the larvae of *S. nonagrioides* which were not killed by the treatment with Cry1Ab in the dose-response bioassay (432 and 491 larvae from the Southwest- and Central Iberia populations, respectively) 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 the present season) (**Table 5**). These results evidenced a magnitude variation of 4.1-fold. Likewise, values of MIC_{50} of laboratory strains were also very uniform, ranging between 7 and 19 ng Cry1Ab/cm^2, which means a magnitude variation of 2.7-fold. In the light of these results, MIC_{50} values obtained during this campaign for the field collected populations and for the laboratory strain are within the range of values got in the past years (**Table 5**).

Table 5. Susceptibility to Cry1Ab toxin of laboratory populations and Iberian field populations of *S. nonagrioides* collected in refuge areas of MON 810 between 2004 and 2012. Bioassays performed during this campaign are shaded.

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

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

[b] 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 .

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

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

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 5- and 6-fold for LC_{50} and MIC_{50} values, respectively (**Table 6**). Taking into consideration MIC_{50} values obtained for both corn borers, larvae of *O. nubilalis* in most cases showed higher susceptibility to the Cry1Ab toxin than *S. nonagrioides*.

Table 6. Susceptibility to Cry1Ab toxin of Iberian field populations of *O. nubilalis* collected in refuge areas of MON 810 between 2004 and 2008, and of laboratory strains between 2004 and 2013. Bioassays performed during this campaign are shaded.

Population	Year	LC_{50}[a] (CI 95%)	LCL_{90}[a] (CI 95%)	MIC_{50}[a] (CI 95%)	MIC_{90}[a] (CI 95%)
Laboratory	2004	4 (2-7)	19 (12-54)	2.1[b]	9.0[b]
Laboratory	2007	2 (1-4)	17 (11-31)	0.6 (0.03-1.2)	2.3 (1.2-3.2)
Laboratory	2008	2 (2-3)	20 (13-33)	0.8 (0.6-1.0)	2.9 (2.3-4.1)
Laboratory	2010	9 (7-11)	26 (19-44)	3.4 (1.6-5.6)	19.0 (10.0-107.3)
Laboratory	2011	10 (8-13)	90 (53-194)	2.0 (1.5-2.5)	6.7 (5.1-10.1)
Laboratory[c]	2012	4 (3-5)	20 (16-28)	2.8[b]	5.0[b]
Laboratory	2013	2.5 (2.0-3.2)	16 (12-25)	1.1 (0.8-1.4)	2.5 (2.0-3.8)
Southwest Iberia (Spain)	2004	6 (4-9)	41 (27-77)	5.4 (4.0-6.9)	19.8 (15.1-29.1)
Southwest Iberia (Portugal)	2005	14 (11-17)	43 (31-67)	9.4 (8.7-10.1)	13.7 (12.4-15.9)
Southwest Iberia (Spain)	2006	6 (4-8)	32(23-54)	1.9 (1.5-2.2)	3.8 (3.3-4.9)
Southwest Iberia	2008	5 (4-7)	32 (24-44)	1.3 (1.0-1.6)	4.0 (1.0-1.6)
Central Iberia	2005	12 (9-15)	57 (41-92)	4.8 (2.9-6.6)	11.8 (8.4- 23.9)
Central Iberia	2006	2 (1-4)	33 (21-68)	1.1[b]	5.2[b]
Central Iberia	2008	3 (2-3)	10 (8-15)	1.3 (0.9-1.6)	3.7 (2.9-5.5)
Northeast Iberia	2004	6 (4-8)	27 (18-56)	2.8 (0.8-4.5)	10.5 (6.2-51.4)
Northeast Iberia	2006	3 (1-5)	42 (22-138)	0.5 (0.05-1.0)	3.2 (1.8-4.8)
Northeast Iberia	2008	9 (7-12)	58 (38-108)	1.6 (1.3-1.9)	4.2 (3.4-5.9)

[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.

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

4. Conclusions

1. It is the fifth time to determine the susceptibility to the Cry1Ab toxin of the field population of *S. nonagrioides* from Southwest Iberia since 2005, and in this period the MIC_{50} value has ranged between 8 and 29 ng Cry1Ab/cm^2. Larvae of this field population have resulted this season about 4-fold less susceptible than those of the laboratory strain.

2. The susceptibility of the field population of *S. nonagrioides* from Central Iberia to the Cry1Ab toxin has been assessed for the fifth time since 2004, ranging the values of MIC_{50} between 7 and 28 ng Cry1Ab/cm^2 during this period. Central Iberian larvae resulted about 2-fold less susceptible than those of the laboratory strain for this season.

3. The diagnostic dose (MIC_{99}) estimated for Iberian field populations of *S. nonagrioides* collected from 2008 to 2012 was 726 ng Cry1Ab/cm^2. This value represents the response of 6,646 larvae.

4. No survivors have been reported among larvae of the F1 generation exposed to MON 810 leaves (surviving larvae from bioassays and larvae produced in the laboratory but not used in the bioassays).

5. The laboratory strain of *O. nubilalis* showed susceptibility levels to the Cry1Ab toxin comparable with those obtained for laboratory strains in previous years. Both LC and MIC values evidenced consistency through time (period 2004-2013), showing 5- and 6-fold variation in both LC_{50} and MIC_{50} values, respectively.

7. The analysis of the historical series of data of susceptibility of *S. nonagrioides* to Cry1Ab has not shown a consistent increase of resistance to this toxin in field populations of *S. nonagrioides* from Southwest and Central Iberia.

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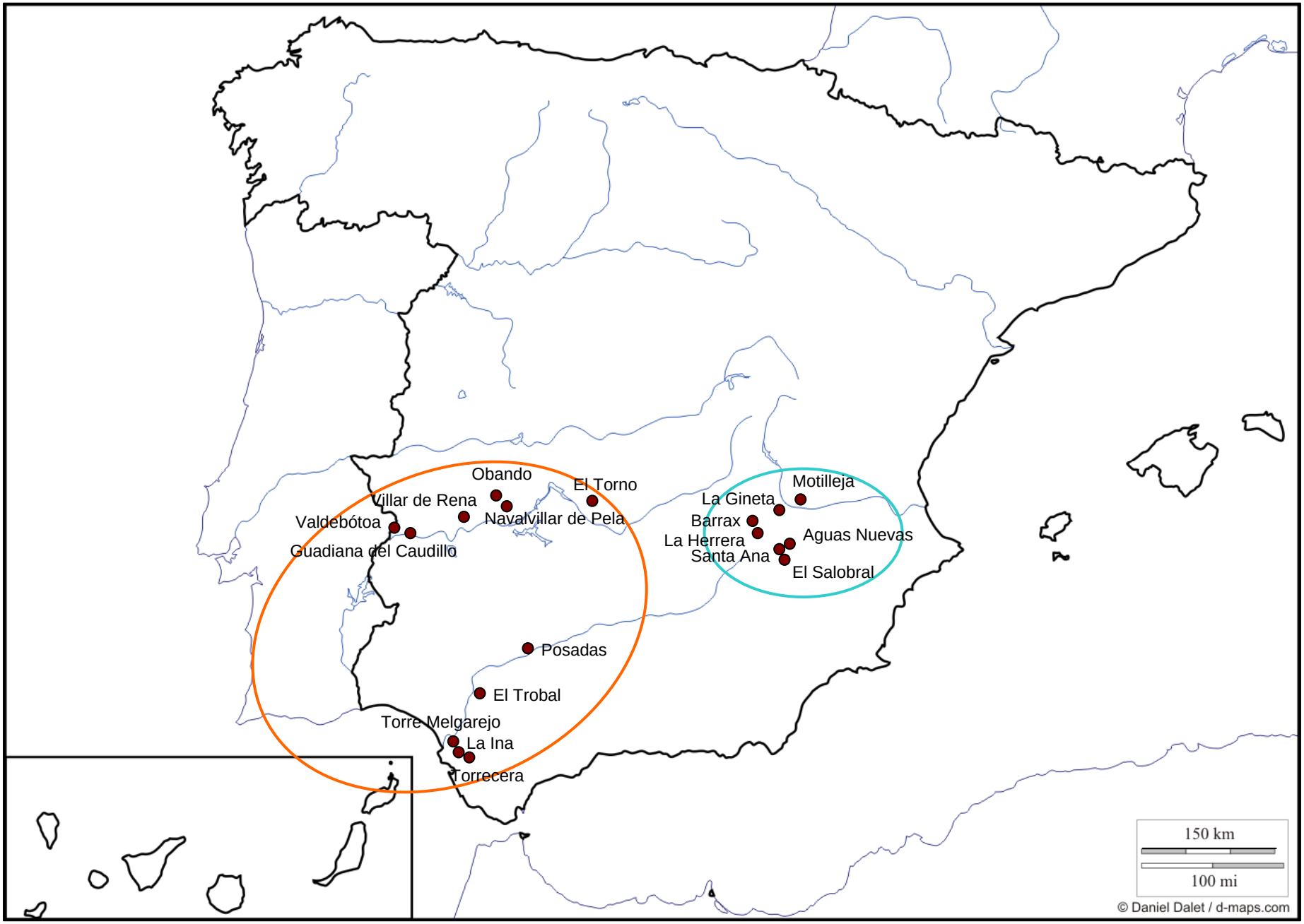
ANNEX I: SAMPLING LOCATIONS IN 2012



ANNEX I: SAMPLING LOCATIONS IN 2012