Appendix 7. Insect Resistance Monitoring in Iberian collections of *Sesamia nonagrioides*: 2015 Season

INSECT RESISTANCE MONITORING REPORT FOR *Sesamia nonagrioides*

ASSOCIATED WITH MON 810 MAIZE CULTIVATION IN THE EU

Season 2015

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 2015 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 be sent to the laboratory BTL GmbH Sagerheide (Germany). 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* aiming at: i) verifying the activity of the batch of protein used in the bioassays with field populations, and ii) calculating, in the case of *S. nonagrioides,* the moult inhibition concentration ratio (MICR) of the field population with respect to the laboratory strain.

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 Northeast-Iberia and larvae of *O. nubilalis* in Central- and Northeast-Iberia.

The process of identifying fields for insect collection starts by contacting local field technicians of seed companies that commercialize MON 810 in Spain. They are asked if they are aware of the presence of corn borers (*Sesamia* and/or *Ostrinia*) in the area to be sampled that year, for which they may consult a number of growers about the detection of corn borer damages in their fields. If yes, the growers are asked for permission to enter their fields during one to three days and break enough number of maize plants to find a minimum of 100 larvae, taking only 1 per plant per corn borer species. This could well mean sampling over 2000 plants depending on the presence of one or two species, the severity of the attack, etc. If the number of larvae per species is not sufficient, the field is not considered and a new field is searched.

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, the objective was to choose three sampling sites separated by at least 50 km. However, as documented in reports from previous years, in most cases more than three sites are sampled due to the insufficient number of larvae found. The history overview of success in corn borer collections is displayed in **Figure 1**.

The samples were collected during September 2015 from refuges and fields of conventional maize adjacent to MON 810 maize. The samples were collected 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, 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 2015. The majority of them had not 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 a variable number of couples (from 5 to 10), in function of the day of adult emergence, 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 utilized in the bioassays.

2.3. Quality of the laboratory strains

To preserve the vigour of the laboratory colonies of *S. nonagrioides* and *O. nubilalis* and to ensure that the populations do not collapse, they are refreshed every one or two years with new individuals collected in non-Bt fields. Infusion of wild individuals in an established laboratory strain is a common practice to increase genetic diversity, which can be lost compared with field populations (Da Silva et al., 2015; Leppla and Ashley, 1989). To this end, the progenies of the same populations collected in the field for the monitoring are used. Firstly, the similarity (no significant differences) between the LC_{50} values of both the laboratory and field populations is checked by susceptibility bioassays. 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. In addition, before introducing the new individuals in the laboratory colony they are maintained separately for two-three generations in the laboratory.

In the season 2015 no new individuals have been incorporated to the laboratory strains.

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 (2015). 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. For the bioassays of this season the batch B3 of Cry1A has been used.

The bioassays were carried out in accordance with the methods described by Farinós et al. (2004), using “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 were comprised between 1 and 128 ng Cry1Ab/cm^2 for the populations of *S. nonagrioides*, and between 0.5 and 128 ng Cry1Ab/cm^2 for *O. nubilalis*. 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. Three replicates were prepared for each concentration and 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. The susceptibility of the laboratory strains of *S. nonagrioides* and *O. nubilalis* to Cry1Ab was assessed using the same stock solution. Then MIC_{50} obtained for *S. nonagrioides* was compared with that of the field population.

From 2010 onward, it was decided to switch from LC to MIC values for measuring susceptibility to Cry1Ab in *S. nonagrioides*, since MIC values proved to be more consistent for this bioassay and species than LCs. Thus, the susceptibility has been determined by MICs in *S. nonagrioides* populations 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 as diagnostic dose (DD) for the population of *S. nonagrioides* collected in Northeast Spain in 2015. Three replicates of 32 neonates each (a total of 96 larvae) were submitted to this concentration, and moult inhibition was registered after 7 days.

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 was harvested for their use in a confirmatory experiment. This experiment was performed to confirm that resistant individuals were not present in the field-collected population, and it consisted of a quick screen of a high number of neonates on Bt maize with the purpose of detecting survivals. With this aim, all surviving larvae from the protein bioassays and a high number of left-over larvae generated from field collections (which were not used in bioassays) were exposed to MON 810 leaves for a period of about 10 days and recording survival. Groups of 50-100 larvae were transferred to plastic boxes provided with new MON 810 maize leaves without the central nerve and they 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 was 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 in the field population was tested by the 95% confidence limits of moult inhibition concentration ratios (MICR) at the MIC_{50} and the MIC_{90} (Robertson et al., 2007). 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 529 last instar larvae of *S. nonagrioides* were collected in Northeast Iberia from three different fields, Alberuela de Tubo, Candasnos and Beire (**Table 3**), in September 2015. It is important to highlight the heavy infestation of *S. nonagrioides* found in Beire, where more than 250 larvae could be collected. Locations where field samplings of *S. nonagrioides* were carried out are displayed in **Annex II.**

Larvae of *O. nubilalis* were collected in sufficient number in Northeast Iberia in only three out of five fields inspected: Alberuela de Tubo, Candasnos and Mélida 2 (**Table 3**), making a total of 376 larvae. Contrary to the case of *S. nonagrioides*, no larvae of *O. nubilalis* were found in Beire. In Central Iberia (province of Albacete) *O. nubilalis* larvae were searched in seven fields, but only in three of them (La Herrera, Motilleja and Santa Ana) enough individuals were found, with a total of 443 larvae. These three locations are not separated by the minimum distance of 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. All the *O. nubilalis* larvae 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 dose-response bioassays

3.2.1. Sesamia nonagrioides

Larvae collected in Northeast Iberian fields resulted in 444 adults that were placed in 28 oviposition cages for mating. The bioassay to evaluate the susceptibility to Cry1Ab of this field population was performed with 920 neonates of the F1 generation in November 2015, since the majority of larvae collected in September were not in diapause. The MIC_{50} and MIC_{90} values for the Northeast larvae were 17 and and 84 ng Cry1Ab/cm^2, respectively (**Table 4A, Figure 2**), which are within the range of values obtained historically since 2005 **(Table 5)**. During these years the susceptibility of this population has shown low variability in MIC_{50} values, ranging between 9 and 22 ng Cry1Ab/cm^2, which resulted in a magnitude variation of 2.4-fold.

For the bioassay with the laboratory population of *S. nonagrioides,* 297 adults were let to mate in 21 oviposition cages. From the F1 progeny, 863 neonates were utilized. The MIC_{50} value for this population was 28 ng Cry1Ab/cm^2, so it resulted less susceptible to the Cry1Ab protein than the population from Northeast Iberia (MICR = 0.6 with respect to the laboratory strain, **Table 4A**). However, no significant differences were found at the MIC_{90} level (MICR = 1.3 (0.-1.8)).

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 6**). These values did not present significant differences from 2011 to 2013 compared to the baseline, regardless the toxin batch used. In the two last campaigns (2014 and 2015) the MIC_{50} ratios have show an increase for the laboratory strain (x 2.2 and x 3.6, respectively) with respect to that of 2010 (**Table 6, Figure 3**).

Variations in laboratory-reared insects have been frequently observed when studying their susceptibility to pesticides or insecticidal proteins and different reasons have been proposed, such as diverse geographical sources of individuals, varying testing personnel, different protein preparations, etc. (Da Silva et al., 2016; Robertson et al., 1995; Marçon et al., 1999; Gaspers et al., 2011). Likewise, differences between laboratory and field colonies have been observed historically during this monitoring program, as well as changes in susceptibility to the toxin Cry1Ab of certains field population in different years (**Table 5**), 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. Ostrinia nubilalis

The susceptibility to Cry1Ab toxin of the laboratory strain of *O. nubilali*s was assessed by LCs and MICs. The LC_{50} and LC_{90} values were 2.4 and 20 ng Cry1Ab/cm^2 respectively, and the MIC_{50} and MIC_{90} were 1.1 and 3.9 ng Cry1Ab/cm^2 respectively (**Table 4B**). LC and MIC values of the control laboratory strain have been in general very consistent in the interval of years examined (2004-2015), being the maximum magnitude of variation 6.8- and 5.7-fold for LC_{50} and MIC_{50} values, respectively (**Table 7, Figure 4**). The values obtained for this season are in the range of values reported previous years and represent a decrease with regard to the higher value observed in the season 2014 (**Table 7, Figure 4**).

3.3. Diagnostic dose

The diagnostic dose of 726 ng Cry1Ab/cm^2 used for the population of *S. nonagrioides* collected in Northeast Iberia in 2015 caused moult inhibition to neonates of 100 %

3.4. Survival of larvae 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 and in the DD bioassays (a total of 574 larvae) could survive after 12 days feeding ad libitum on MON 810 tissue. Additionally, there was no survivor among the spare neonate larvae (about 1400) that were exposed to MON 810 leaves for 10 days.

4. Conclusions

1. The susceptibility to the Cry1Ab toxin of the field population of *S. nonagrioides* from **Northeast Iberia** has been determined for the sixth time since 2005. The MIC_{50} obtained for this season was 17 ng Cry1Ab/cm^2. During the period 2005-2015 MIC_{50} values have ranged between 9 and 22 ng Cry1Ab/cm^2, therefore the maximum magnitude of variation was 2.4-fold. When the field and the laboratory populations were tested with the same toxin batch, the former resulted more susceptible to the Cry1Ab protein (MICR = 0.6 with respect to the laboratory strain). It is likely that the decrease in susceptibility of the lab population could be due to variations that occur in laboratory-reared insects produced by a variety of reasons, as previously observed in different studies.

2. The moult inhibition of *S. nonagrioides* larvae coming from Northeast Iberia collections was 100% when a diagnostic dose (MIC_{99}) of 726 ng Cry1Ab/cm^2 was used in the bioassay. Likewise, no survivors have been reported in about 1400 larvae of the F1 generation of this population exposed to MON 810 leaves.

3. A total of 376 and 443 larvae of *O. nubilalis* were collected in Northeast and Central Iberia, respectively and sent to the laboratory BTL GmbH Sagerheide (Germany) for testing their susceptibility to the Cry1Ab protein expressed in MON 810 varieties.

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

In short, the analysis of the historical series of data of susceptibility to Cry1Ab of the populations of *S. nonagrioides* from Northeast 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 2015 season

Species	Area	Fields (Province)[a]	Postal Code	Date	Surface (Ha) [b]	Distance to the nearest MON810 field (m) [c]	No of larvae collected
S. nonagrioides	Northeast Iberia	Alberuela de Tubo (HU)	22212	21/09/2015	16	10	107
		Candasnos (HU)	22591	22/09/2015	6	2000	162
		Beire (NA)	31393	23/09/2015	2	800	260
O. nubilalis	Central Iberia	La Herrera (AB)	02162	07/09/2015	40	0	106[d]
		Aguas Nuevas 1 (AB)	02049	07/09/2015	3	0	0
		Aguas Nuevas 2 (AB)	02049	08/09/2015	4	0	0
		Albacete (AB)	02220	07/09/2015	40	0	0
		Motilleja (AB)	02220	08/09/2015	20	0	178[d]
		Santa Ana (AB)	02328	08/09/2015	3.5	0	159[d]
		El Salobral (AB)	02140	08/09/2015	4.5	0	0
	Northeast Iberia	Alberuela de Tubo (HU)	22212	21/09/2015	16	10	132[d]
		Candasnos (HU)	22591	22/09/2015	6	2000	144[d]
		Beire (NA)	31393	23/09/2015	2	800	0
		Mélida 1 (NA)	31382	23/09/2015	8	15	0
		Mélida 2 (NA)	31382	23/09/2015	3	700	100[d]

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

[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] Larvae sent to Germany after removing those that were damaged or seemed to have some pathogen.

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 2015 campaign.

A) *Sesamia nonagrioides*

Population	Season	Toxin batch	n	Slope ± SE	$\chi 2$	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	2015	B3	863	3.3 ± 0.2	84.5	19	28 (21-36)	1	67 (50-110)	1
Northeast	2015	B3	920	1.8 ± 0.2	28.5	22	17 (13-21)	0.6 (0.5-0.8)*	84 (63-124)	1.3 (0.9-1.8)

B) *Ostrinia nubilalis*

Population	Season	Toxin batch	n	Slope ± SE	$\chi 2$	d.f.	LC_{50}[a] (CI 95%)	LC_{90}[a] (CI 95%)
Laboratory	2015	B3	996	1.4 ± 0.1	60	24	2.4 (1.6-3.4)	20.3 (13.3-36.5)
							MIC_{50}[a] (CI 95%)	MIC_{90}[a] (CI 95%)
Laboratory	2015	B3	747	2.4 ± 0.2	115	16	1.1 (0.6-1.7)	3.9 (2.4-10.6)

[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.

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 2015. 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)
Laboratory	2015	B3	28 (21-36)	67 (50-110)
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)
Northeast Iberia	2015	B3	17 (13-21)	84 (63-124)

[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.

[c] New batch of Cry1Ab protein (B4) provided by Monsanto.

Table 6. 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)
2014	B3	MIC	-	2.2 (1.6-3.1)*
2015	B3	MIC	-	3.6 (2.7-4.8)*

[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).

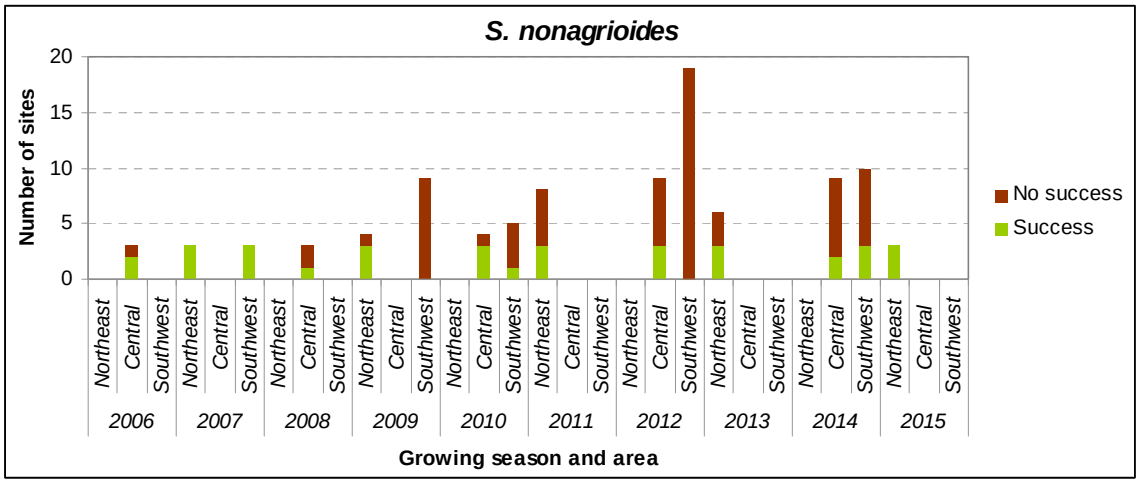
Table 7. Susceptibility to Cry1Ab toxin and a laboratory strain of *O. nubilalis* between the season 2004 and 2015. The bioassay performed during this campaign is shaded.

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)
Laboratory	2015	B3	2.4 (1.6-3.4)	20 (13-36)	1.1 (0.6-1.7)	3.9 (2.4-10.6)

[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. History overview of the success in field collections of the corn borers *S. nonagrioides* and *O. nubilalis* in three Iberian areas since 2006. A collection in a site is considered successful when a minimum of 100 larvae are gathered.



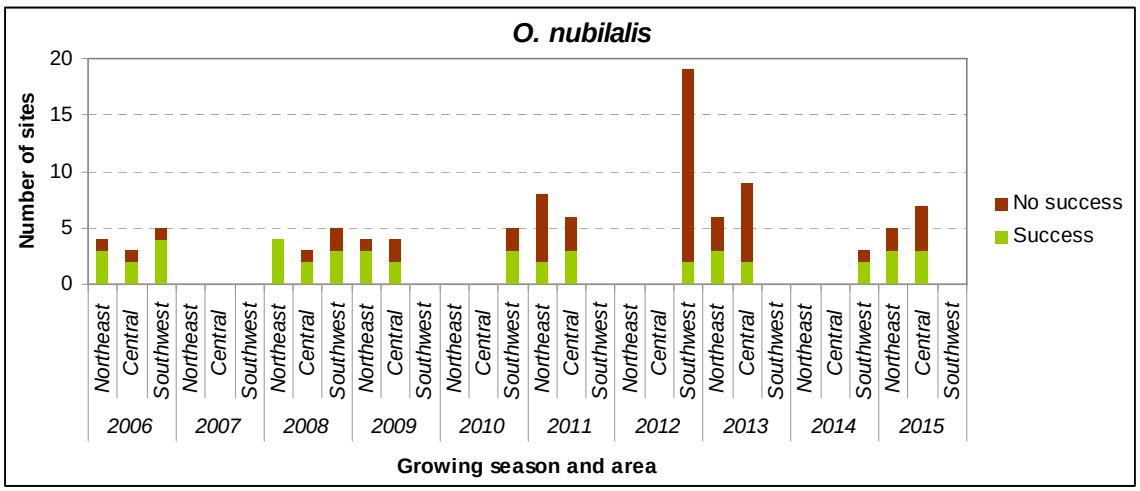
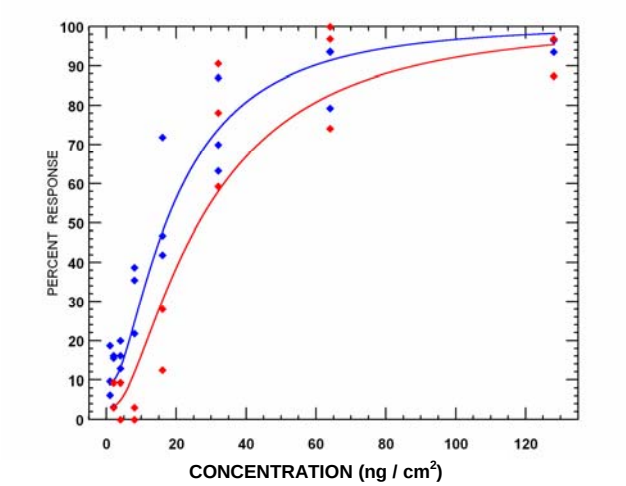


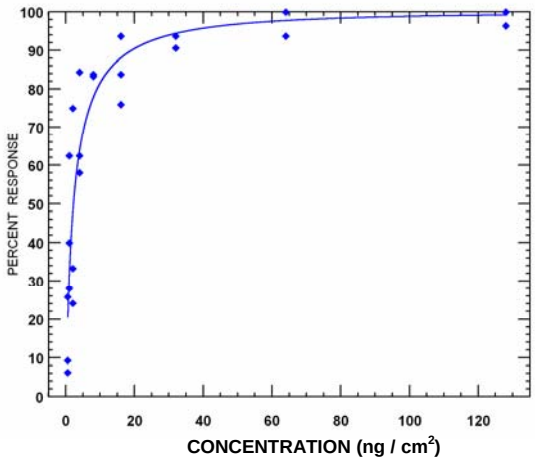
Figure 2. Fitted curves of susceptibility to the toxin Cry1Ab (PoloPlus, LeOra Software, 2002-2016).
A: Laboratory colony (red) and a field population from Northeast Iberia (blue) of *Sesamia nonagrioides*. Slopes of individual population lines were constrained to be parallel. Response is moult inhibition after seven days feeding on treated diet.
B: Laboratory colony of *Ostrinia nubilalis*. Response is mortality (B1) or molt inhibition (B2) after seven days feeding on treated diet.

A) *Sesamia nonagrioides*



B) *Ostrinia nubilalis*

B1. Lethal Concentration



B2. Molt Inhibition Concentration

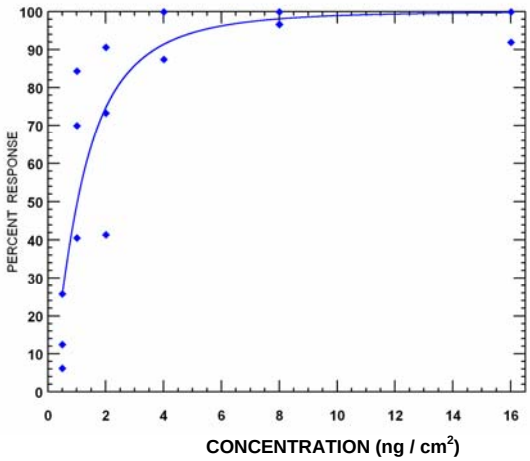


Figure 3. Susceptibility to Cry1Ab toxin, measured by MIC_{50} values, of a laboratory population of *S.nonagrioides*. Colors indicate the B1 (blue), B2 (red) and B3 (green) toxin batches.

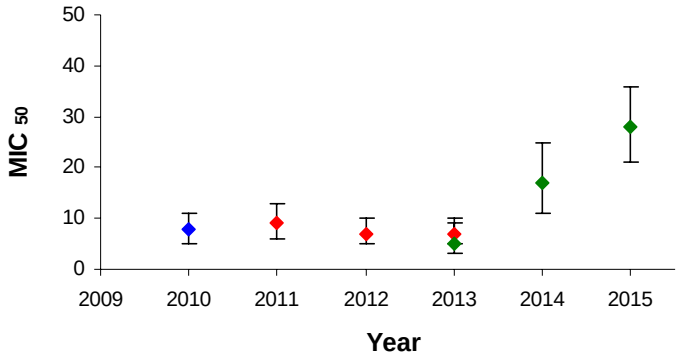
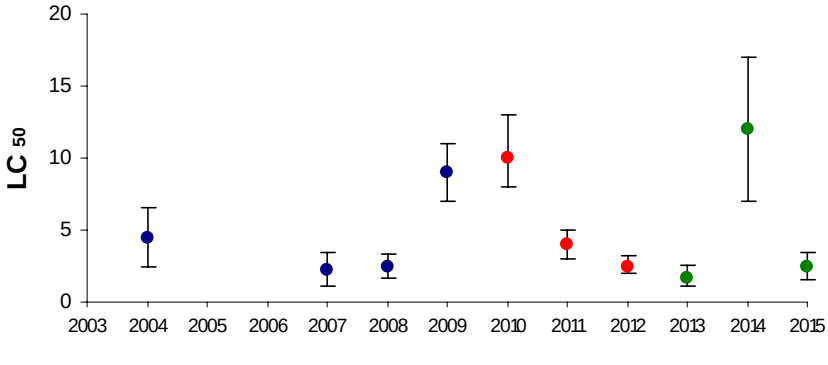
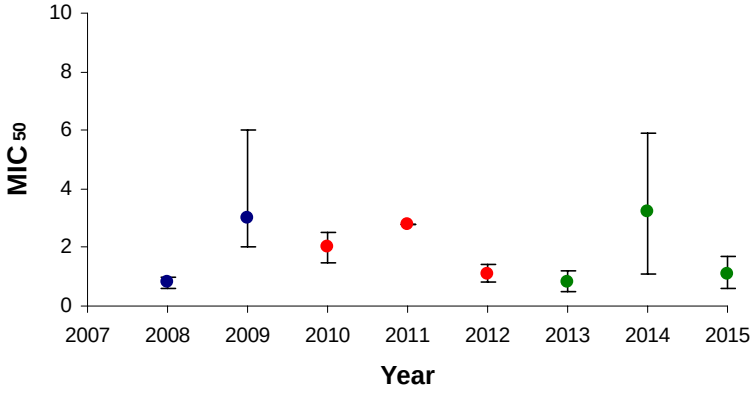
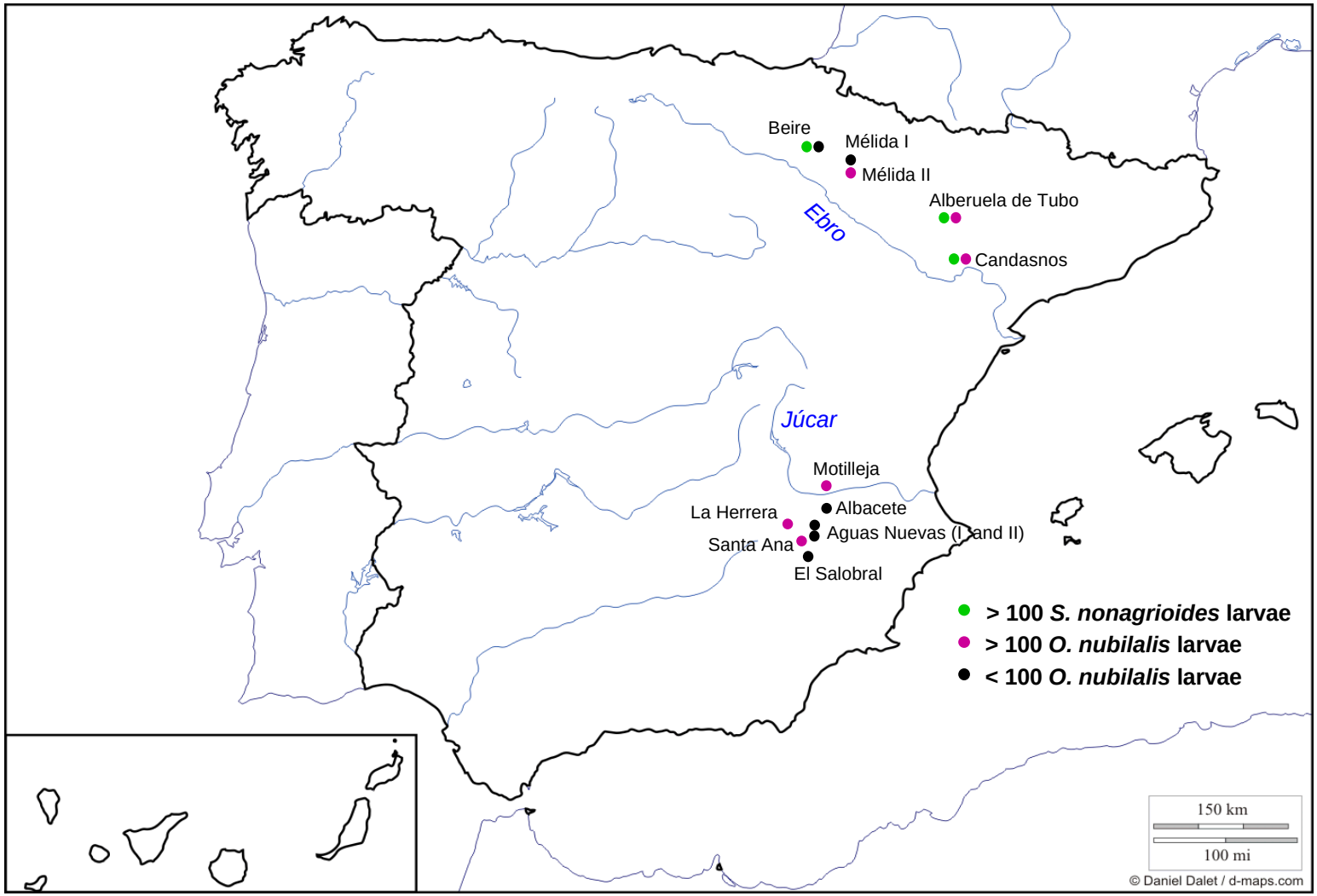


Figure 4. Susceptibility to Cry1Ab toxin, measured by LC_{50} and MIC_{50} values, of a laboratory population of *O. nubilalis.* Colors indicate the B1 (blue), B2 (red) and B3 (green) toxin batches.





ANNEX II: SAMPLING LOCATIONS FOR *S. nonagrioides* AND *O. nubilalis* IN 2015*



* Geographical coordinates are not provided in order not to violate farmers' privacy rights.