INSECT RESISTANCE MONITORING REPORT FOR *Sesamia nonagrioides*

ASSOCIATED WITH MON 810 MAIZE CULTIVATION IN THE EU

Season 2016

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; 2017), 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*. The objective is to detect, in a timely manner, the potential development of resistance that could result in inadequate protection against the target species. 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), routine monitoring for changes in the susceptibility of EU field populations of *S. nonagrioides* to the Cry1Ab protein was carried out in the period 2004-2015 (see Annual Monitoring reports) and compared with previous baseline susceptibility data established for this species (González-Núñez et al., 2000, Farinós et al., 2004). The plan covered the three maize-growing areas in the EU where MON 810 hybrids have been grown and *S. nonagrioides* is present: Northeast Iberia, Central Iberia and Southwest Iberia. Baselines were also gathered for other areas in Europe but no further samplings were performed in these areas because the adoption rate of Bt maize is less than 20% and monitoring resistance is not necessary according to the EuropaBio Harmonized IRM

plan (EuropaBio, 2012). In Iberia, each target field population was monitored every two years, but for practical reasons they were divided into two groups so that each year sampling was carried out in one of the groups. However, this harmonised IRM plan has been recently updated to accommodate the updates in the regulatory framework, and to incorporate the available scientific information and new learnings gained from this and other IRM plans (EuropaBio 2017). The revised plan establishes that sampling for resistance monitoring will take place in areas where the Bt maize adoption is over 60% and where the target pest is present. Currently, this situation only occurs in the area of the Ebro valley (Northeast of Spain) within the EU. Since *S. nonagrioides* and *O. nubilalis* are multivoltine species, the new plan proposes that monitoring for these corn borers in this area should be carried out on an annual basis.

The susceptibility of *S. nonagrioides* field populations to the Cry1Ab protein expressed in MON 810 maize varieties has been estimated so far by means of dose-response bioassays. Measured endpoints of the tests were mortality (lethal concentration, LC) and moulting inhibition (moulting inhibition concentration, MIC). From 2010 onward, it was decided to switch from LC to MIC values because it was observed that variations in the susceptibility were better reflected in MIC_{50} values than in LC_{50} values, given the characteristics of the bioassay and the biology of the species. In both cases, MIC_{50} and LC_{50} values of field populations were statistically tested with respect to a susceptible laboratory strain assayed with the same batch of toxin and compared with the range of values obtained for this species in previous years.

Two main recommendations have been provided in the Scientific Opinion of EFSA on the annual post-market environmental monitoring (PMEM) report on the cultivation of genetically modified maize MON 810 in 2015 from Monsanto Europe S.A. (EFSA GMO Panel 2017): (1) "*to increase sampling efforts and ensure that as many field-collected larvae as possible are represented in the laboratory assays as F1 larvae"* in order to provide sufficient detection sensitivity (i.e. 3% resistance allele frequency); and (2) "*focusing the monitoring activities in north-east Iberia (i.e. the Ebro valley), where field resistance to Cry1Ab is more likely to develop. In that geographical area, insects should be collected annually from three sampling zones of approximately 10 km x 10 km where adoption rate of maize MON 810 has been higher than 60% for at least three consecutive years*".

Accordingly with these two recommendations, and following the new harmonized IRM plan (EuropaBio 2017), during the 2016 season the collection of field larvae has been concentrated in the Ebro valley, where the number of larvae collected has considerably

increased. Moreover, we have developed a diagnostic concentration bioassay (Sims et al., 1997; Marçon et al., 2000) to monitor for changes in susceptibility to the Cry1Ab protein in *S. nonagrioides* field populations from Northeast Spain. The use of this approach permits a higher number of field-collected individuals to be represented in the laboratory assays as F1 larvae, thereby helping to decrease the current 5% detection limit for resistance allele frequency.

The tasks carried out in the 2016 maize growing season were the following:

1. Collection of larvae of *S. nonagrioides* in three different zones from Northeast of Spain (Ebro valley) to be used in diagnostic concentration bioassays.

2. Collection of larvae of *O. nubilalis* in three different zones from Northeast of Spain (Ebro valley) 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. Analysis of the susceptibility to Cry1Ab of laboratory strains of *S. nonagrioides and O. nubilalis* by means of moulting inhibition concentration (MIC) values, aiming at verifying the activity of the batch of protein used in the bioassays.

2. Materials and Methods

2.1. Insect collection

Last instar larvae of *S. nonagrioides* and *O. nubilalis* were collected from three sampling zones for each species within the Ebro valley area, in the Northeast of Spain (NE Spain), each zone comprising at least three maize fields in the smallest possible surface. Taking into consideration a potentially reduced target pest pressure and consequently a potential low larvae abundance, approximately 1000 larvae were targeted for collection per area, about 350 larvae collected in each of the three sampling zones and, if possible, a minimum of 100 larvae per maize field. For each species, if the number of individuals collected within a zone was not sufficient, the zone was disregarded and a new one was searched.

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, 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 to collect larvae, which requires destructive sampling of the maize stalk. The amount of maize plants used depends on the presence of one or two species, the severity of the attack, etc.

Last instar larvae of both corn borers were collected following standard operative procedures (SOP) of each species (EuropaBio, 2017). The samples were collected at the end of the maize-growing season, during September and October 2016, 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.

2.2. Insect culture

Field collected larvae were brought to the laboratory, dipped in a solution containing 1% bleach to avoid contamination by pathogens and placed in 21x16x4 cm plastic boxes (50 larvae of *S. nonagrioides* or 100 larvae of *O. nubilalis*). Both species were fed on an artificial diets established from that described by Poitout and Buès (1970) with some modifications (**Tables 1, 2**). Immediately after asepsis, collected larvae of *O. nubilalis* were sent to BTL GmbH Sagerheide (Germany) to be analyzed there.

The majority of larvae of *S. nonagrioides* had entered diapause at the time of collection, so they were placed on a rearing chamber (Sanyo MLR-350 H, Sanyo, Osaka, Japan) at 15 ± 1ºC, 70 ± 5% relative humidity and a photoperiod of 12:12 hours (L:D). They were kept at these conditions during 2 months, after which larvae were placed under conditions 28 ± 1ºC, 70 ± 5% relative humidity and continuous light to interrupt diapause, until pupation. The sex was determined at the pupal stage and a variable number of couples from the same zone (it normally ranged between 3 and 6), 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 at standard rearing conditions (25 ± 1ºC, 70 ± 10% relative humidity and a photoperiod of 16:8 hours (L:D). 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 (< 24 h old) were utilized in the bioassays.

2.3. Quality of the laboratory strains

Reference susceptible strains of *S. nonagrioides* and *O. nubilalis* have been maintained under laboratory conditions to serve as control in this study. A minimum of

350 adults are crossed every generation. Neonate larvae are collected from all the ovipositional cages formed with the adults of the previous generation (unless some cage has evidences of some disease). 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). Prior to the addition of new individuals, certain precautions are taken. 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.

Even when these precautions were taken, the laboratory population of *O. nubilalis* showed signs of weakening in 2016, so a new reference strain was required. One hundred and forty-five diapausing larvae were collected on December 2015 in non-Bt maize fields located in the region of Galicia (North-west of Spain), where Bt-maize has never been cultivated. They were sent to BTL Bio-Test Labor GmbH Sagerheide (Germany) and maintained at diapausing conditions. The first adults emerged in May 2016 and insects were reared at conditions 27°C (± 2 °C), humidity of 90% (± 1%) and a photoperiod of 20:4h (L:D). In December 2016 eggs masses of this population were sent to the CIB and established as the new reference laboratory strain.

2.4. Cry1Ab protein

Two batches of Cry1Ab protein have been used since the start of the MON 810 monitoring plan (2004) to the last season (2016). 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) (concentration 1.8 mg/ml in 50 mM sodium bicarbonate buffer, pH 10.25; purity 91%) has been provided at different times: B2-1 was sent in October 2011, B2-2 in February 2014 and B2-3 in April 2016. 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 batch B2-3 of Cry1A has been used for the bioassays of this season.

2.5. Bioassays

2.5.1. Susceptibility of the reference strains of S. nonagrioides *and* O. nubilalis *to the Cry1Ab protein in dose-response bioassays*

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 endpoint of the test in both species was moulting inhibition (moulting inhibition concentration, MIC) relative to the negative control after 7 days of exposure, where moulting inhibition equals larvae that have either died or not moulted to the 2nd instar after 7 days.

The concentration ranges were comprised between 1 and 128 ng Cry1Ab/cm^2 for both species. To determine the susceptibility of each population, 7 to 10 different concentrations resulting in mortality or moulting 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 batch B2-3 of protein. The MIC_{50} value obtained for *S. nonagrioides* was compared with those of the same population in previous years. The MIC_{50} value was established for first time for the new reference strain of *O.nubilalis*.

2.5.2. Susceptibility of S. nonagrioides *to the Cry1Ab protein in diagnostic concentration bioassays*

A diagnostic concentration (DC) intended to cause moulting inhibition between 99 and 100% to first instar larvae of *S. nonagrioides* has been estimated. Given that starting from 2016 only NE Iberian populations will be recorded, the DC was calculated with data from larvae collected in NE Spain over four previous seasons: 2009, 2011, 2013

and 2015. The result represents the response of more than 4300 larvae in four dose-response bioassays with these populations. The obtained MIC_{99} (and 95% confidence intervals) with the pool of data was 734 (531-1091) ng Cry1Ab/cm^2 (PoloPlus). The value selected as DC is the upper limit of 95% CI of the MIC_{99} (Crespo et al., 2008; Alcantara et al, 2011), to obtain levels of moulting inhibition between 99 and 100%. Thus, the DC to be applied hereafter for *S. nonagrioides* is 1091 ng Cry1Ab/cm^2.

The susceptibility to the protein Cry1Ab by the use of DC bioassays was tested on F1 progeny of the field population collected in NE Spain in 2016 and on the reference laboratory strain of *S. nonagrioides*. The methodology of the bioassay was the same as that explained above (Section 2.4.1.), with the exception that only DC (1091 ng Cry1Ab/cm^2) and control (sodium bicarbonate buffer solution) are tested. A minimum of 1000 neonates per field population (zone) were treated, and 100 neonates per zone were used as controls. Moulting inhibition was recorded after 7 days.

To ensure that as many field collected individuals as possible were represented in the bioassay, and to calculate the detection limit for resistance allele frequency in this study, the following parameters were quantified: number of field collected larvae, number of emerged adults, number of oviposition cages settled and used in bioassays and number of neonates used in the bioassays from each oviposition cage (treated and controls). The number of egg masses used for the bioassays was maximized by doing the bioassay at a daily basis, whenever neonates of an oviposition cage emerged. This procedure forced to (i) estimate in advance the number of oviposition cages to be set, (ii) determine the approximate number of neonates to be used from each oviposition cage (treated and controls), and (iii) prepare a lot of small bioassay arenas and to conduct the DC bioassay little by little, so that the bioassays extended for about 2 months.

2.5.3. 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 survival was recorded. Groups of 200-300 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. This experiment was performed at the same conditions of insect culture: 25 ± 1ºC, 70 ± 10% relative humidity and a photoperiod of 16:8 hours (light: dark). If necessary, fresh tissue was added every 2-3 days.

2.6. Statistical analysis

The results of moulting inhibition at different concentrations of Cry1Ab (dose-response bioassays) were adjusted by probit weighted regression lines. The moulting inhibition concentrations (MICs) for 50% (MIC_{50}) and 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. Plots showing the percent response to the different concentrations of the Cry1Ab protein were performed with the program PoloPlus 1.0 (LeOra Software, 2002-2017).

The average percentage of moulting inhibition of neonates after treatment at the diagnostic concentration (DC) was tested to determine if it was significantly lower than the expected value of 99% and than the percentage of moulting inhibition observed in the reference strain after treatment at the same DC. In both cases, a one-sample t-test and a one-tailed probability distribution (IBM SPSS Statistics 23) was used. Moulting inhibition values of each zone were corrected with Abbott's formula (Abbott, 1925) prior to analysis.

3. Results and Discussion

3.1. Collection of larvae

During 2016 growing season, we significantly increased our sampling efforts and expanded the scale of the bioassays. Three technicians with long years of experience were involved in the collection of field larvae for *S. nonagrioides* and *O. nubilalis*. To ensure the collection of at least 1000 larvae for each species in the Ebro valley, the technicians spent in total over 380 working hours within a span of 14 days and travelled over 3550 km. Each technician spent 10 working hours per day in the field to maximize the use of sunlight during the field larvae collections. The technicians made in total four

rounds of trips to four different field zones to collect adequate larvae for the bioassay. As a result, they were able to achieve a successful collection of the field larvae for the bioassay by covering the total distance of 3550 km in 14 days within four trips in the four zones. Regardless, it should be noted that in one field zone the technicians did not succeed in collecting sufficient larvae for *O. nubilalis* as required for the bioassay. Therefore, this required an additional trip totaling 28 hours of larvae collection in about 550 km in another field zone to ensure the collection of sufficient larvae for this species.

A total of 1364 last instar larvae of *S. nonagrioides* were collected between September and October 2016 from three different zones in NE Spain (428, 524 and 412 larvae from the zones 1, 2 and 3, respectively; **Table 3**). A map showing the sampling points for *S. nonagrioides* is displayed in **Annex IIa.** In the zones 1 and 2 more than three fields were sampled due to the insufficient number of larvae found, whereas larvae came from only two fields in the zone 3 (**Figure 1 and Annex IIb**). The maximum distance between successfully sampled fields within a zone was always below 15 Km (**Annex IIb**).

Larvae of *O. nubilalis* were collected between September and October 2016 from three zones in the Northeast of Spain, yielding a total of 1111 larvae (478, 393 and 240 larvae from the zones 1, 3 and 4, respectively; **Table 4a**). Larvae were searched in 12 fields in the zone 2, but only 51 larvae could be gathered (**Table 4b**, **Annex IIIc**). Thus, this zone was disregarded, the larvae collected were not used in the bioassays and an additional zone 4 was searched for this species. A map showing the samplings points for *O. nubilalis* is displayed in **Annex IIIa.** More than three fields were sampled in the zones 1 and 4 to complete the required number of larvae, whereas and in the zone 3 larvae came from only two fields (**Figure 1**, **Annex IIIb**). The maximum distance between successfully sampled fields within a zone was below 10 Km (**Annex IIIb**).

In the light of the results of this season, the collection of the targeted 1000 larvae per species in the Ebro valley in the same 10 km x 10 km zones and with the same effort is largely unpredictable in future growing seasons.

3.2. Susceptibility of the reference strains to the Cry1Ab protein in dose-response bioassays

The bioassay to evaluate the susceptibility to Cry1Ab of the laboratory population of *S. nonagrioides* was performed with 862 neonates. The MIC_{50} value (30 ng Cry1Ab/cm^2;

Table 5, Figure 2a) is within the range of values obtained since 2011 with the same batch of toxin, and very similar to that obtained last year (**Figure 3**). During these years MIC_{50} values of this population have varied slightly, ranging between 5 and 30 ng Cry1Ab/cm^2.

The susceptibility to Cry1Ab toxin of the new laboratory strain of *O. nubilali*s was assessed for the first time using 958 neonates. The MIC_{50} value obtained was 5 ng Cry1Ab/cm^2 (**Table 5, Figure 2b**). This result is higher than the MIC_{50} value observed last year with the previous reference strain (1 ng Cry1Ab/cm^2), but within the range of values obtained since 2010 with the same batch of toxin (**Figure 4**).

Variations in laboratory-reared insects regarding their susceptibility to pesticides or insecticidal proteins, such as we have observed historically during this monitoring program, are not unusual. 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). Even so, MIC values of the control laboratory strains have been in general very consistent in the interval of years examined using the same batch of toxin (B2), being the maximum magnitude of variation 6- and 5-fold for *S. nonagrioides* (**Table 6**) and *O. nubilalis*, respectively.

3.3. Diagnostic concentration bioassays

From the 1364 larvae of *S. nonagrioides* collected in the field from three different zones in the NE Spain in 2016, 960 adults (70%) emerged, which were placed in 90 oviposition cages for mating. The offspring of 95% of these adults (911) was used in the bioassays and treated with the diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2. These values mean that about 67% of the field collected larvae were represented in the DC bioassays (**Table 7**). Therefore, the detection limit for resistance allele frequency in 2016 is 0.033 (3.3%). This has been calculated considering the model developed by Andow and Ives (2002) regarding the potential monitoring methods to study resistance evolution, where the statistical detection limit using larval screen is $1/(N)^{1/2}$ for recessive alleles.

More than 3000 F1 neonates originated from the field collected larvae were used in the bioassays. The DC (1091 ng Cry1Ab/cm^2) caused a mean (± S.E.) moulting inhibition of 97.96% ± 0.71% (98.86%, 98.47% and 96.56% in larvae from zone 1, zone 2 and zone 3, respectively; **Table 7**). This value was not significantly lower than the expected

value of 99% (t = -1.459, df = 2, p = 0.141). The same DC applied to neonates of the laboratory strain of *S. nonagrioides* caused moulting inhibition of 99.20% (**Table 7**). Yet again, the average value obtained with field F1 neonates (97.96%) was not significantly lower from the moult inhibition value of the reference strain (t = -1.740, df = 2, p = 0.112). Since this is the first time that DC bioassays are performed for measuring susceptibility, the moult inhibition value could not be compared with values of previous years.

3.4. Survival of larvae on MON 810 leaves

None of the F1 spare first instar larvae of *S. nonagrioides* (more than 10,000), not used in bioassays, from the populations collected in three different zones in the Ebro valley in 2016 could survive after 10 days feeding ad libitum on MON 810 tissue.

Additionally, there was no survivor among the larvae not killed by the treatment with Cry1Ab in the DC bioassays that were exposed to MON 810 leaves for 10 days.

4. Summary of results

1. Monitoring for changes in the susceptibility of EU field populations of *S. nonagrioides* and *O. nubilalis* to the Bt Cry1Ab in 2016 has been exclusively focused for the first time in the Ebro valley, located in the Northeast of Spain (NE Spain), the place in the EU with the highest level of adoption of this transgenic crop. A total of 1364 last instar larvae of *S. nonagriodes* and 1111 larvae of *O. nubilalis* have been collected in three sampling zones for each species within the Ebro valley in 2016. Larvae of *O. nubilalis* were sent to the laboratory BTL GmbH Sagerheide (Germany) for testing their susceptibility to the Cry1Ab protein.
2. The susceptibility to the Cry1Ab toxin of the field populations of *S. nonagrioides* from NE Spain has been determined by the use of diagnostic-concentration bioassays, instead of dose-response bioassays, as in previous years. With this purpose, a diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2, intended to cause molting inhibition $\geq$ 99% to first instar larvae of *S. nonagrioides*, was estimated with data from larvae collected from NE Spain in previous seasons (2009, 2011, 2013 and 2015).
3. From the 1364 larvae of *S. nonagrioides* collected, 960 adults (70%) emerged,

and the offspring of 95% of these adults (911) was used in the bioassays and treated with the DC of 1091 ng Cry1Ab/cm^2. These values indicate that about two thirds of the field collected larvae were represented in the DC bioassays. Thus, the detection limit for resistance allele frequency in field populations of *S. nonagrioides* in 2016 is 0.033 (3.3%). This number considerably improves those of previous seasons.

4. The treatment with the DC caused moulting inhibition of 97.96% (S.E. 0.71%) to F1 neonates from the field collected larvae of the NE Spain. This outcome was not significantly lower than the expected value of 99%, and than the percentage of moulting inhibition observed in the reference strain after treatment at the same DC.
5. No survivors have been reported in the more than 10,000 spare larvae of the F1 generation of the field collected populations and survivors of DC bioassays that were exposed to MON 810 leaves.
6. A new reference strain of *O. nubilalis* has been established in the laboratory, coming from larvae collected in December 2015 from Galicia (Northwest of Spain), where Bt maize has never been cultivated.
7. The laboratory strain of *S. nonagrioides* and the newly established laboratory strain of *O. nubilalis* showed susceptibility levels to the batch B2-3 of the Cry1Ab toxin (MIC_{50} values of 30 and 5 ng Cry1Ab/cm^2, respectively) comparable with those obtained for laboratory strains in previous years.

5. Concluding remarks

The moulting inhibition caused to F1 neonates of *S. nonagrioides* from larvae collected in the Ebro valley in 2016 after treatment at a diagnostic concentration was not significantly lower than the expected value of 99%. Thus, no decrease in the susceptibility of *S. nonagrioides* to the Cry1Ab protein has been observed.

In the light of the results of this season, the collection of the targeted 1000 larvae per species in the Ebro valley in the same 10 km x 10 km zones and with the same effort is largely unpredictable in future growing seasons.

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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	Sigma-Aldrich

Table 3. *Sesamia nonagrioides* larvae collection details for the 2016 season in the Ebro valley (NE Spain)

Zone	Field	Province [a]	Postal Code	Surface (Ha) [b]	Distance to the nearest MON810 field (m) [c]	Date	No of larvae collected
1	Lanaja 1	HU	22250	3	300	12-15 Sep 2016	0
	Lanaja 2	HU	22250	2	200	12-15 Sep 2016	0
	Lanaja 3	HU	22250	25	150	12-15 Sep 2016	176
	Lanaja 4	HU	22250	5	0	12-15 Sep 2016	0
	Lanaja 5	HU	22250	3	50	12-15 Sep 2016	142
	Sariñena 1	HU	22200	3	50	12-15 Sep 2016	110
	Sariñena 2	HU	22200	5	50	12-15 Sep 2016	0
							Total: 428
2	Candasnos 1	HU	22591	2	40	19-22 Sep 2016	149
	Candasnos 2	HU	22591	10	20	19-22 Sep 2016	0
	Candasnos 3	HU	22591	5	0	19-22 Sep 2016	0
	Candasnos 4	HU	22591	8	30	19-22 Sep 2016	175
	Candasnos 5	HU	22591	10	0	19-22 Sep 2016	0
	Candasnos 6	HU	22591	8	0	19-22 Sep 2016	0
	Candasnos 7	HU	22591	15	400	19-22 Sep 2016	0
	Candasnos 8	HU	22591	9	0	19-22 Sep 2016	0
	Peñalba 1	HU	22592	5	0	19-22 Sep 2016	186
	Peñalba 2	HU	22592	5	0	19-22 Sep 2016	14
	Ontiñena	HU	22232	8	unknown	19-22 Sep 2016	0
	Bujaraloz	Z	50177	16	0	19-22 Sep 2016	0
							Total: 524
3	La Almunia de Doña Godina 1	Z	50100	45	unknown, >1000	3-5 Oct 2016	200
	La Almunia de Doña Godina 2	Z	50100	30	unknown, >1000	3-5 Oct 2016	0
	La Almunia de Doña Godina 3	Z	50100	15	unknown, >1000	3-5 Oct 2016	212
							Total: 412

Grand total: 1364

[a] Provinces: HU = Huesca; NA = Navarra; Z = Zaragoza
[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.

Table 4. *Ostrinia nubilalis* larvae collection details for the 2016 season in the Ebro valley (NE Spain)

a) Zones where larvae collection has been successful

Zone	Field	Province [a]	Postal Code	Surface (Ha) [b]	Distance to the nearest MON810 field (m) [c]	Date	No of larvae collected [d]
1	Lanaja 1	HU	22250	3	300	12-15 Sep 2016	166
	Lanaja 2	HU	22250	2	200	12-15 Sep 2016	0
	Lanaja 3	HU	22250	25	150	12-15 Sep 2016	78
	Lanaja 4	HU	22250	5	0	12-15 Sep 2016	0
	Lanaja 5	HU	22250	3	50	12-15 Sep 2016	112
	Sariñena 1	HU	22200	3	50	12-15 Sep 2016	122
	Sariñena 2	HU	22200	5	50	12-15 Sep 2016	0
							Total: 478
3	La Almunia de Doña Godina 1	Z	50100	45	unknown, >1000	3-5 Oct 2016	200
						20 Oct 2016	154
	La Almunia de Doña Godina 2	Z	50100	30	unknown, >1000	3-5 Oct 2016	0
	La Almunia de Doña Godina 3	Z	50100	15	unknown, >1000	3-5 Oct 2016	39
							Total: 393
4	Mendigorria 1	NA	31150	7.5	200	6-7 Oct 2016	172
	Mendigorria 2	NA	31150	4	100	6-7 Oct 2016	20
	Mendigorria 3	NA	31150	6	0	6-7 Oct 2016	0
	Mendigorria 4	NA	31150	22	400	6-7 Oct 2016	0
	Artajona 1	NA	31140	4	300	6-7 Oct 2016	48
	Artajona 2	NA	31140	5	0	6-7 Oct 2016	0
	Puente la Reina	NA	31100	10	400	6-7 Oct 2016	0
							Total: 240

Grand total: 1111

Table 4 (Cont.)

b) Zones where larvae collection has failed

Zone	Field	Province [a]	Postal Code	Surface (Ha) [b]	Distance to the nearest MON810 field (m) [c]	Date	No of larvae collected
2	Candasnos 1	HU	22591	2	40	19-22 Sep 2016	7
	Candasnos 2	HU	22591	10	20	19-22 Sep 2016	0
	Candasnos 3	HU	22591	5	0	19-22 Sep 2016	2
	Candasnos 4	HU	22591	8	30	19-22 Sep 2016	3
	Candasnos 5	HU	22591	10	0	19-22 Sep 2016	0
	Candasnos 6	HU	22591	8	0	19-22 Sep 2016	0
	Candasnos 7	HU	22591	15	400	19-22 Sep 2016	2
	Candasnos 8	HU	22591	9	0	19-22 Sep 2016	0
	Peñalba 1	HU	22592	5	0	19-22 Sep 2016	6
	Peñalba 2	HU	22592	5	0	19-22 Sep 2016	4
	Ontiñena	HU	22232	8	unknown	19-22 Sep 2016	12
	Bujaraloz	Z	50177	16	0	19-22 Sep 2016	15
							Total: 51

[a] Provinces: HU = Huesca; NA = Navarra; Z = Zaragoza
[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 from zones 1, 3 and 4 were sent to Germany after removing those that were damaged or seemed to have some pathogen.

Table 5. Susceptibility to Cry1Ab toxin of the reference laboratory populations of *S.nonagrioides* and *O. nubilalis*.

Species	Toxin batch	n	Slope ± SE	χ^2	d.f.	MIC_{50}[a] (FL 95%)	MIC_{90}[a] (FL 95%)
S. nonagrioides	B2-3	862	2.9 ± 0.2	61.2	19	30 (24-38)	83 (62-132)
O. nubilalis	B2-3	958	1.9 ± 0.1	58.7	22	5 (4-7)	26 (18-44)

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

Table 6. Susceptibility to Cry1Ab toxin of laboratory populations of *S. nonagrioides* between 2004 and 2015. The bioassays performed during this campaign is 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	B2-1	9 (6-13)	68 (45-127)
Laboratory	2012	B2-1	7 (5-10)	62 (41-107)
Laboratory	2013	B2-1	7 (5-10)	48 (31-88)
Laboratory	2013	B2-2	5 (3-9)	42 (26-87)
Laboratory	2014	B2-2	17 (11-25)	91 (57-209)
Laboratory	2015	B2-2	28 (21-36)	67 (50-110)
Laboratory	2016	B2-3	30 (24-38)	83 (62-132)

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

Table 7. Tracking of *S. nonagrioides* from the NE Spain populations and from the laboratory population used in the diagnostic concentration bioassays, and outcomes of the bioassays.

	Tracking of the larvae used in the diagnostic concentration bioassays							Diagnostic concentration bioassays				
Population	Fields	Last instar larvae collected	Adults emerged[a]	Oviposition cages	Oviposition cages used in bioassays[b]	Adults used in bioassays (M-F)[c]	Total adults whose offspring was used[d]	Nº larvae treated in bioassays	MI (%)[e]	Nº larvae control	MI in control (%)[e]	Corrected MI (%)[f]
Northeast Spain	Zone 1	428	288 (67%)	25	24	148-134	282 (66%) (98%)	1024	98.93	160	5.63	98.86
	Zone 2	524	376 (72%)	36	34	167-183	350 (67%) (93%)	1004	98.51	191	2.09	98.47
	Zone 3	412	296 (72%)	29	27	139-140	279 (68%) (94%)	1202	96.67	221	3.17	96.56
	Total	1364	960 (70%)	90	85	454-457	911 (67%) (95%)	3230	97.96	572	3.50	97.88
Laboratory	-	-	378	27	18	138-116	254	783	99.23	192	4.69	99.20

[a] Adults from field collected larvae emerged between 2nd December 2016 and 16th January 2017. Those emerged before or after these dates were discarded. The percentage with respect to the number of larvae collected is in brackets.
[b] Cages were discarded when eggs hatched during the weekend or when the fecundity and/or fertility was too low
[c] M, males; F, females
[d] The percentages with respect to the number of collected larvae and with respect to the number of emerged adults are in brackets (in this order)
[e] MI, moulting inhibition: larvae that have not reached the second larval instar
[f] Calculated using Abbot's formula (Abbot, 1925)

Figure 1. Success in field collections of *S. nonagrioides* and *O. nubilalis* in the four different zones (Z1, Z2, Z3 and Z4) searched in the NE Spain in 2016. A collection at a field within a zone was considered successful if at least 20 larvae were gathered.

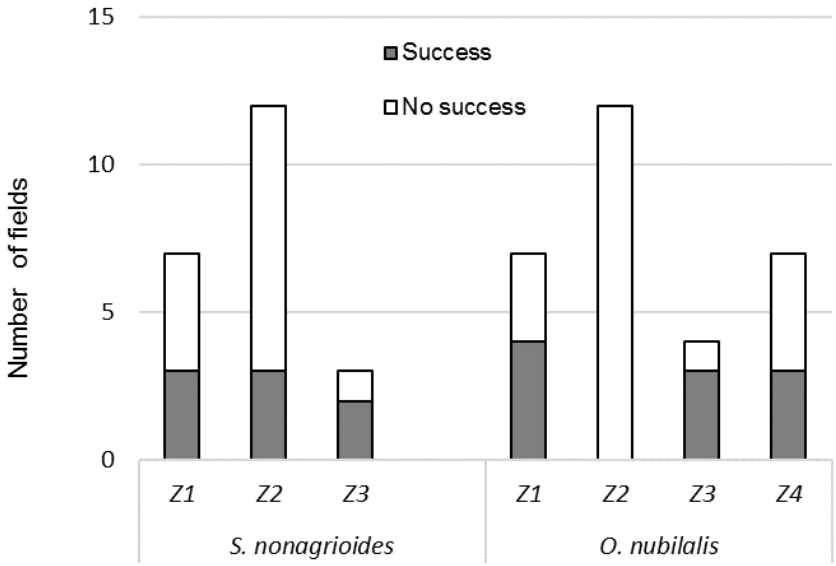


Figure 2. Fitted curves of susceptibility to the toxin Cry1Ab of the laboratory populations of *S. nonagrioides* and *O. nubilalis* (PoloPlus. LeOra Software. 2002-2016). Response is moulting inhibition after seven days feeding on treated diet. **a:** *S. nonagrioides*. **b:** *O. nubilalis*.

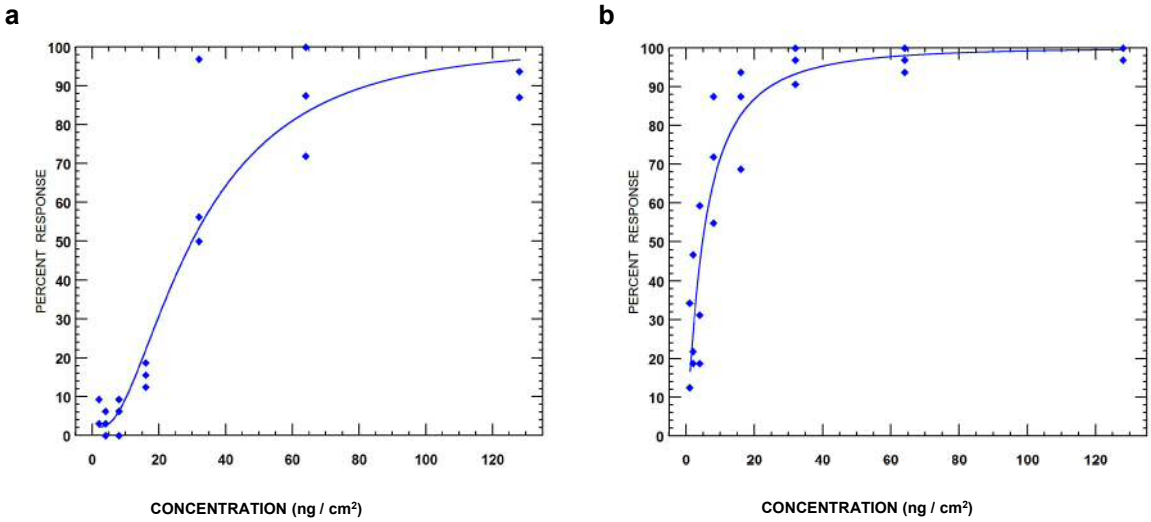


Figure 3. Susceptibility to Cry1Ab toxin. measured by MIC_{50} values. of a laboratory population of *S. nonagrioides*. Colors indicate the B1 (blue) and B2 (pink) toxin batches.

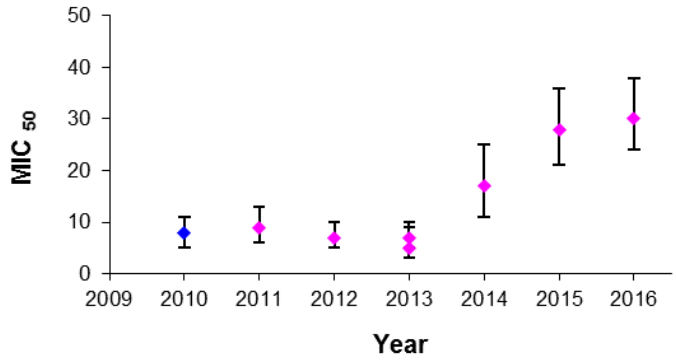
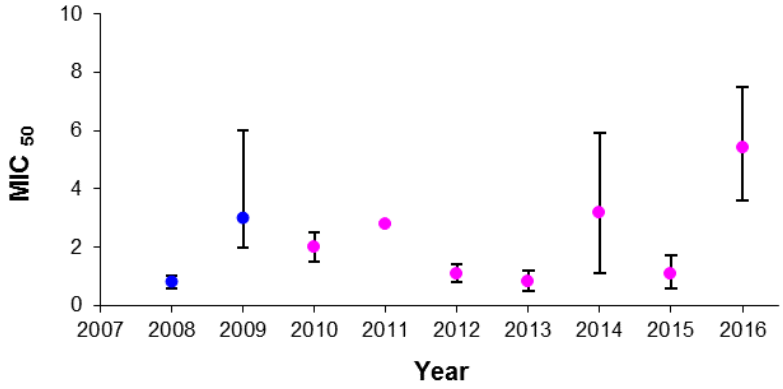


Figure 4. Susceptibility to Cry1Ab toxin. measured by LC_{50} and MIC_{50} values of a laboratory population of *O. nubilalis.* Colors indicate the B1 (blue) and B2 (pink) toxin batches.



ANNEX IIa. Collection of *S. nonagrioides* larvae in the Ebro valley in 2016

ANNEX IIb. Collection of *S. nonagrioides* larvae in the Ebro valley in 2016

Successful field-Zone 1

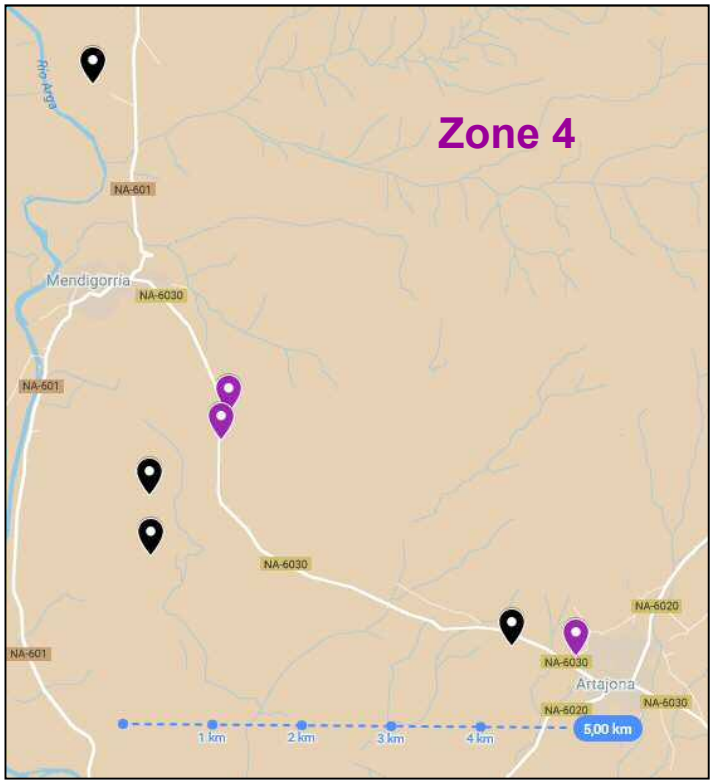
Successful field-Zone 2

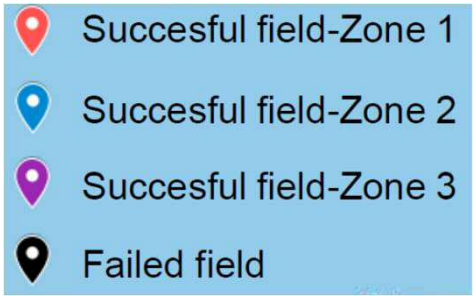
Successful field-Zone 3

Failed field

ANNEX IIIa. Collection of *O. nubilalis* larvae in the Ebro valley in 2016

ANNEX IIIb. Collection of *O. nubilalis* larvae in the Ebro valley in 2016





ANNEX IIIc. Collection of *O. nubilalis* larvae in the Ebro valley in 2016