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

Season 2017

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1. Introduction

Maize containing event MON 810 is transgenic improved maize expressing the Cry1Ab protein derived from *Bacillus thuringiensis* subsp. *kurstaki*, and conferring protection against certain lepidopteran insect pests such as *Ostrinia nubilalis* and *Sesamia nonagrioides*. Resistance development in targeted lepidopteran pests is a potential concern arising from the widespread cultivation of MON 810 maize varieties. In order to maintain the benefits obtained from growing MON 810 maize varieties, Monsanto, following directions described in the industry IRM (Insect Resistance Management) working group guidelines proposed to the competent authority (EU Commission), available since 2003 but published in 2007 (Alcalde et al., 2007) and subsequently updated as the EuropaBio harmonised IRM plan (EuropaBio, 2012; 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.

Routine monitoring for changes in the susceptibility of EU field populations of *S. nonagrioides* to the Cry1Ab protein has been carried out in the period 2004-2016[1]. During the period 2004-2015 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

[1] https://ec.europa.eu/food/plant/gmo/reports_studies_en (Post-market environmental monitoring)

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. The susceptibility of *S. nonagrioides* field populations to the Cry1Ab protein expressed in MON 810 maize varieties was estimated 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 compared with previous baseline susceptibility data established for this species or with a susceptible laboratory strain assayed with the same batch of toxin (González-Núñez et al., 2000; Farinós et al., 2004, 2018)

The harmonised IRM plan was 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; Farinós et al., 2018; Thieme et al., 2018). 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 revised plan proposes that monitoring for these corn borers in this area should be carried out on an annual basis. This revised plan was put in practice for first time during the season of 2016, being this season (2017) the second time.

In addition, the EFSA Scientific Opinion (EFSA GMO Panel, 2017) and Statement (EFSA, 2018) on the annual post-market environmental monitoring (PMEM) report on the cultivation of genetically modified maize MON 810 in 2015 and 2016, respectively, included two main recommendations: *(1) annual sampling of target pests in the Ebro Valley, the area where deployment of Bt-maize is the highest and where resistance is likely to evolve more quickly; and (2) setting a maximum detection threshold for resistance allele frequency at 3% to enable the early detection of resistance so that alternative management measures can be implemented in time to delay the development of resistance.*

Accordingly with these recommendations and following the revised harmonized IRM plan (EuropaBio, 2017), in the seasons 2016 and 2017 the collection of field larvae has been concentrated in the Ebro valley (including the Autonomous Communities of

Aragón, Cataluña and Navarra), where the adoption rate of Bt maize is over 60%. Moreover, a diagnostic concentration bioassay (Sims et al., 1997; Marçon et al., 2000) has been used to monitor for changes in susceptibility to the Cry1Ab protein in *S. nonagrioides* field populations from Northeast Spain. This methodology allows the use of 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 2017 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, and comparison of the susceptibility value obtained with that of the susceptible laboratory strain and with the hypothetical value of 99%.

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), which 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. 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.

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 2017, 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 diet 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.

Larvae of *S. nonagrioides* were in 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 (from 4 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 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. As a general

rule, these populations are formed from individuals collected in non-Bt fields, although from areas where Bt maize is grown (always avoiding the Ebro valley). A minimum of 300 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, in which case it is removed. Populations that are maintained for many years in the laboratory typically suffer excessive inbreeding. 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 with new individuals collected in non-Bt fields after taking some precautions: i) the similarity (no significant differences) between the LC_{50} values of both the laboratory and field populations is checked by susceptibility bioassays; ii) the absence of pathogens (namely *Nosema* sp.) in the new population is verified by inspecting a number of larvae in slides under the microscope in every generation; and iii) new individuals are maintained separately for two-three generations in the laboratory before introducing the laboratory colony. To the best of our knowledge, this has been proved to be the best practice to avoid the collapse that we have experienced in the past working with these species.

Through 2018, larvae of *S. nonagrioides* have been requested to the Misión Biológica de Galicia (MBG, CSIC) in Galicia, Spain, where Bt maize has never been cultivated. After some generations in the lab, they are now adapted to feed on an artificial diet and to laboratory conditions (Hoffmann & Ross, 2018). Our objective is to establish a population from Galicia as the reference population from next season onwards, and refresh it yearly with new individuals from the same region. This will facilitate the availability of a susceptible population that has never been exposed to Bt. In the same way, a new population of *O. nubilalis* coming from Galicia has been used from the 2016 season as a reference 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 (2017). 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, B2-3 in April 2016 and B2-4 in July 2017. 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 B2-4 of Cry1Ab 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 4 and 128 ng Cry1Ab/cm^2 for *S. nonagrioides* and between 1 and 128 ng Cry1Ab/cm^2 for *O. nubilalis*. To determine the susceptibility of each population, 6 to 8 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 B2-4 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 determined for the second for the new reference strain of *O. nubilalis*, established in the laboratory last year.

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

A diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2, calculated with data from larvae collected in NE Spain over the seasons 2009, 2011, 2013 and 2015, was used for DC bioassays to measure susceptibility to the Cry1Ab protein. This DC is intended to cause moulting inhibition between 99 and 100% to first instar larvae of *S. nonagrioides*.

The susceptibility to the protein Cry1Ab by the use of DC bioassays was tested on F1 progeny of the field populations collected in NE Spain in 2017 and on the reference laboratory strain of *S. nonagrioides,* which served as control. 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 development on MON 810 tissue

A experiment was performed to verify that resistant individuals were not present in the field-collected populations even if some larvae had moulted to the 2nd larval instar in the DC bioassay, following a stepwise approach.

Two-hundred neonates (not used in the DC bioassays) of each oviposition cage of the F1 generation were exposed to MON 810 fresh leaves and about 10 larvae of each cage, which served as control, were exposed to conventional maize leaves. Larvae

were kept in plastic boxes provided with new maize leaves without the central nerve and they were allowed to feed ad libitum. If necessary, fresh tissue was added every 2-3 days. Moulting to the 2nd larval instar was recorded after 10 days. After that time, larvae that moulted to the 2nd larval instar were maintained continuously on Bt maize, and their siblings from the same oviposition cage (which had not been used in the F1 bioassay) were reared on artificial diet to perform the same bioassay with MON 810 tissue in the following generation (F2). Again, moulting to the 2nd larval instar was recorded after 10 days.

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).

2.6. Statistical analysis

The results of moulting inhibition of laboratory populations 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 PoloPlus 1.0 (LeOra Software, 2002-2018). 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 average percentage of moulting inhibition of neonates after treatment at the diagnostic concentration (DC) was estimated to determine if it was significantly lower than (i) the percentage of moulting inhibition observed in the susceptible reference strain after treatment at the same DC and (ii) the expected generical value of 99%. Values were compared by a one-sample t-test and a one-tailed probability distribution (IBM SPSS Statistics 23). Previously, moulting inhibition values of each zone had been corrected with Abbott's formula (Abbott, 1925).

3. Results and Discussion

The stepwise approach followed to do the bioassays is showed in **Annex I**.

3.1. Collection of larvae

Three technicians were involved in the collection of field larvae for *S. nonagrioides* and *O. nubilalis* during 2017 growing season. They spent in total about 370 hours of fieldwork and travelled over 3100 km, and made in total three rounds of trips to three different field zones to collect a sufficient number of larvae for the bioassay.

A total of 1452 last instar larvae of *S. nonagrioides* were collected between September and October 2017 from three different Zones in NE Spain (504, 493 and 455 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.** Larvae were collected in three fields in the Zone 1, whereas six and ten fields had to be sampled in the Zones 2 and 3, respectively, to get sufficient number of larvae (**Figure 1, Annex IIb**). The maximum distance between successfully sampled fields was about 10, 20 and 5 Km within the Zone 1, 2 and 3, respectively (**Annex IIb**).

Larvae of *O. nubilalis* were collected between September and October 2017 from three Zones in the Northeast of Spain, yielding a total of 1111 larvae (452, 319 and 340 larvae from the Zones 1, 2 and 3, respectively; **Table 4**). A map showing the samplings points for *O. nubilalis* is displayed in **Annex IIIa.** Despite the number of fields sampled (three, six and ten fields in the Zone 1, 2 and 3, respectively), larvae were mainly gathered in two fields separated by 1 Km in the Zone 1, one field in the Zone 2 and two fields separated by 4 Km in the Zone 3 (**Figure 1**, **Annex IIIb**). Sampling during this season has revealed the increasing difficulty to find different fields attacked by *O. nubilalis* among those available for sampling.

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 767 neonates. The MIC_{50} value (24 ng Cry1Ab/cm^2; **Table 5, Figure 2a**) is slightly lower than that obtained last year, and within the range of values obtained since 2011 with the same batch of toxin. Since 2004 MIC_{50} values of this population have varied slightly, ranging between 5 and 30 ng Cry1Ab/cm^2 (**Table 6**).

The susceptibility to Cry1Ab toxin of the laboratory strain of *O. nubilali*s received in 2016 was assessed for the second time using 850 neonates. The MIC_{50} value obtained was 3.9 ng Cry1Ab/cm^2 (**Table 5, Figure 2b**), similar to the MIC_{50} value observed last year (5.4 ng Cry1Ab/cm^2) and within the range of values (0.8-5.4 ng Cry1Ab/cm^2)

obtained since 2010 with the previous reference strain and the same batch of toxin (**Figure 3**).

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. (Robertson et al., 1995; Marçon et al., 1999; Da Silva et al., 2016 ; Farinós et al., 2018). 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 7-fold for *S. nonagrioides* (**Table 6**) and *O. nubilalis*, (**Figure 3),** respectively.

3.3. Diagnostic concentration bioassays

From the 1452 last instar larvae of *S. nonagrioides* collected in the field from three different zones in the NE Spain in 2017, 589 (41%, combining larvae and pupae) died in the process of rearing in the laboratory and 75 adults (5%) did not emerge in the date range for oviposition cages **(Table 7).** Thus, 788 adults (54%), emerged between 21th December 2017 and 5th February 2018, were placed in 62 oviposition cages for mating. The offspring of 95% of these adults (749) was used in the bioassays and treated with the diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2. These values mean that about 52% of the field collected larvae were represented in the DC bioassays (**Table 8**). Therefore, the detection limit for resistance allele frequency in 2017 is 0.0365 (3.7%). 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.

Of the total F1 neonates originated from the field collected larvae, 3333 were used in the bioassays. The DC (1091 ng Cry1Ab/cm^2) caused a mean (± S.E.) moulting inhibition of 94.14% ± 1.4% (91.65%, 96.50% and 94.28% in larvae from zone 1, zone 2 and zone 3, respectively; **Table 8**). This value was significantly lower than the expected value of 99% (t = -3.4647, df = 2, p = 0.037). However, the same DC applied to neonates of the laboratory strain of *S. nonagrioides* caused moulting inhibition of 97.69% (**Table 8**). In this case, the average value obtained with field F1 neonates

(94.14%) was not significantly lower from the moult inhibition value of the reference strain (t = -2.5373, df = 2, p = 0.063).

Note that the value of moult inhibition obtained with the laboratory susceptible strain is below the expected value of 99%. This result contrasts with that obtained in 2016, the first time that DC bioassays were implemented in resistance monitoring for *S. nonagrioides*, in which the percentage of moult inhibition in the laboratory strain at the DC was 99.20% (**Table 9**). Fluctuations of about 6-fold for both LC_{50} and MIC_{50} were also found in the laboratory strain during the period that monitoring was performed by means of dose-response bioassays (2004–2016), but no trends were observed over time. Indeed, though the MIC_{50} for the control strain in 2017 was within the range obtained in previous years, the MIC_{90} was the highest of the historical record (**Table 6**). To account for these fluctuations apparently related to experimental conditions (protein batch, testing conditions, etc.), MIC_{50} and LC_{50} values of field populations were compared with the susceptible laboratory strain (Farinós et al., 2018).

This finding highlights the importance of maintaining a susceptible laboratory strain against which the field populations should be compared, enabling the correct interpretation of the results.

3.4. Confirmatory experiment: Development of larvae on MON 810 leaves

10,650 F1 spare first instar larvae of *S. nonagrioides*, not used in bioassays, from the populations collected in three different zones in the Ebro valley in 2017, were fed ad libitum on MON 810 tissue. As a control, 426 neonates were reared on conventional maize. After 10 days, 406 (95.31%) of the control larvae had moulted to the 2nd instar, whereas 10 larvae (0.09%) of those reared on Bt maize could moult to the 2nd larval instar **(Table 10)**. The 10 surviving larvae came from the same oviposition cage. They were forced to feed continuously on Bt maize and all of them died before moulting to the 3rd larval instar.

To verify that these 10 larvae were not resistant to Bt maize, their siblings, coming from the same oviposition cage, were kept for one more generation. They were reared on artificial diet under laboratory conditions. One hundred adults (44 males and 56 females) were allocated to 5 oviposition cages and 1000 neonates of the F2 generation were fed ad libitum on MON 810 tissue, in a new confirmatory experiment. In this case, no larvae moulted to the 2nd larval instar after 10 days, and all of them died.

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 2017 has been focused for the second time in the Ebro valley, in the Northeast (NE) of Spain, where the adoption rate of Bt maize in 2017 was over 60%. A total of 1452 last instar larvae of *S. nonagriodes* and 1111 larvae of *O. nubilalis* were collected in three sampling zones for each species. 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 in bioassays by the use of a diagnostic-concentration (DC) of 1091 ng Cry1Ab/cm^2, intended to cause molting inhibition $\geq$ 99% to first instar larvae of *S. nonagrioides*. This DC was estimated with data from larvae collected from NE Spain in four previous seasons: 2009, 2011, 2013 and 2015.
3. From the 1452 larvae of *S. nonagrioides* collected, 788 adults (54%) emerged, and the offspring of 95% of these adults (749) was used in the bioassays and treated with the DC of 1091 ng Cry1Ab/cm^2. These values indicate that just over half 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 2017 is 0.0365 (3.7%). This number is slightly higher than that of last season (3.3%), even though 6% more larvae were collected in this season.
4. The treatment with the DC caused moulting inhibition of 94.10% (S.E. 1.4%) to F1 neonates from the field collected larvae of the NE Spain. This outcome was significantly lower than the hypothetical value of 99% (t=-3.4647, p=0.037), although not significantly different (t=-25373, p=0.063) to the percentage of moulting inhibition observed in neonates of the susceptible laboratory strain (97.69%) after treatment at the same DC.
5. Among the 10,650 neonates of the F1 generation of the field collected populations that were exposed to MON 810 leaves, ten larvae coming from the same oviposition cage moulted to the 2nd larval instar, but none of them reached the 3rd larval instar. Moreover, none of the 1,000 neonates of the F2 generation coming from the same parents survived when they were fed with MON 810 tissue.

6. The susceptible laboratory strains of *S. nonagrioides* and strain of *O. nubilalis* showed susceptibility levels to the batch B2-4 of the Cry1Ab toxin (MIC_{50} values of 24 and 4 ng Cry1Ab/cm^2, respectively) comparable with those obtained for laboratory strains in previous years.

5. Concluding remarks

The collection in 2017 of a higher number of last instar larvae of *S. nonagrioides* than in previous seasons has not been translated into an improvement of the detection limit for resistance allele frequency, given the higher proportion of individuals lost in the process of rearing, especially during the diapause period.

The moulting inhibition (94.1%) caused to F1 neonates of *S. nonagrioides* from larvae collected in the Ebro valley in 2017 after treatment at a diagnostic concentration (DC) was significantly lower than the hypothetical value of 99%. However, the value obtained was not significantly different than that caused to susceptible neonates of the laboratory strain (97.7%) treated with the same DC. This finding highlights the importance of maintaining a susceptible laboratory strain against which the field populations should be compared, enabling correct interpretation of the results. It also underlines the dilemma of how to choose an appropriate comparator to assess variations in the susceptibility of field populations to the Cry1Ab toxin.

The results obtained are in line with those reported in Camargo et al. (2018) after carrying out a new F2 screen. This study concludes that the frequency of resistance alleles in 2016 is higher but not statistically different from the value obtained in 2004–2005, and therefore resistance in the Ebro Valley does not seem to be evolving faster than predicted by a *S. nonagrioides* resistance evolution model (Castañera et al., 2016).

6. References

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7. 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 2017 season in the Ebro valley (NE Spain)

Zone	Field	Province [a]	Postal Code	Date	Surface (Ha) [b]	Distance to the nearest MON810 field (m) [c]	No of larvae collected [d]
1	2017-Lanaja 1	HU	22250	12-14 Sep 2017	27	5	171
	2017-Lanaja 2	HU	22250	12-14 Sep 2017	7	100	152
	2017-Lanaja 3	HU	22250	12-14 Sep 2017	40	200	181
	Total						**504**
2	2017-Candasnos 1	HU	22591	26-28 Sep 2017	13	0	126
	2017-Candasnos 2	HU	22591	26-28 Sep 2017	40	15	22
	2017-Candasnos 3	HU	22591	26-28 Sep 2017	23	60	179
	2017-Candasnos 4	HU	22591	26-28 Sep 2017	10	14	12
	2017-Candasnos 5	HU	22591	26-28 Sep 2017	10	60	9
	2017-Ontiñena	HU	22591	26-28 Sep 2017	13	10	145
	Total						**493**
3	2017-Artajona 1	NA	31140	03-05 Oct 2017	3,5	0	163
	2017-Artajona 2	NA	31140	03-05 Oct 2017	2	0	25
	2017-Artajona 3	NA	31140	03-05 Oct 2017	20	50	98
	2017-Artajona 4	NA	31140	03-05 Oct 2017	8	0	3
	2017-Falces 1	NA	31370	03-05 Oct 2017	1,5	0	5
	2017-Falces 2	NA	31370	03-05 Oct 2017	2,5	0	0
	2017-Mendigorría 1	NA	31150	03-05 Oct 2017	2	0	8
	2017-Mendigorría 2	NA	31150	03-05 Oct 2017	1,9	0	12
	2017-Mendigorría 3	NA	31150	03-05 Oct 2017	6	0	141
	2017-Miranda de Arga	NA	31253	03-05 Oct 2017	2,3	0	0
	Total						**455**
	Grand total						**1452**

[a] Provinces: HU = Huesca; NA = Navarra.
[b] Data are approximate
[c] There could be other nearer fields that are not known by the technician and/or the farmer. "0" means that it is adjacent to a MON 810 field.

Table 4. *Ostrinia nubilalis* larvae collection details for the 2017 season in the Ebro valley (NE Spain)

Zone	Field	Province [a]	Postal Code	Date	Surface (Ha) [b]	Distance to the nearest MON810 field (m) [c]	No of larvae collected [d]
1	2017-Lanaja 1	HU	22250	12-14 Sep 2017	27	5	237
	2017-Lanaja 2	HU	22250	12-14 Sep 2017	7	100	215
	2017-Lanaja 3	HU	22250	12-14 Sep 2017	40	200	0
	Total						**452**
2	2017-Candasnos 1	HU	22591	26-28 Sep 2017	13	0	26
	2017-Candasnos 2	HU	22591	26-28 Sep 2017	40	15	0
	2017-Candasnos 3	HU	22591	26-28 Sep 2017	23	60	280
	2017-Candasnos 4	HU	22591	26-28 Sep 2017	10	14	0
	2017-Candasnos 5	HU	22591	26-28 Sep 2017	10	60	0
	2017-Ontiñena	HU	22591	26-28 Sep 2017	13	10	13
	Total						**319**
3	2017-Artajona 1	NA	31140	03-05 Oct 2017	3,5	0	239
	2017-Artajona 2	NA	31140	03-05 Oct 2017	2	0	4
	2017-Artajona 3	NA	31140	03-05 Oct 2017	20	50	0
	2017-Artajona 4	NA	31140	03-05 Oct 2017	8	0	4
	2017-Mendigorria 1	NA	31150	03-05 Oct 2017	2	0	0
	2017-Mendigorria 2	NA	31150	03-05 Oct 2017	1,9	0	0
	2017-Mendigorria 3	NA	31150	03-05 Oct 2017	6	0	93
	2017-Miranda de Arga	NA	31253	03-05 Oct 2017	2,3	0	0
	2017-Falces 1	NA	31370	03-05 Oct 2017	1,5	0	0
	2017-Falces 2	NA	31370	03-05 Oct 2017	2,5	0	0
	Total						**340**
	GRAND TOTAL						**1111**

[a] Provinces: HU = Huesca; NA = Navarra
[b] Data are approximate
[c] There could be other nearer fields that are not known by the technician and/or the farmer. "0" means that it is adjacent to a MON 810 field.

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

Species	Toxin batch	n	Slope ± SE	X^2	d.f.	MIC_{50}[a] (FL 95%)	MIC_{90}[a] (FL 95%)
S. nonagrioides	B2-4	767	1.6 ± 0.2	27.9	16	24 (16-35)	162 (100-363)
O. nubilalis	B2-4	850	2.0 ± 0.16	44.8	19	3.9 (2.8-5.1)	16.5 (12.0-26.2)

[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 2017. The bioassay 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)
Laboratory	2017	B2-4	24 (15-35)	162 (100-363)

[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. Individuals of *S. nonagrioides* lost in the process of rearing or discarded for susceptibility bioassays. Percentages are shown with respect to the number of field larvae collected in each zone.

Fields	Field larvae collected	Dead larvae and pupae	Adults out of the date range for oviposition cages [a]
Zone 1	504	167 (33.1%)	35 (6.9%)
Zone 2	493	240 (48.7%)	15 (3.0%)
Zone 3	455	182 (40.0%)	25 (5.5%)
Total	1452	589 (40.6%)	75 (5.2%)

[a] Adults that did not emerge between 21st December 2017 and 5th February 2018.

Table 8. 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	504	302 (60%)	22	20	124-161	285 (57%) (94%)	1048	91,79	175	1,71	91,65
	Zone 2	493	238 (48%)	20	18	112-119	231 (47%) (97%)	1111	97,03	159	15,09	96,50
	Zone 3	455	248 (55%)	20	18	107-126	233 (51%) (94%)	1174	94,63	160	6,25	94,28
	All zones[g]	1452	788 (54%)	62	56	343-406	749 (52%) (95%)	3333	94,54	494	7,49	94,10
Laboratory	-	-	257	18	12	92-105	197	654	98,01	157	14,01	97,69

[a] Adults from field collected larvae emerged between 21st December 2017 and 5th February 2018. 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] For field populations, 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)
[g] Results obtained pooling the data of the three zones.

Table 9. Moulting inhibition of the F1 neonates of the NE Spain population and the susceptible laboratory strain, reported 7 days after treatment with a diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2.

Year	Moulting inhibition at DC (%)		*p*-values [a]	
	NE Spain	Lab strain	Observed Lab strain	Expected 99%
2016	97.96 ± 0.71	99.20	0.112	0.141
2017	94.14 ± 1.40	97.69	0.063	0.037

[a] *p*-values of one-sample t-test analyses performed to compare the percentage of moulting inhibition of the field population (NE Spain) with respect to the observed moulting inhibition obtained with the susceptible laboratory strain and with the expected theoretical value of 99%.

Table 10. Larval growth of neonates of the F1 generation of *S. nonagrioides* after 10 days feeding on Bt (MON 810) or not-Bt (conventional) maize tissue.

	ZONE 1		ZONE 2		ZONE 3		TOTAL	
	MON 810	Conventional	MON 810	Conventional	MON 810	Conventional	MON 810	Conventional
Nº of F0 oviposition cages used [a]	17	11	18	14	18	14	53	39
Nº of F1 neonates exposed [b]	3450	110	3600	156	3600	160	10650	426
Nº moulted larvae (≥ L2)	0	107	0	146	10	153	10	406
% moulting (≥ L2)	0.00	97.27	0.00	93.59	0.28	95.63	0.09	95.31

[a] F0 is the generation collected in the field.
[b] F1 and F2 neonates were < 24 h.

Figure 1. Successful field collections of *S. nonagrioides* and *O. nubilalis* in three different zones (Z1, Z2 and Z3) searched in the NE Spain in 2017. A collection at a field within a zone was considered successful if at least 50 larvae were gathered.

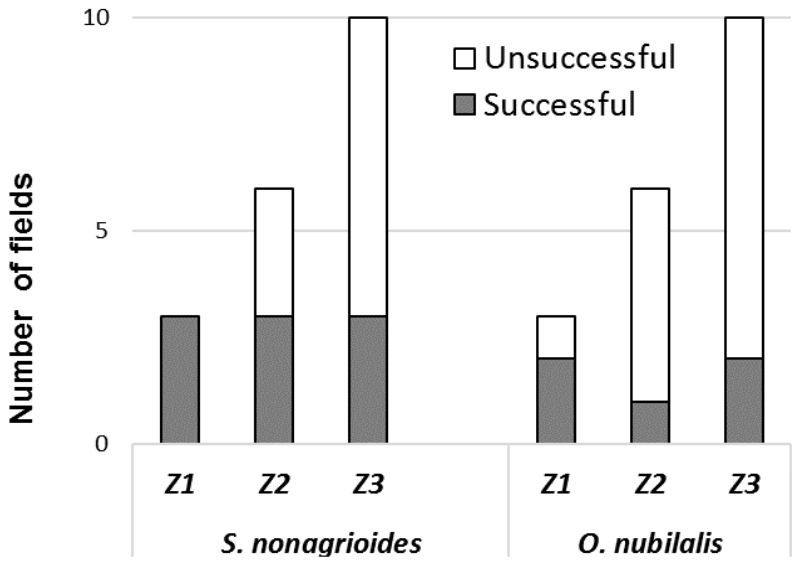

Figure 2. Fitted curves of susceptibility to the toxin Cry1Ab of the laboratory populations of *S. nonagrioides* and *O. nubilalis* (PoloPlus 1.0, LeOra Software 2002-2018). Response is moulting inhibition after seven days feeding on treated diet. **a:** *S. nonagrioides*. **b:** *O. nubilalis*.

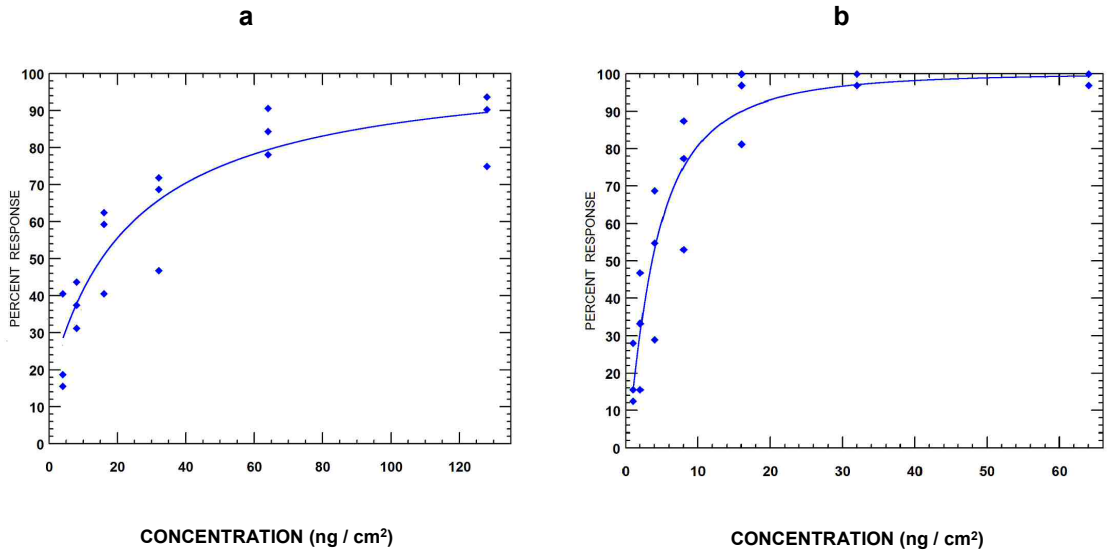

Figure 3. Susceptibility to Cry1Ab toxin measured by MIC_{50} values of a laboratory population of *O. nubilalis.* Colors indicate the B1 (blue) and B2 (pink) toxin batches.