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

Season 2023

CONTENTS

1. Introduction		2
2. Materials and Methods		4
	2.1. Insect collection	4
	2.2. Insect rearing	5
	2.3. Laboratory strains	6
	2.4. Cry1Ab protein	7
	2.5. Bioassays	7
	2.5.1. Susceptibility of the reference strains of *S. nonagrioides* and *O. nubilalis* to the Cry1Ab protein in concentration-response (CR) bioassays	7
	2.5.2. Susceptibility of *S. nonagrioides* to the Cry1Ab protein in diagnostic concentration (DC) bioassays	8
	2.5.3. Larval development on MON 810 tissue: Plant bioassays	9
	2.5.4. Confirmatory experiments	9
	2.6. Statistical analysis	10
3. Results and Discussion		11
	3.1. Collection of larvae and insect rearing	11
	3.2. Susceptibility of the reference strains to the Cry1Ab protein in concentration-response bioassays	13
	3.3. Diagnostic concentration bioassays	13
	3.4. Larval development on MON 810 tissue: plant bioassays	15
	3.5 Confirmatory experiments	15
4. Summary of results		15
5. Concluding remarks		17
6. References		18
7. Tables and figures		21-32
Annex I	Stepwise approach followed to do bioassays with *S. nonagrioides*	33
Annex II	Collection of *S. nonagrioides* larvae in NE Spain in 2023	34-35
Annex III	Collection of *O. nubilalis* larvae in NE Spain in 2023	36-37

1. Introduction

Maize containing event MON 810 is transgenic improved maize expressing the Cry1Ab protein derived from *Bacillus thuringiensis* subsp. *Kurstaki* (Bt maize), 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 cultivation of MON 810 maize varieties. In order to maintain the benefits obtained from growing MON 810 maize varieties, Bayer established an insect resistance monitoring program across Europe, focused on areas where MON 810 maize is currently or planned to be commercially grown for the control of the European targeted pests *O. nubilalis* and *S. nonagrioides*. The objective is to detect, in a timely manner, the potential evolution of resistance that could result in inadequate protection against the target species. To achieve this goal, Bayer follows 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 harmonized IRM plan (EuropaBio, 2012, 2017, 2019; CropLife Europe, 2021, 2023). This report focuses on the monitoring plan for *S. nonagrioides* in the 2023 growing season.

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; Eizaguirre and Fantinou, 2012; 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 very 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. Second generation larvae infest older maize plants, damaging stems and ears, also causing significant yield losses (Velasco et al., 2004).

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 2004-2023[1]. During the period 2004-2015, the plan covered the three maize-growing areas in the EU where MON 810 hybrids were been grown and *S. nonagrioides* was present: north-

[1] https://ec.europa.eu/food/plant/gmo/post_authorisation/plans_reports_opinions_en

eastern Iberia, central Iberia and south-western 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 harmonized IRM plan (EuropaBio, 2012). In Iberia, each target field population was initially monitored every two years. The susceptibility of *S. nonagrioides* field populations to the Cry1Ab protein expressed in MON 810 maize varieties was estimated by means of concentration-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 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 harmonized IRM plan has been subsequently updated (EuropaBio, 2017, 2019; CropLife Europe, 2021, 2023) to accommodate upgrades in the regulatory framework, and to incorporate new available scientific information and learnings gained from this and other IRM plans (Farinós et al., 2018; Thieme et al., 2018; Bertho et al., 2020; García et al., 2023). The current revised plan establishes that sampling for resistance monitoring should take place in areas where the Bt maize adoption is over 60% and where the target pests are present. Presently, this situation only occurs in north-eastern (NE) Spain within the EU. Therefore, in accordance to that plan and since *S. nonagrioides* and *O. nubilalis* are multivoltine species in this area, monitoring should be carried out annually. Furthermore, the EFSA Scientific Opinion (EFSA GMO Panel, 2017) and Statement (EFSA, 2018) on the annual post-market environmental monitoring (PMEM) reports on the cultivation of genetically modified maize MON 810 in 2015 and 2016, respectively, included the recommendations to (1) perform annual sampling of target pests in NE Spain, the area where deployment of Bt-maize is the highest and where resistance is likely to evolve more quickly; and (2) set maximum detection threshold for resistance allele frequency at 3% to enable the early detection of resistance so that alternative management measures could be implemented in time to delay the development of resistance.

In accordance with these recommendations and following the revised harmonized IRM plans (EuropaBio, 2017, 2019; CropLife Europe, 2021, 2023), from the 2016 season

onwards the collection of field larvae has been done annually focusing in NE Spain (including the Autonomous Communities of Aragon, Catalonia and Navarre), where the adoption rate of Bt maize is the highest in the EU, exceeding 60% in some years. Moreover, to decrease the detection threshold for resistance allele frequency, 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 NE Spain. The aim of using this methodology is that a high number of field-collected individuals are represented in the laboratory bioassays as F1 larvae.

The tasks carried out in the 2023 maize growing season were the following:

1. Collection of larvae of *S. nonagrioides* in three different zones from NE Spain to be used in: i) diagnostic concentration bioassays, to compare the susceptibility (in terms of moult inhibition) of the field populations with that of a susceptible laboratory strain and with the hypothetical value of 99%; and ii) plant bioassays, to compare larval mortality when feeding on MON 810 maize *vs.* conventional maize.

2. Collection of larvae of *O. nubilalis* in three different zones from NE Spain 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 concentration-response bioassays, 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 (Zone 1: Huesca, Zone 2: Girona and Zone 3: Navarra) for each species in NE Spain (NE Spain), each zone comprising at least three different maize fields.

A minimum of 1000 larvae were initially targeted for collection per species, about 350 larvae collected in each of the three sampling zones and, if possible, a minimum of 50 larvae per maize field. However, due to the mortality rates observed in field larvae

when kept in the laboratory, an effort has been made to collect as many larvae as possible to achieve the target for maximum detection threshold for resistance allele frequency of 3%.

The process of identifying fields for insect collection starts by contacting local field technicians of seed companies that commercialize MON 810 maize varieties in Spain. They are asked if they are aware of the presence of corn borers in the area to be sampled, for which they may consult a number of growers about the detection of corn borer damage in their fields. When that is the case, the growers are asked for permission to enter their fields 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 the standard operative procedures (SOP) of each species (CropLife Europe, 2023). The samples were collected at the end of the maize-growing season, during September and October 2023, from refuges and fields of conventional maize close to MON 810 maize. This is carried out cutting the stalk of the maize plants and taking only one larvae of each species per plant to avoid collecting siblings.

2.2. Insect rearing

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

Larvae of *S. nonagrioides* that were in diapause at the time of collection were placed on a rearing chamber (Sanyo MLR-350 H, Sanyo, Osaka, Japan) at 14 ± 1ºC, 70 ± 5% relative humidity and a photoperiod of 12:12 hours (L:D). They were kept at these conditions until the larvae showed signs of diapause break. Then, larvae were placed under conditions 28 ± 1ºC, 70 ± 5% relative humidity and continuous light until pupation. Larvae that were not in diapause at the time of collection were maintained at standard rearing conditions (25 ± 1ºC, 70 ± 10% relative humidity and a photoperiod of 16:8 hours (L:D) until pupation. The sex was determined at the pupal stage and a variable number of couples from the same zone (normally from 3 to 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. After 7 days, egg masses were collected, placed into ventilated plastic boxes containing wet filter paper and incubated under the same conditions. Neonates (larvae < 24 h old) were utilized in the bioassays.

2.3. Laboratory strains

Reference susceptible strains of *S. nonagrioides* and *O. nubilalis* maintained under laboratory conditions serve as control in this study. As a general rule, these populations are formed from individuals collected in conventional non-Bt fields from areas where Bt maize has never been commercially grown or has a low adoption rate, and therefore have not been subjected to selection pressure. Individuals coming from the field are used as reference populations after adaptation to the artificial diet and to laboratory conditions (Hoffmann and Ross, 2018). In the laboratory, a minimum of 300 adults of each species are crossed every generation. Neonate larvae are taken from all the oviposition cages formed with the adults of the previous generation, unless any of them have symptoms of any disease, in which case it is removed. In addition, the larvae are periodically checked for the presence of pathogens (namely *Nosema* sp.) by inspecting a number of them in slides under the microscope and by molecular methods (PCR). It is important to bear in mind that populations maintained for many years in the laboratory typically suffer excessive inbreeding (Roush, 1986), which may influence bioassay results.

The current reference population of *O. nubilalis* originated from field larvae collected in three locations of Pontevedra province (Galicia, north-western Spain) in 2015, with the assistance of La Misión Biológica de Galicia (MBG, CSIC) staff. This field strain has been maintained and used as reference strain by the BTL GmbH Sagerheide laboratory (Germany) since then, and a part of it was sent to the CIB-CSIC laboratory (Spain) in 2022 to use it also as a reference population. For *S. nonagrioides*, a new reference population for 2024 has been collected in Portugal, since the former colony (originated from larvae collected in Galicia in 2020) was affected by *Nosema* sp. infection after 26 generations in the laboratory. Besides, the former population from Galicia, used as reference strains since 2018, presented high natural variability in Cry1Ab susceptibility. This new Portuguese reference population was formed from 402 adults, 62.9% of the 639 larvae collected in Coimbra. As this sampling could not be carried out until June 2024, the population did not have a period of adaptation to the

laboratory prior to its use for bioassays. Therefore, the results obtained with this population in this season should be taken with caution. Nevertheless, all indications are that the population is adapting well to laboratory conditions.

2.4. Cry1Ab protein

Two batches of Cry1Ab protein have been used since the start of the MON 810 monitoring plan in 2004. The first batch (B1) was provided by Bayer in 2003 (2.03 mg/ml in sodium bicarbonate buffer, pH 10.5; purity 95%), and it was used until 2010. The second batch (B2) (1.8 mg/ml in 50 mM sodium bicarbonate buffer, pH 10.25; purity 91%) has been provided at different times: B2-1, B2-2, B2-3, B2-4, B2-6, B2-7, B2-8, B2-9, B2-10 and B2-11 were received in 2011, 2014, 2016, 2017, 2018, 2019, 2020, 2021, 2022 and 2023, respectively. 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 mM) with pH 10.25 was used.

2.5. Bioassays

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

The bioassays were carried out in accordance with the methods described by Farinós et al. (2004), using “Bio-Assay Tray-128 Cells (BAW128)” plastic trays (Frontier Scientific Services Agriculture, DE, USA). 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 (<24 h) was placed in each well using a fine paintbrush and it was covered with a breathing adhesive cover “Bio-Assay Tray Lid-16 Cells (BACV16)” (Frontier Scientific Services Agriculture, DE, USA). 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 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 2^{nd} larval instar (L2) after 7 days.

To determine the susceptibility of each population, 5 to 7 different concentrations resulting in moulting inhibition higher than 0% and below 100% were used. The concentration ranges were comprised between 2 and 128 ng Cry1Ab/cm^2 for *S. nonagrioides* and between 0.25 and 16 ng Cry1Ab/cm^2 for *O. nubilalis*. At least three replicates were prepared for each concentration and the control for both species. In general, a replicate consisted of 16 to 32 larvae per concentration (32 to 64 for controls). For each replicate, neonates from different oviposition cages were used. The MIC_{50} values obtained for both *O. nubilalis* and *S. nonagrioides* were compared with those obtained with the reference populations in previous years.

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

A diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2, intended to cause moulting inhibition between 99 and 100% to first-instar (L1) larvae of *S. nonagrioides*, was used for DC bioassays to measure susceptibility to the Cry1Ab protein. The value of the DC was estimated by using all the available data of MIC bioassays performed with larvae collected in NE Spain over the seasons 2009, 2011, 2013 and 2015. Hence, the resulting value represented the response of more than 4300 larvae in four concentration-response bioassays. This DC has been used from the 2016 campaign onwards, as no new concentration-response bioassays of field populations have been done since then.

Susceptibility to the Cry1Ab protein by the use of DC bioassays was tested on F1 progeny of the field populations collected in NE Spain in 2023 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 2.5.1.), 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 a minimum of 100 neonates per zone were used as controls. Moulting inhibition was recorded after 7 days.

It was ensured that as many field individuals as possible were represented in the bioassays. To this end, individuals of *S. nonagrioides* from each of the three zones were tracked and the following variables were recorded: 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 (infertile oviposition cages are not represented in the bioassay). 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 to conduct the bioassay little by little. All this, together with the fact that the larvae from Girona were not in diapause, meant that the DC bioassays were conducted over a period of 18 weeks.

2.5.3. Larval development on MON 810 tissue: Plant bioassays

Plant bioassays were performed to verify that there were no resistant individuals in the field-collected populations even if some larvae from the DC bioassay had moulted to the 2nd larval instar.

About two-hundred neonates (not used in the DC bioassays) of each oviposition cage of the F1 generation coming from the three NE Spain zones and of the laboratory strain 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 and survival was recorded after 10 days.

It was ensured that all the Bt plants used in the bioassay expressed Cry1Ab by means of ImmunoStrip® for Bt-Cry1Ab/1Ac (Agdia Inc., Elkhart, IN).

2.5.4. Confirmatory experiments

Different experiments aimed at confirming that survivors of DC and plant bioassays from field-collected populations were not resistant individuals were performed when necessary after the F1 bioassays.

Firstly, all L2 larvae recovered alive after 7 days in the DC bioassay were placed in plastic boxes of 9 cm in diameter and 3 cm height, with those coming from the same oviposition cage grouped together. Then, they were fed *ad libitum* on Bt maize leaves, following the same procedure of section 2.5.3. If any of these larvae fed on MON 810 were able to moult to the 3rd larval instar and they were alive after 10 days, their siblings (not used in the F1 bioassays) were fed on an artificial diet and reared until the

next generation (F2) to perform new DC and plant bioassays, as explained in sections 2.5.2. and 2.5.3.

In the case of plant bioassays, if any neonate fed on MON 810 were able to moult to the 2nd larval instar and they were alive after 10 days, their siblings (not used in the F1 bioassays) were fed on an artificial diet and reared until the next generation (F2) to perform a new DC bioassay and plant bioassay, as explained in sections 2.5.2. and 2.5.3.

The confirmatory experiments were carried out under the same conditions of temperature, humidity and light as the insect culture and bioassays described above.

2.6. Statistical analysis

The results of moulting inhibition of laboratory populations at different concentrations of Cry1Ab (concentration-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, 2002-2024). 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 obtained MIC_{50} was 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 value of 99%. Moulting inhibition values of each zone were corrected with Abbott's formula (Abbott, 1925) and logit transformed before analyses. Values were compared by a one-sample t-test and a one-tailed probability distribution, and trends over time in moulting inhibition values (2016-2023 period) were assessed by linear regression (IBM SPSS Statistics 29).

Additionally, moulting inhibition of neonates after treatment at the diagnostic concentration (DC) of field populations and laboratory strains were compared by a test of equivalence, following the "Statistical considerations for the safety evaluation of GMOs" proposed by the EFSA (EFSA GMO Panel, 2010). The set of laboratory susceptible populations from the years 2016-2022 has been defined as the reference

for comparison. Two-sided 90% confidence intervals were calculated for each year since 2016, assuming a simple two-group design of the experiment (field populations of a given year *vs* reference) and a lognormal distribution for the observations in each group. As the definition of suitable ranges in this area have not been established, asymmetric equivalence limits of 20, 10 and 5% (maximum acceptable percent difference at logarithmic scale) have been used. The calculated confidence intervals and equivalence limits were plotted and interpreted following EFSA recommendations (EFSA GMO Panel, 2010).

3. Results and Discussion

The stepwise approach followed to perform the bioassays is shown in **Annex I**. In addition to the recommendations made in the CropLife Europe document (2023), this approach considers the recommendations made by the United States of America Environmental Protection Agency, which specifies the follow-up steps to be initiated in the event that the resistance monitoring bioassays detect a population with unusually low sensitivity to a Bt toxin (US EPA, 2010; 2018). In the present approach, the unusual low susceptibility/sensitivity to Bt toxin is measured in the diagnostic and plant bioassays, and referred to specific oviposition cages.

3.1. Collection of larvae and insect rearing

This campaign, the technicians involved in the collection of field larvae for *S. nonagrioides* and *O. nubilalis* carried out about 186 hours of fieldwork, travelled over 3380 km and made a total of three round trips, one for each different field zone.

A total of 1803 last-instar larvae of *S. nonagrioides* were collected in September and October 2023 from three different Zones in NE Spain (568, 573 and 662 larvae from Zones 1, 2 and 3, respectively; **Table 3**). A map showing the sampling points for *S. nonagrioides* is displayed in **Annex IIa.** Fourteen, five and nine fields in Zones 1, 2 and 3, were searched, respectively, but larvae were successfully collected in four fields of Zones 1 and 2 and three fields in Zone 3 **(Figure 1, Annex IIb**). The maximum distance between successfully sampled fields was about 39, 19 and 7 Km within Zones 1, 2 and 3, respectively (**Annex IIb**). Thus, the minimum of 1000 larvae targeted for collection could be fulfilled.

Larvae of *O. nubilalis* were collected between September and October 2023 from the

same three Zones in the NE Spain, yielding a total of 556 larvae (299, 88 and 169 larvae from Zones 1, 2 and 3, respectively; **Table 4**). A map showing the sampling points for *O. nubilalis* is displayed in **Annex IIIa.** Larvae were successfully collected in two fields in Zone 1 and Zone 3 and one field in Zone 2 (**Figure 1, Annex IIIb**), although the number of fields surveyed was higher (fourteen in Zone 1, five in Zone 2 and nine in Zone 3). The maximum distance between successfully sampled fields was about 2 Km within Zone 1 and 5 km within Zone 3 (**Annex IIIb**). Even though 28 maize fields have been surveyed, the minimum of 1000 larvae targeted for collection could not be reached for this species.

Attempts are made to collect more larvae than the minimum target number so that the highest possible number of field individuals would be represented in the bioassays and thus reach the maximum detection threshold of 3% of the resistance allele frequency. However, this is not always achieved, due to lack of larvae in the prospected fields and/or to high mortality rates of field larvae reared in the laboratory. There are different reasons that could explain the high mortality of both lepidopteran species during the diapause period, which usually lasts at least 3 months (García et al., 2023). In the case of *S. nonagrioides*, larval mortality in the laboratory has been found to be higher when reared under diapause conditions than that observed when reared under normal maintenance conditions (Fantinou and Tsitsipis, 1999). In addition, mortality during the diapause time may increase due to the transmission of diseases or to the emergence of parasitoids from larvae carried from the field (Eizaguirre et al., 1990; Fantinou and Tsitsipis, 1999; Monetti et al., 2003). Another cause could be a low adaptation to artificial breeding conditions (Hoffmann and Ross, 2018), which may be different depending on the population, even if they are of the same species (Carpenter and Bloem, 2002). To reduce these effects as much as possible in *S. nonagrioides*, the following measures are taken: (a) a low number of larvae per box is maintained to reduce mortality by limiting disease spread (Fantinou and Tsitsipis, 1999); (b) the vermiculite of the boxes is frequently renewed during the diapause period to prevent fungal growth; (c) those larvae from boxes suspected of containing larvae with pathogens go through additional asepsis (dipping of larvae in 1% bleach solution); and (d) the diet in the rearing boxes is renewed only once a week in order not to disturb diapause conditions.

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

The susceptibility to Cry1Ab protein of the laboratory population of *S. nonagrioides* in a concentration-response bioassay was performed with 576 neonates. One of the three replicates conducted was not taken into account for the calculation of MIC values, since mortality in the controls was higher than 25%, which is the maximum value allowed for a replicate to be considered valid. The bioassay resulted in a MIC_{50} value of 15 (4-28) ng Cry1Ab/cm^2 (**Table 5**, **Figure 2a**), which is in the range of MIC_{50} values obtained with laboratory populations in previous years with the same batch of toxin (between 5 and 30 ng Cry1Ab/cm^2; **Table 6**).

A number of 1437 neonates of the *O. nubilalis* laboratory strain were used for the Cry1Ab susceptibility assessment bioassay. The MIC_{50} value obtained with the reference strain was 4.2 ng Cry1Ab/cm^2 (**Table 5**, **Figure 2b**), in the range of values obtained with laboratory strains with the same batch of toxin (0.8 to 5.4 ng Cry1Ab/cm^2; **Figure 3**).

Variations in laboratory-reared insects regarding their susceptibility to pesticides or insecticidal proteins, 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; García et al., 2023). 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 1803 *S. nonagrioides* last-instar larvae collected, 773 (42.9%) larvae and pupae died in the process of rearing in the laboratory, mainly during the diapause period. In addition, 58 adults (3.2%) did not emerge in the date range for oviposition cages (between 28th September 2023 and 19th February 2024) or had some malformation upon emergence, so they were not used in the bioassays (**Table 7**)**.** Thus, of the 1030 adults that emerged, 972 were placed in 97 oviposition cages for mating. The offspring of 925 of these adults was used in the bioassays and treated with the diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2 (**Table 8**). Therefore, the

detection limit for resistance allele frequency in 2023 is 0.033 (3.3%), calculated considering the model developed by Andow and Ives (2002).

Of the total F1 neonates originated from the field collected larvae, 3455 were used in the bioassays. The DC (1091 ng Cry1Ab/cm^2) caused a corrected moulting inhibition (MI) of 95.95%, 93.06% and 98.39% in Zones 1, 2 and 3, respectively (**Table 8**). For the reference strain, 1232 neonates were tested, causing the inhibition of moulting in 98.85% of them.

Statistically significant differences were observed between field-collected populations MI value (95.80 ± 1.54%) and the expected MI value of 99% (t = 2.94, df = 2, p = 0.049), but no statistically significant differences were found between field-collected populations and the laboratory strain (98.85%) MI values (t = 2.62, df = 2, p = 0.060) (**Table 9**). Likewise, significant differences between the MI value of the field populations and the expected MI value were obtained in 2017, 2019 and 2022, but only in 2017 the MI value of the field populations was significantly lower than that of the laboratory strain (**Table 9**).

The MI value for field populations obtained in 2023 (95.80%) is in the range of previously obtained values (93.65% - 98.65%). Only two years (2016 and 2021) out of eight (from 2016 to 2023), and only with the laboratory strain, the obtained MI values were over the expected 99% value (**Table 9; Figure 4a**). Besides, when the MI values were assessed over time by lineal regression, no significant trends were obtained (R^2 = 0.039, p = 0.353), supporting no evidence of field resistance in terms of changes in the susceptibility of populations from NE Spain to the Cry1Ab protein **(Figure 4b)**. The annual fluctuations of these values could be due to the great natural and intrapopulation variability in susceptibility to the toxin shown by *S. nonagrioides*, as occurs with other lepidopteran pests (Ferré and Van Rie, 2002; Han et al., 2006; Ostrem et al., 2016), being a challenge, in many cases, to make accurate estimates of susceptibility to Cry toxins and making it difficult to detect biologically significant changes. These variations are also evident in control strains, among which fluctuations of up to 6-fold have been found (**Table 6**). This underlines the importance of comparing field populations with a reference population, which enables a correct interpretation of the results.

The interpretation of the results of the test of equivalence has been made based on the directions of EFSA GMO Panel (2010; Section 4.1. and Figure 1). The confidence

intervals for differences in moulting inhibition of neonates after treatment at the diagnostic concentration (DC) lie within the 10% equivalence limits for the 8 years analyzed (2016-2023). Therefore, it can be concluded that the field and laboratory populations in those years are equivalent in their susceptibility to Cry1Ab toxin, based on moult inhibition data. Being more restrictive, using the 5% equivalence limits, it can be observed that in the years 2016, 2018, 2019, 2020 and 2021 the field and laboratory populations are also equivalent. In 2017, 2022 and 2023 the confidence intervals overlapped with one of these limits; in these cases, non-equivalence cannot be rejected, but it should be concluded that equivalence between the two populations (field and laboratory) is more likely than not (**Figure 5**).

3.4. Larval development on MON 810 tissue: plant bioassays

16610 F1 first-instar larvae of *S. nonagrioides*, from the populations collected in three zones in NE Spain in 2023, and 4260 from the laboratory strain were fed *ad libitum* on MON 810 tissue to test if they were able to moult to the 2nd larval instar within ten days. As a control, 800 neonates of these field-collected populations were reared on conventional maize. No controls of the reference strain could be assayed due to the lack of conventional maize plants at the time of the bioassay as a result of a technical failure of the chamber in which they were growing. During the assay none of the larvae from the field-collected populations nor from the laboratory strain were able to moult to the 2nd larval instar and survive after 10 days feeding on MON 810 maize. Most larvae fed on conventional maize from the field populations (94.4%) moulted at least to 2nd larval instar after 10 days **(Table 10)**.

3.5 Confirmatory experiments

A total of 140 (4.05%) larvae from 38 oviposition cages reached the 2nd larval instar in the F1 DC bioassay (**Table 11**). They were collected and grouped into boxes, according to the oviposition cage of origin, and then fed on MON 810 leaves. None of these larvae moulted to the 3rd larval instar and survived 10 days feeding on Bt maize leaves (**Table 11**). Thus, no additional confirmatory assays were carried out.

4. Summary of results

1. Monitoring for changes in the susceptibility of EU field populations of *S. nonagrioides* and *O. nubilalis* to Cry1Ab protein in 2023 has been focused for the eighth time in NE

Spain, where the adoption rate of MON 810 maize is highest of Europe. In 2022 adoption rate of MON 810 maize on the three NE Spanish regions was 31.6% in Catalonia, 44.6% in Aragon and 31.4% in Navarre (MAPA, 2024a, 2024b).

A total of 1803 larvae of *S. nonagrioides* and 556 larvae of *O. nubilalis* were collected in three sampling zones. The minimum of 1000 larvae targeted for collection could be fulfilled for *S. nonagrioides*, but not for *O. nubilalis,* due to the low levels of larval infestation in most of the sampled fields. Larvae of *O. nubilalis* were sent to the laboratory BTL GmbH Sagerheide (Germany) for testing their susceptibility to the Cry1Ab protein.

2. From the 1803 larvae of *S. nonagrioides* collected, 1030 adults emerged, of whom 972 mated. The offspring of 90% of the emerged adults was used in the bioassays and treated with the diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2, intended to cause moulting inhibition $\geq$ 99% to first-instar larvae of *S. nonagrioides*. The implementation of best practices in larvae rearing therefore allowed 51% of the collected larvae to be represented in the DC bioassays. The detection limit for resistance allele frequency in field populations of *S. nonagrioides* in 2023 is 0.033 (3.3%).

3. The values of the detection limit for resistance allele frequency in field populations of *S. nonagrioides* in the eight last seasons, (3.3, 3.7, 4.2, 3.4, 3.6, 3.0, 2.9 and 3.3 for the range of years 2016-2023, respectively) *vs.* the number of larvae collected in the field each year (1364, 1452, 1490, 1644, 1569, 1699, 1967 and 1803, respectively), highlight the technical difficulties that can be encountered, depending on different factors, in each campaign, regardless of the number of larvae collected.

4. The treatment with the DC caused mean moulting inhibition of 95.80% (S.E. 1.54%) to F1 neonates from the field collected larvae of the NE Spain. This outcome was significantly different from the expected value of 99% ($t = 2.94$, $df = 2$, $p = 0.049$) but not from the laboratory strain moulting inhibition value (98.85%) ($t = 2.62$, $df = 2$, $p = 0.060$). Besides, no significant trends over time were obtained for moulting inhibition values in the period 2016-2023 ($R^2 = 0.039$, $p = 0.353$).

5. The test of equivalence between field populations collected in 2023 and reference laboratory strains of *S. nonagrioides* (2016-2022) showed that the confidence intervals for moulting inhibition lie within the 10% equivalence limits, indicating that populations are equivalent in their susceptibility to Cry1Ab toxin, based on moult inhibition data.

6. None of the 16610 neonates of the F1 generation of the field collected populations was able to moult to the 2nd larval instar and survive after 10 days feeding on MON 810 leaves.

7. Laboratory *S. nonagrioides* and *O. nubilalis* strains showed susceptibility levels to the batch B2-11 of the Cry1Ab protein (MIC_{50} values of 15 and 4.2 ng Cry1Ab/cm^2, respectively) comparable with those obtained from laboratory strains in previous years. However, it should be noted that offspring's mortality of this season's reference population of *S. nonagrioides* has been irregular among oviposition cages. This is probably because the first field generation was used for the bioassays.

5. Concluding remarks

Considerable effort was made to collect from the field a high number of last instar larvae of *S. nonagrioides* (>1800), which allowed a detection limit of 3.3% to be reached. These numbers highlight the technical difficulties encountered in meeting the 3% target (García et al., 2023).

The moult inhibition (95.80%) of *S. nonagrioides* F1 neonates from NE Spain in 2023, treated with a diagnostic concentration (DC), was significantly different than the hypothetical value of 99%, but not from the moult inhibition value (95.85%) caused to neonates of a laboratory strain with the same DC. The results obtained in recent years, as well as the experience accumulated in the more than 15 years of MON 810 monitoring, highlight the importance of maintaining a susceptible laboratory strain against which the field populations should be compared, enabling correct interpretation of the results.

Plant bioassays resulted in a 100% mortality rate for both field and laboratory larvae fed on MON 810 maize tissue. In addition, no L2 larvae surviving from the DC bioassays survived when transferred to MON 810 maize leaves. According to these results this is the first year that no F2 confirmatory experiments were necessary.

We have further analyzed the moult inhibition values of field populations for the period 2016-2023, for an insightful assessment of these aggregated data. When the moult inhibition values were assessed over time by lineal regression, no significant trends were obtained. Moreover, the test of equivalence confirms that field populations from 2016 onwards and laboratory populations (2016-2022) are equivalent in their susceptibility to Cry1Ab.

In summary, the results obtained show no evidence of field resistance of *S. nonagrioides* to MON 810 maize in NE Spain. These results are in line with those revealed in the farmers' surveys, in which no evidence of any unexpected adverse effect associated with the cultivation of MON 810 was found (Bertho et al., 2020).

6. References

Abbott, W.S. 1925. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18:265–267.

Alcalde, E., Amijee, F., Blache, G., Bremer, C., Fernandez, S., Garcia-Alonso, M., Holt, K., Legris, G., Novillo, C., Schlotter, P., Storer, N. and Tinland, B. 2007. Insect Resistance Monitoring for Bt Maize Cultivation in the EU: Proposal from the Industry IRM Working Group. *J. Verbr. Lebensm.* 2, Supplement 1: 47-49.

Andow, D.A. and Ives, A.R. 2002. Monitoring and adaptive resistance management. *Ecol Appl* 12:1378–1390.

Anglade, P. 1972. Les Sesamia, pp. 1389-1401. In A. S. Balachowsky (ed.), *Entomologie appliquée à l'agriculture*, Tome II, Lépidoptères, vol. 2. Masson et Cie, Paris, France.

Bertho L., Schmidt K., Schmidtke J., Brants I., Cantón R.F., Novillo C., and Head, G. 2020. Results from ten years of post-market environmental monitoring of genetically modified MON 810 maize in the European Union. *PLoS ONE* 15(4): e0217272.

Carpenter, J.E. and Bloem, S. 2002. Interaction between insect strain and artificial diet in diamondback moth development and reproduction. *Entomol. Exp. Appl.* 102: 283–294.

Castañera, P. 1986. *Plagas del Maíz*. IV Jornadas técnicas sobre el maíz. Lérida. Plagas: 1-24. Ministerio de Agricultura, Pesca y Alimentación.

Castañera, P., Farinós, G.P., Ortego, F. and Andow, D.A. 2016. Sixteen years of Bt maize in the EU hotspot: Why has resistance not evolved? *PLoS ONE.* 11(5): e0154200.

CropLife Europe. 2021; 2023. Harmonised insect resistance management (IRM) plan for cultivation of Bt maize (single insecticidal traits) in the EU.

Da Silva, K.F., Spencer, T.A., Camargo Gil, C., Siegfried, B.D. and Walters, F.S. 2016. Impact of *Spodoptera frugiperda* neonate pretreatment conditions on Vip3Aa19 insecticidal protein activity and laboratory bioassay variation. *Pest Manag.* Sci. 72: 837–844.

EFSA (European Food Safety Authority), Álvarez, F., Devos, Y., Georgiadis, M., Messéan, A. and Waigmann, E. 2018. Statement on annual post-market environmental monitoring report on the cultivation of genetically modified maize MON 810 in 2016. *EFSA Journal* 2018;16(5):5287, 34 pp. https://doi.org/10.2903/j.efsa.2018.5287

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). 2010. Statistical considerations for the safety evaluation of GMOs. *EFSA Journal*, 8(2), 1250.

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). 2017. Scientific Opinion 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 Journal* 2017;15(5):4805, 27 pp. https://doi.org/10.2903/j.efsa.2017.4805

Eizaguirre, M., Albajes, R. and Galichet, P.F. 1990. A note on the presence in Catalonia of a parasitic system bound to the tachinid fly *Lydella thompsoni* Herting, a parasitoid of corn borers. *Investigación Agraria*, Producción y Protección Vegetales 5:345-348.

Eizaguirre, M. and Fantinou, A.A. 2012. Abundance of *Sesamia nonagrioides* (Lef.) (Lepidoptera: Noctuidae) on the edges of the Mediterranean basin. *Psyche*, doi:10.1155/2012/854045.

6. References

EuropaBio. 2012; 2017; 2019. Harmonised insect resistance management (IRM) plan for cultivation of Bt maize (single insecticidal traits) in the EU.

Fantinou, A.A. and Tsitsipis, J.A. 1999. Effect of larval density on development and diapause of *Sesamia nonagrioides* (Lef.)(Lep., Noctuidae) under laboratory conditions. *J. Appl. Entomol.* 123(3), 187-190.

Farinós, G.P., De la Poza, M., Hernández-Crespo, P., Ortego, F. and Castañera, P. 2004. Resistance monitoring of field populations of the corn borers *Sesamia nonagrioides* and *Ostrinia nubilalis* after five years of Bt maize cultivation in Spain. *Entomol. Exp. Appl.* 110: 23-30.

Farinós, G.P., De la Poza, M., Ortego, F. and Castañera, P. 2012. Susceptibility to the Cry1F toxin of field populations of *Sesamia nonagrioides* (Lepidoptera: Noctuidae) in Mediterranean maize cultivation regions. *J. Econ. Entomol.* 105: 214-221.

Farinós, G.P., Hernández-Crespo, P., Ortego, F. and Castañera, P. 2018. Monitoring of *Sesamia nonagrioides* resistance to MON 810 maize in the European Union: lessons from a long-term harmonized plan. *Pest Manag.* Sci. 74: 557-568.

Ferré, J., and Van Rie, J. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annu. Rev Entomol.* 47 (1), 501-533.

García, M., García-Benítez, C., Ortego, F., Farinós G.P. 2023. Monitoring insect resistance to Bt maize in the European Union: Update, challenges and future prospects. *J. Econ. Entomol.* 116: 275-288.

González-Núñez, M., Ortego, F. and Castañera, P. 2000. Susceptibility of Spanish populations of the corn borers *Sesamia nonagrioides* (Lepidoptera: Noctuidae) and *Ostrinia nubilalis* (Lepidoptera: Crambidae) to a *Bacillus thuringiensis* endotoxin. *J. Econ. Entomol.* 93: 459-463.

Han, L. Z., Wu, K. M., Peng, Y. F., Wang, F., and Guo, Y. Y. 2006. Evaluation of transgenic rice expressing Cry1Ac and CpTI against *Chilo suppressalis* and intrapopulation variation in susceptibility to Cry1Ac. *Environ. Entomol*. 35 (5), 1453-1459.

Hoffmann, A.A. and Ross, P.A. 2018. Rates and patterns of laboratory adaptation in (mostly) insects. *J. Econ. Entomol*. 111: 501-509.

LeOra Software. 2002-2024. *Polo Plus 1.0 Probit and Logit analysis.* LeOra Software, Petaluma, California.

MAPA (Ministerio de Agricultura, Pesca y Alimentación). 2024a. Registro Público de Organismos Modificados Geneticamente (OMG). Ministry of Agriculture, Fisheries and Food. Public Registry of Genetically Modified Organisms (GMO). https://www.mapa.gob.es/es/agricultura/temas/biotecnologia/omg/registro-publico-omg/default.aspx.

MAPA (Ministerio de Agricultura, Pesca y Alimentación). 2024b. Avances de superficies y producciones de cultivos. https://www.mapa.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/avances-superficies-producciones-agricolas/.

Marçon, P.C.R.G., Young, L.J., Steffey, K.L. and Siegfried, B.D. 1999. Baseline susceptibility of European corn borer (Lepidoptera: Crambidae) to *Bacillus thuringiensis* toxins. *J. Econ. Entomol.* 92: 279–285.

Marçon, P.C.R.G., Siegfried, D.G., Spencer, T. and Hutchison, W.D. 2000. Development of diagnostic concentrations for monitoring *Bacillus thuringiensis* resistance in European corn borer (Lepidoptera: Crambidae). *J. Econ. Entomol.* 93:925-930.

Monetti, L., Malvar, R.A., Ordas, A. and Cordero-Rivera, A. 2003. Parasitoids incidence and diversity on maize stem borers *Sesamia nonagrioides* Lefevre and *Ostrinia nubilalis* Hubner in NW Spain. *Maydica*, 48(2), 133-140.

Ostrem, J. S., Pan, Z., Flexner, J. L., Owens, E., Binning, R., and Higgins, L. S. 2016. Monitoring susceptibility of western bean cutworm (Lepidoptera: Noctuidae) field populations to *Bacillus thuringiensis* Cry1F protein. *J. Econ. Entomol.* 109 (2), 847-853.

Poitout, S. and Bùes, R. 1970. Elevage de plusieurs espèces de Lépidoptères Noctuidae sur milieu artificiel simplifié. *Ann. Zool. Ecol. Anim.* 2: 79-91.

Robertson, J.L., Preisler, H.K., Ng, S.S., Hickle, L.A. and Gelernter, W.D. 1995. Natural variation: a complicating factor in bioassays with chemical and microbial pesticides. *J. Econ. Entomol.* 88:1–10.

Roush, R.T. 1986. Inbreeding depression and laboratory adaptation in *Heliothis virescens* (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. Am.* 79: 583-587.

Sims, S.R., Greenplate, J.T., Stone, T.B., Caprio, M. and Gould, F. 1997. Monitoring strategies for early detection of Lepidoptera resistance to *Bacillus thuringiensis* insecticidal proteins. R*esistant Pest Management Newsletter*, 9: 21-24.

Thieme, T.G.M., Buuk, C., Gloyna, K., Ortego, F. and Farinós, G.P. 2018. Ten years of MON 810 resistance monitoring of field populations of *Ostrinia nubilalis* in Europe. *J. Appl. Entomol.* 142:192-200.

US EPA (United States Environmental Protection Agency), 2010. Biopesticide Registration Action Document: Cry1Ab and Cry1F *Bacillus thuringiensis* (Bt) corn plant-incorporated protectants.

US EPA (United States Environmental Protection Agency), 2018. White paper on resistance in lepidopteran pests of *Bacillus thuringiensis* (Bt) plant incorporated protectants in the United States.

Velasco, P., Soengas, P., Revilla, P., Ordás, A., and Malvar, R.A. 2004. Mean generation analysis of the damage caused by *Sesamia nonagrioides* (Lepidoptera: Noctuidae) and *Ostrinia nubilalis* (Lepidoptera: Crambidae) in sweet corn ears. *J. Econ. Entomol.* 97: 120-126.

7. Tables and figures

Table 1. Artificial diet used for *S. nonagrioides*.

Components	Amount	Provider
Distilled H_2O	1 l	
Agar	26 g	Condalab
Maize flour	160 g	El Granero
Wheat germ	40 g	Santiveri
Yeast	43 g	Santiveri
Ascorbic acid	6 g	Panreac
Benzoic acid	1.25 g	Merck
Nipagin (Methyl p-hidroxibenzoato)	1 g	Sigma-Aldrich
Wesson's salts mixture	1.55 g	Sigma-Aldrich

Table 2. Artificial diet used for *O. nubilalis*.

Components	Amount	Provider
Distilled H_2O	1 l	
Agar	24 g	Condalab
Maize flour	168 g	El Granero
Wheat germ	42 g	Santiveri
Yeast	45 g	Santiveri
Ascorbic acid	9 g	Panreac
Benzoic acid	3 g	Merck
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 2023 season in north-eastern (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]	GPS coordinates
1	2023-Huesca 1	HU	22591	16-18/10/2023	15.0	n.d.	0	41.496013,0.061209
	2023-Huesca 2	HU	22591	16-18/10/2023	37.3	n.d.	0	41.488206,0.092408
	2023-Huesca 3	**HU**	**22591**	**16-18/10/2023**	**37.6**	**n.d.**	**81**	**41.524315,0.077226**
	2023-Huesca 4	HU	22200	16-18/10/2023	26.8	n.d.	0	41.508681,0.089912
	2023-Huesca 5	**HU**	**22200**	**16-18/10/2023**	**10.4**	**n.d.**	**199**	**41.825426,-0.189914**
	2023-Huesca 6	HU	22200	16-18/10/2023	28.1	n.d.	0	41.799426,-0.192094
	2023-Huesca 7	**HU**	**22200**	**16-18/10/2023**	**105.7**	**n.d.**	**98**	**41.747814,-0.111269**
	2023-Huesca 8	HU	22200	16-18/10/2023	49.0	n.d.	0	41.838120,-0.134264
	2023-Huesca 9	HU	22200	16-18/10/2023	7.5	n.d.	0	41.838120,-0.134264
	2023-Huesca 10	HU	22200	16-18/10/2023	17.7	n.d.	0	41.836657,-0.124428
	2023-Huesca 11	HU	22200	16-18/10/2023	15.7	n.d.	0	41.840290,-0.127958
	2023-Huesca 12	HU	22200	16-18/10/2023	25.3	n.d.	0	41.740434,-0.145944
	2023-Huesca 13	HU	22200	16-18/10/2023	15.6	n.d.	0	41.721127,-0.146165
	2023-Huesca 14	**HU**	**22200**	**16-18/10/2023**	**28.0**	**n.d.**	**190**	**41.828784, -0.140922**
	Total Zone 1						**568**	
2	2023-Girona 1	GI	17142	18-21/09/2023	1.9	10	0	42.223380,3.041330
	2023-Girona 2	**GI**	**17142**	18-21/09/2023	**1.8**	**10**	**74**	**42.218085,3.049482**
	2023-Girona 3	**GI**	**17142**	18-21/09/2023	**2.0**	**0**	**262**	**42.172612,3.098528**
	2023-Girona 4	**GI**	**17142**	18-21/09/2023	**2.2**	**0**	**70**	**42.152803,3.083590**
	2023-Girona 5	**GI**	**17142**	18-21/09/2023	**0.7**	**0**	**167**	**42.048815,3.100922**
	Total Zone 2						**573**	
3	2023-Navarra 1	NA	31140	03-05/10/2023	2.3	0	4	42.618455,-1.847133
	2023-Navarra 2	NA	31140	03-05/10/2023	3.4	0	0	42.629065,-1.846889
	2023-Navarra 3	NA	31140	03-05/10/2023	6.5	300	0	42.596410,-1.843550
	2023-Navarra 4	**NA**	**31140**	**03-05/10/2023**	**5.4**	**200**	**211**	**42.573999,-1.808188**
	2023-Navarra 5	NA	31140	03-05/10/2023	5.1	0	0	42.606476,-1.773599
	2023-Navarra 6	NA	31140	03-05/10/2023	10.6	0	27	42.622427,-1.809168
	2023-Navarra 7	NA	31140	03-05/10/2023	4.5	0	24	42.603157,-1.829824
	2023-Navarra 8	**NA**	**31140**	**03-05/10/2023**	**37.9**	**0**	**238**	**42.605894,-1.812622**
	2023-Navarra 9	**NA**	**31140**	**03-05/10/2023**	**9.0**	**20**	**158**	**42.565535,-1.749314**
	Total Zone 3						**662**	
	GRAND TOTAL						**1803**	

[a] Spanish provinces: HU = Huesca; GI = Girona; NA = Navarra
[b] Area of the entire maize field (Bt maize plus refuge area). Data are approximate.
[c] There could be other Bt maize fields closer to the refuge fields, but they were unknown to the technician/farmer. "0" means that it is adjacent to a MON 810 field; "n.d." means that this data is not available.

Table 4. *Ostrinia nubilalis* larvae collection details for the 2023 season in north-eastern (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]	GPS coordinates
1	2023-Huesca 1	HU	22591	16-18/10/2023	15.0	n.d.	0	41.496013,0.061209
	2023-Huesca 2	HU	22591	16-18/10/2023	37.3	n.d.	0	41.488206,0.092408
	2023-Huesca 3	**HU**	**22591**	**16-18/10/2023**	**37.6**	**n.d.**	**223**	**41.524315,0.077226**
	2023-Huesca 4	**HU**	**22200**	**16-18/10/2023**	**26.8**	**n.d.**	**76**	**41.508681,0.089912**
	2023-Huesca 5	HU	22200	16-18/10/2023	10.4	n.d.	0	41.825426,-0.189914
	2023-Huesca 6	HU	22200	16-18/10/2023	28.1	n.d.	0	41.799426,-0.192094
	2023-Huesca 7	HU	22200	16-18/10/2023	105.7	n.d.	0	41.747814,-0.111269
	2023-Huesca 8	HU	22200	16-18/10/2023	49.0	n.d.	0	41.838120,-0.134264
	2023-Huesca 9	HU	22200	16-18/10/2023	7.5	n.d.	0	41.838120,-0.134264
	2023-Huesca 10	HU	22200	16-18/10/2023	17.7	n.d.	0	41.836657,-0.124428
	2023-Huesca 11	HU	22200	16-18/10/2023	15.7	n.d.	0	41.840290,-0.127958
	2023-Huesca 12	HU	22200	16-18/10/2023	25.3	n.d.	0	41.740434,-0.145944
	2023-Huesca 13	HU	22200	16-18/10/2023	15.6	n.d.	0	41.721127,-0.146165
	2023-Huesca 14	HU	22200	16-18/10/2023	28.0	n.d.	0	41.828784, -0.140922
	Total Zone 1						**299**	
2	2023-Girona 1	GI	17142	18-21/09/2023	1.9	10	0	42.223380,3.041330
	2023-Girona 2	GI	17142	18-21/09/2023	1.8	10	0	42.218085,3.049482
	2023-Girona 3	GI	17142	18-21/09/2023	2.0	0	0	42.172612,3.098528
	2023-Girona 4	GI	17142	18-21/09/2023	2.2	0	0	42.152803,3.083590
	2023-Girona 5	**GI**	**17142**	**18-21/09/2023**	**0.7**	**0**	**88**	**42.048815,3.100922**
	Total Zone 2						**88**	
3	2023-Navarra 1	NA	31140	03-05/10/2023	2.3	0	4	42.618455,-1.847133
	2023-Navarra 2	NA	31140	03-05/10/2023	3.4	0	0	42.629065,-1.846889
	2023-Navarra 3	NA	31140	03-05/10/2023	6.5	300	0	42.596410,-1.843550
	2023-Navarra 4	**NA**	**31140**	**03-05/10/2023**	**5.4**	**200**	**74**	**42.573999,-1.808188**
	2023-Navarra 5	NA	31140	03-05/10/2023	5.1	0	0	42.606476,-1.773599
	2023-Navarra 6	NA	31140	03-05/10/2023	10.6	0	0	42.622427,-1.809168
	2023-Navarra 7	NA	31140	03-05/10/2023	4.5	0	0	42.603157,-1.829824
	2023-Navarra 8	NA	31140	03-05/10/2023	37.9	0	0	42.605894,-1.812622
	2023-Navarra 9	**NA**	**31140**	**03-05/10/2023**	**9.0**	**20**	**91**	**42.565535,-1.749314**
	Total Zone 3						**169**	
	GRAND TOTAL						**556**	

[a] Spanish provinces: HU = Huesca; GI = Girona; NA = Navarra
[b] Area of the entire maize field (Bt maize plus refuge area). Data are approximate.
[c] There could be other Bt maize fields closer to the refuge fields, but they were unknown to the technician/farmer. "0" means that it is adjacent to a MON 810 field; "n.d." means that this data is not available.

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] (CI 95%)	MIC_{90} [a] (CI 95%)
S. nonagrioides	B2-11	576	1.3 ± 0.2	27.6	12	15 (4-28)	144 (71-978)
O. nubilalis	B2-11	1437	4.4 ± 0.3	111	40	4.2 (3.5-4.8)	8.2 (6.9-10.5)

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

Table 6. Susceptibility to Cry1Ab toxin of laboratory populations of *S. nonagrioides* between 2004 and 2023. The bioassay performed during the present campaign is shaded. The bioassays carried out under the current revised plan (from 2016 onwards) are below the grey dotted line.

Population	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)
Laboratory	2018	B2-6	19 (13-26)	116 (76-224)
Laboratory	2019	B2-7	27 (16-40)	233 (133-656)
Laboratory	2020	B2-8	14 (10-19)	93 (59-180)
Laboratory	2021	B2-9	25 (14-40)	292 (139-1336)
Laboratory	2022	B2-10	28 (20-38)	158 (103-321)
Laboratory	2023	B2-11	15 (4-28)	144 (71-978)

[a] 50% and 90% moulting inhibition concentration (MIC_{50} and MIC_{90}) and their 95% confidence intervals (CI 95%) 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 not used for mating [a]
Zone 1	568	201 (35.4%)	22 (3.9%)
Zone 2	573	177 (30.9%)	21 (3.7%)
Zone 3	662	395 (59.7%)	15 (2.3%)
Total	1803	773 (42.9%)	58 (3.2%)

[a] Adults that did not emerge between 28th September 2023 and 19th February 2024, and adults having some malformation upon emergence.

Table 8. Tracking of *S. nonagrioides* populations from north-eastern (NE) Spain and from the laboratory used in diagnostic concentration (DC) bioassays, and bioassay results.

	Tracking of the larvae used in the DC bioassays							Results of DC bioassays				
Population	**Fields**	**Last-instar larvae collected**	**Adults emerged [a]**	**Adults mated [b]**	**Oviposition cages**	**Oviposition cages used in bioassays [c]**	**Adults used in bioassays [d]**	**Nº larvae treated in bioassays**	**MI (%) [e]**	**Nº larvae control**	**MI in control (%) [e]**	**Corrected MI (%) [f]**
NE Spain	Zone 1	568	367 (65%)	345 (61%)	36	31	322 (57%) (88%)	1072	96.18	268	5.60	95.95
	Zone 2	573	396 (69%)	375 (65%)	37	34	361 (63%) (91%)	1307	93.65	283	8.48	93.06
	Zone 3	662	267 (40%)	252 (38%)	24	21	242 (37%) (91%)	1076	98.51	176	7.39	98.39
	All zones [g]	1803	1030 (57%)	972 (54%)	97	86	925 (51%) (90%)	3455	95.95	727	7.15	95.64
Laboratory	-	-	-	402	23	23	402	1232	99.03	183	15.30	98.85

[a] The percentage with respect to the number of larvae collected is in brackets
[b] Adults that mated, after excluding those that did not emerge 28th October 2023 and 19th February 2024, and those that presented malformations. The percentage with respect to the number of larvae collected is in brackets.
[c] Oviposition cages were discarded when the fecundity and/or fertility was too low.
[d] Adults used in the bioassays, after excluding those that laid infertile eggs. For field populations, percentages with respect to the number of collected larvae and with respect to the number of emerged adults, respectively, are in brackets.
[e] MI, moulting inhibition: larvae that have not reached the 2nd larval instar.
[f] Calculated using Abbot's formula (Abbot, 1925).
[g] Results obtained pooling the data of the three zones.

Table 9. Moulting inhibition values of F1 neonates of the north-eastern (NE) Spain population of *S. nonagrioides* compared with those of the laboratory population and with the expected value of 99%.

Year	Moulting inhibition at DC (%) [a]			*p*-values [b]	
	NE Spain	Lab strain	Expected	Lab strain	Expected
2016	97.96 ± 0.71	99.20	99	0.066	0.107
2017	94.14 ± 1.40	97.69	99	**0.038***	**0.011***
2018	98.65 ± 0.40	97.75	99	0.081	0.253
2019	97.97 ± 0.36	97.02	99	0.067	**0.029***
2020	98.31 ± 0.39	98.67	99	0.291	0.113
2021	98.27 ± 1.02	99.20	99	0.355	0.429
2022	93.65 ± 2.25	96.77	99	0.116	**0.018***
2023	95.80 ± 1.54	98.85	99	0.060	**0.049***

[a] Moulting inhibition values reported 7 days after treatment with a diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2.
[b] *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%. Moulting inhibition values were previously logit transformed.

Table 10. Larval growth of neonates of the F1 generation of the north-eastern (NE) Spain population of *S. nonagrioides* compared with those of the laboratory population after 10 days feeding on Bt (MON 810) or non-Bt (conventional) maize tissue.

Population	**Field**	**Nº of F0 oviposition cages used [a]**	**Maize leaves**	**Nº of F1 neonates exposed [b]**	**Nº of moulted larvae (≥ L2)**	**% moulting**
NE Spain	Zone 1	31	MON 810	5960	0	0.00
			Conventional	290	263	90.69
	Zone 2	34	MON 810	6450	0	0.00
			Conventional	300	287	95.67
	Zone 3	21	MON 810	4200	0	0.00
			Conventional	210	205	97.62
	All zones	86	MON 810	16610	0	0.00
			Conventional	800	755	94.38
Laboratory	-		MON 810	4260	0	0.00

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

Table 11. Larvae of the north-eastern (NE) Spain population of *S. nonagrioides* and of the laboratory population that moulted to the 2nd larval instar (L2) in the DC bioassay and then moulted to at least the 3rd larval instar (L3) when fed MON 810 maize leaves.

Population	Fields	Nº larvae treated in DC bioassays	L2 [a] (%)	L3 [b] (%)
NE Spain	Zone 1	1072	41 (3.82)	0 (0.00)
	Zone 2	1307	83 (6.35)	0 (0.00)
	Zone 3	1076	16 (1.49)	0 (0.00)
	All zones	3455	140 (4.05)	0 (0.00)
Laboratory	-	1232	12 (0.97)	0 (0.00)

[a] Number of larvae that moulted to L2 in the DC bioassay, and then were fed on MON 810 maize. Percentages with respect to the number of treated larvae.
[b] Number of larvae that moulted to L3 – L6 after feeding on MON 810 maize leaves. Percentages with respect to the number of treated larvae.

Figure 1. Successful field collections of *S. nonagrioides* and *O. nubilalis* in three different zones (Z1, Z2 and Z3) searched in the north-eastern Spain in 2023. A collection at a field within a zone was considered successful if at least 50 larvae were gathered.

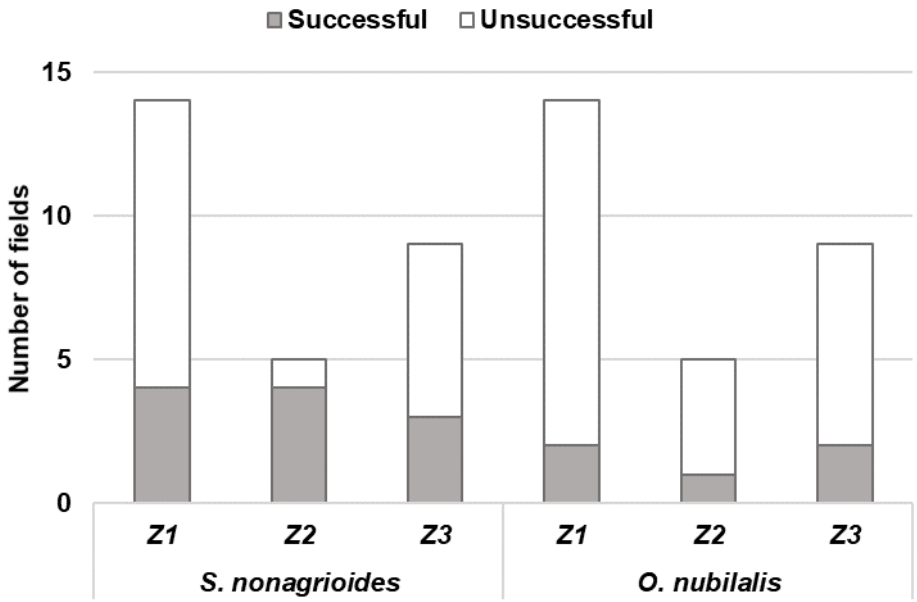


Figure 2. Fitted curves of susceptibility to the Cry1Ab protein of the laboratory populations of *S. nonagrioides* and *O. nubilalis* (PoloPlus 1.0, LeOra Software 2002-2024). Response is moulting inhibition after seven days feeding on treated diet. **a)** *S. nonagrioides*; **b)** *O. nubilalis*.

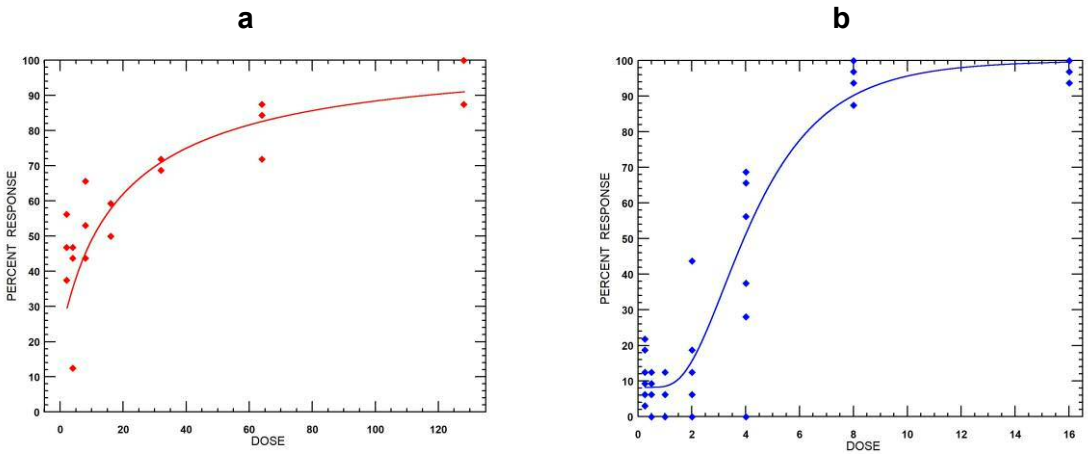


Figure 3. Susceptibility to Cry1Ab toxin measured by MIC_{50} values of a laboratory population of *O. nubilalis.* Colours indicate the B1 (blue) and B2 (pink) toxin batches.

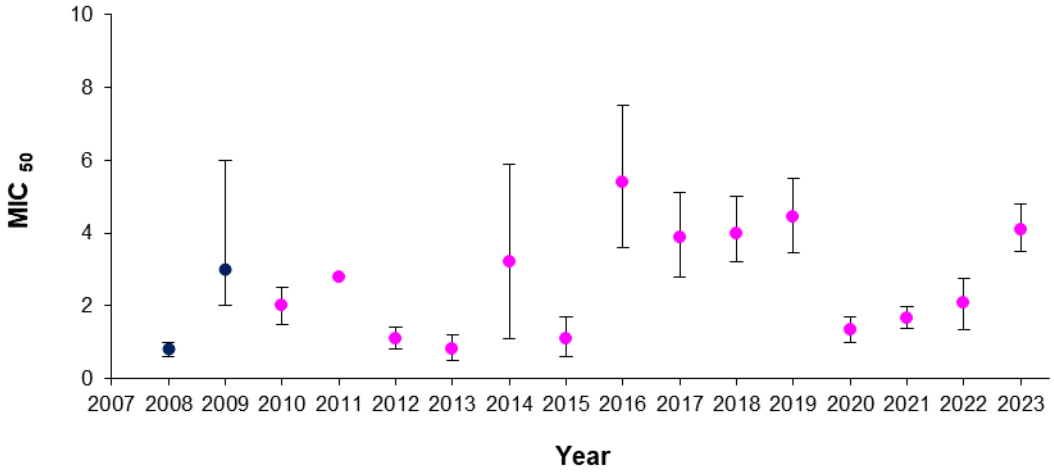


Figure 4. Moulting inhibition (MI) of *S. nonagrioides* from field populations of north-eastern Spain and the laboratory population, treated with a diagnostic concentration (DC) of 1091 ng/cm^2 bioassays: **a)** Annual comparison between field populations (mean ± SE) and laboratory strains. The dotted black line represents the expected 99% MI value; **b)** Lineal regression (dotted grey line) of field population MI values (light green circles) over time. Dark green triangles represent the laboratory population MI values.

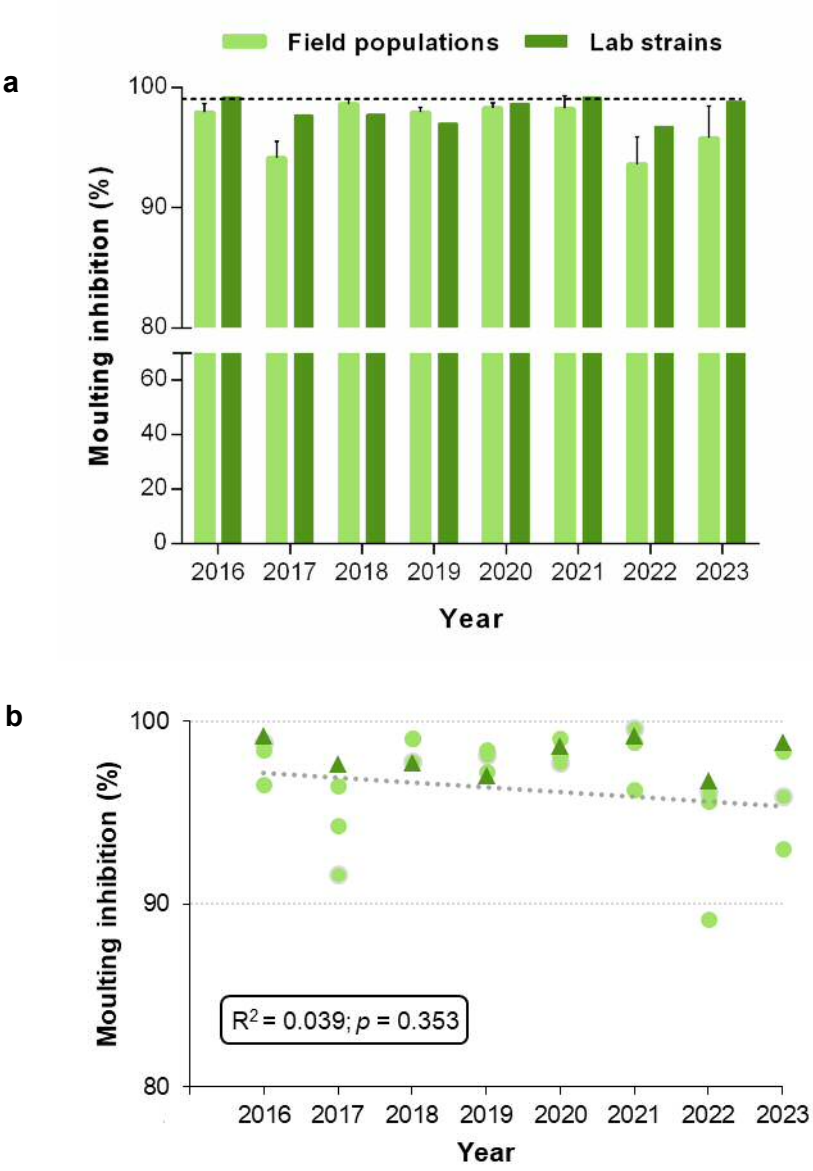
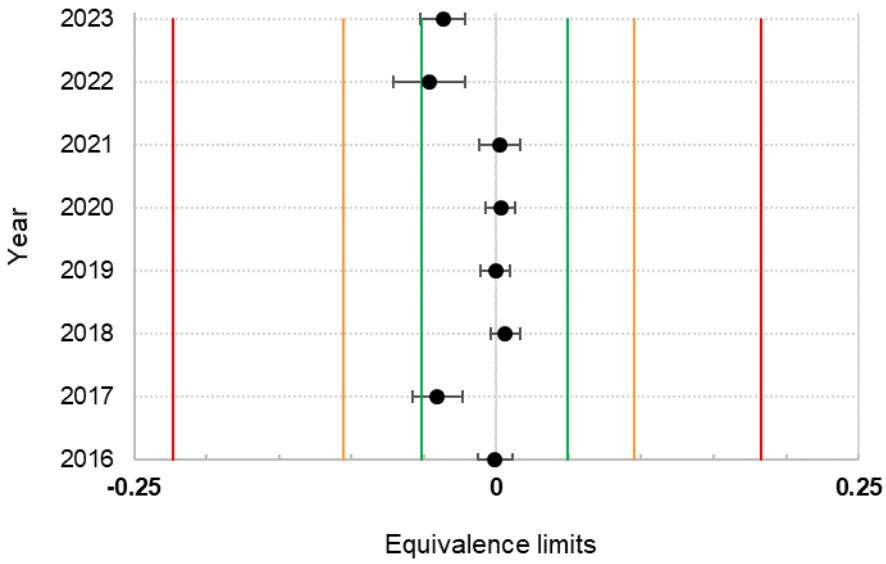
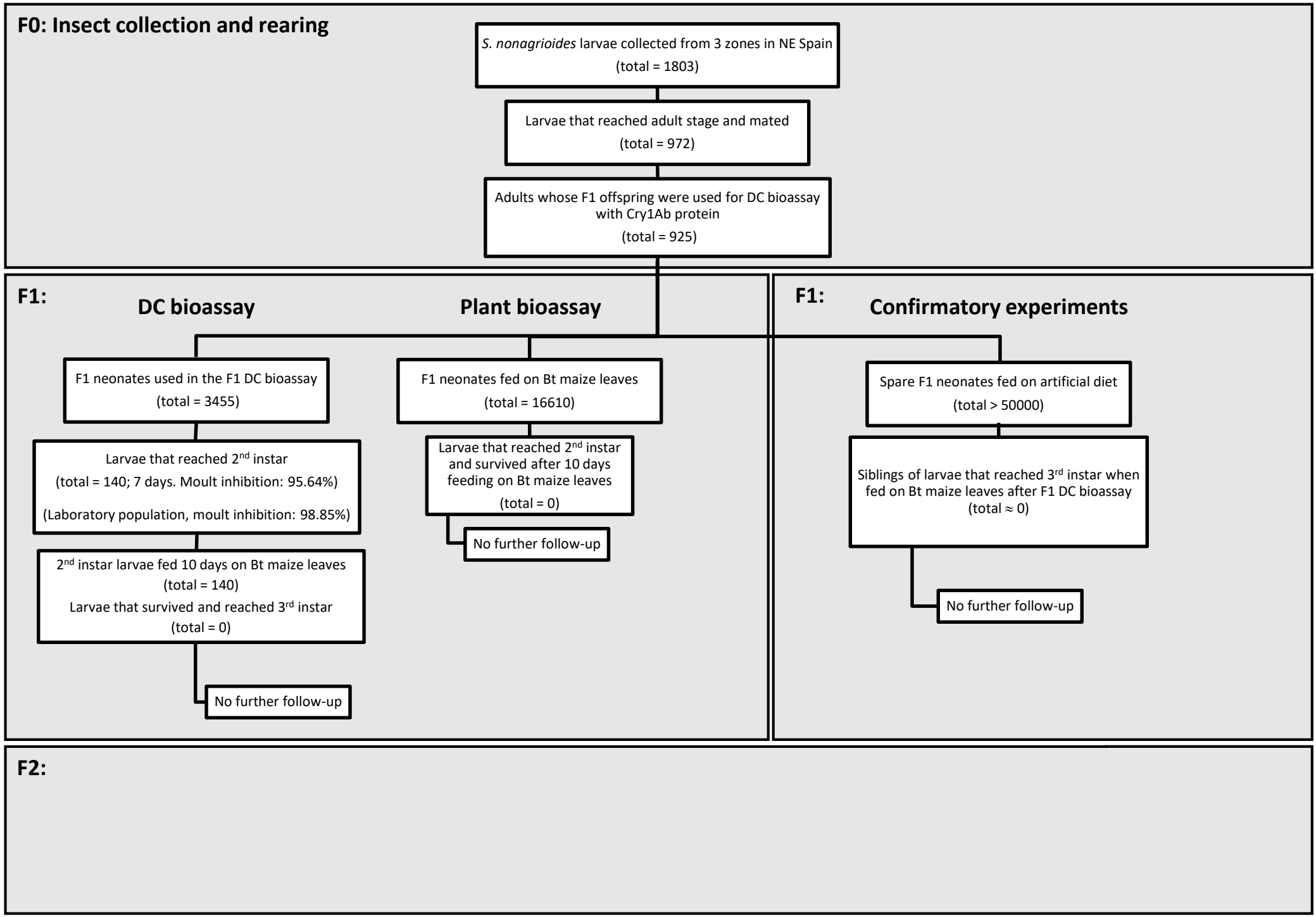


Figure 5. Graph for assessing the outcome of the equivalence test between field populations and reference laboratory strains of *S. nonagrioides.* A simple two-group design of the experiment (field populations of a given year *vs* the set of susceptible laboratory populations for the period 2016-2022) was assumed. Values represented are the mean (black dots) and two-sided 90% confidence intervals (horizontal lines) for moulting inhibition after treatment at the diagnostic concentration (DC). Equivalence limits are represented with vertical colored lines: ± 5% (green lines), ± 10 % (yellow lines) and ± 20% (red lines).



Annex I. Stepwise approach followed to do bioassays with *Sesamia nonagrioides* (season 2023)

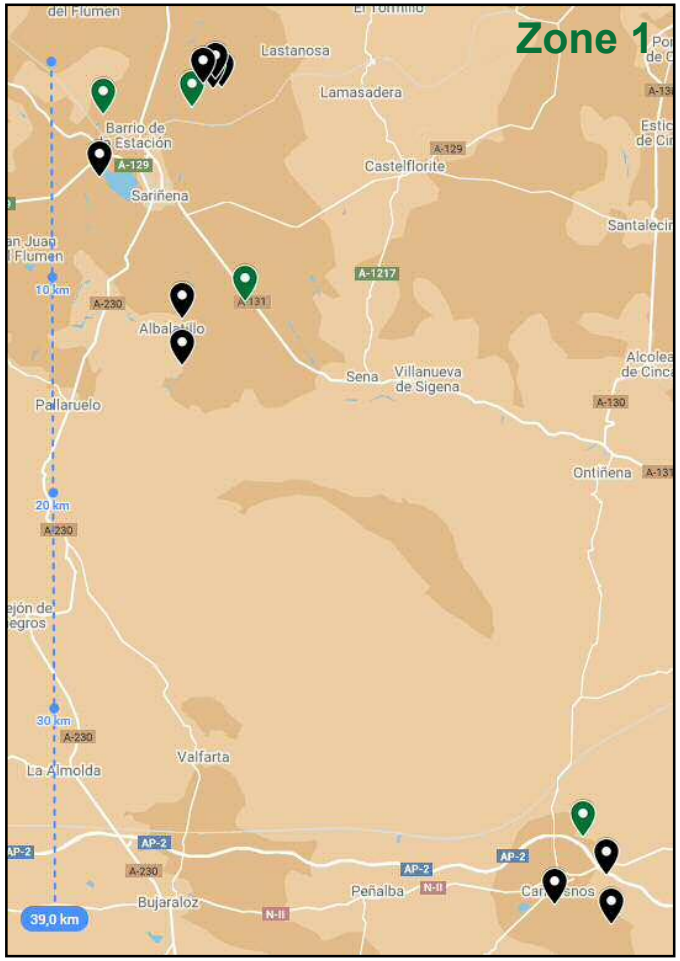


ANNEX IIa. Collection of *S. nonagrioides* larvae in NE Spain in 2023

Successful sampling site: at least 50 larvae were gathered.

Unsuccessful sampling site: less than 50 or no larvae were gathered.

ANNEX IIb. Collection of *S. nonagrioides* larvae in NE Spain in 2023



Successful sampling sites-Zone 1

Successful sampling sites-Zone 2

Successful sampling sites-Zone 3

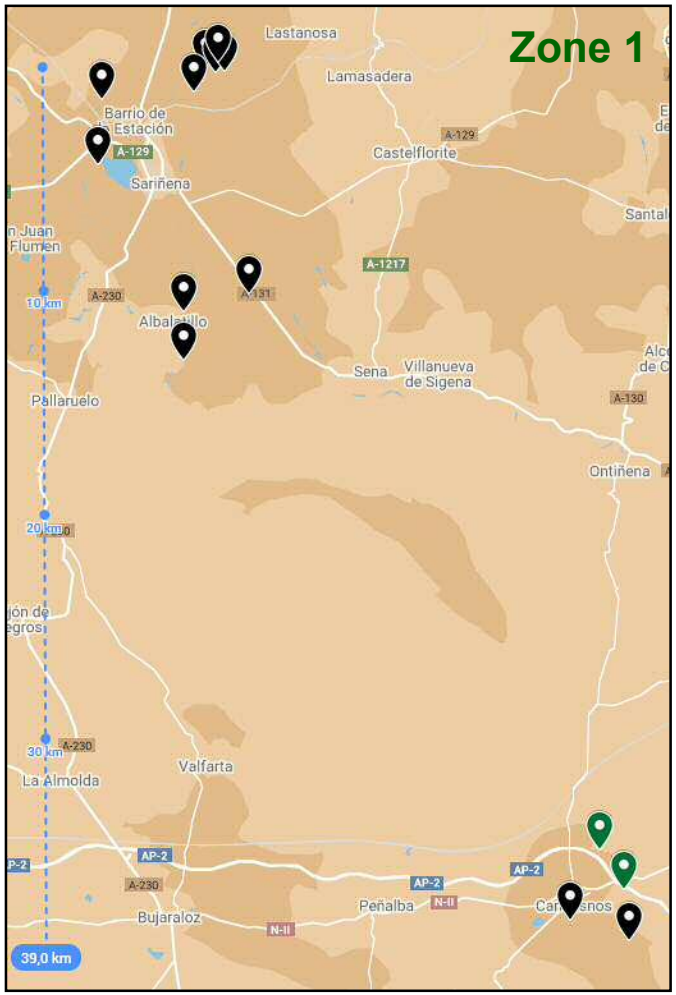
Unsuccessful sampling sites

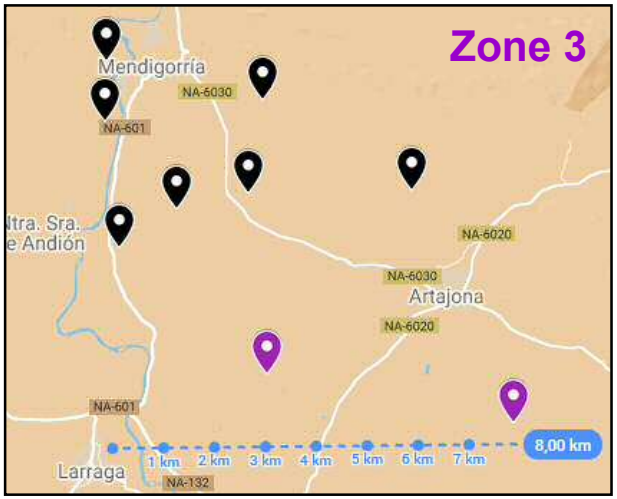
ANNEX IIIa. Collection of *O. nubilalis* larvae in NE Spain in 2023

Successful sampling site: at least 50 larvae were gathered.

Unsuccessful sampling site: less than 50 or no larvae were gathered.

ANNEX IIIb. Collection of *O. nubilalis* larvae in NE Spain in 2023





Successful sampling sites-Zone 1

Successful sampling sites-Zone 2

Successful sampling sites-Zone 3

Unsuccessful sampling sites