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

Season 2022

CONTENTS

1. Introduction

Maize containing event MON 810 is transgenic improved maize expressing the Cry1Ab protein derived from *Bacillus thuringiensis* subsp. *kurstaki*, and conferring protection against certain lepidopteran insect pests such as *Ostrinia nubilalis* and *Sesamia nonagrioides*. Resistance development in targeted lepidopteran pests is a potential concern arising from the 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 2022 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 2004-2022[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:

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

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 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 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 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 last 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. Currently, this situation only occurs in North-eastern Spain within the EU. Additionally, since *S. nonagrioides* and *O. nubilalis* are multivoltine species in this area, monitoring should be carried out annually.

In addition, 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 North-eastern Spain, the area where deployment of Bt-maize is the highest and where resistance is likely to evolve more quickly; and (2) set 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.

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 North-eastern 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 North-eastern 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 2022 maize growing season were the following:

1. Collection of larvae of *S. nonagrioides* in three different zones from North-eastern 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 Bt maize *vs.* conventional maize.

2. Collection of larvae of *O. nubilalis* in three different zones from North-eastern 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 dose-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 North-eastern Spain (NE Spain), each zone comprising at least three different maize fields. This is the first campaign in which samples are taken from Girona (new Zone 2) because since 2016 the percentage of Bt maize in the province of Girona has been around 60% of the total maize cultivated (MAPA, 2023b), so it should be considered a hotspot for the evolution of corn borers' resistance to Bt maize

(CropLife Europe, 2023; EFSA, 2018). The inclusion of this new sampling point is also in line with the proposal of the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) that it should be possible to modify the sampling program “based on changes in pest importance and/or the adoption levels of lepidopteran-resistant Bt corn” (US EPA, 2010). Thus, to include this area of interest in the current monitoring program, the former Zone 1 (Lanaja, Huesca) and 2 (Candasnos, Huesca) have been merged into the new Zone 1.

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 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 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 2022, from refuges and fields of conventional maize adjacent 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**). Immediately 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* 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 from Galicia (Northwest of Spain), where Bt maize has never been commercially grown. Thus, corn borers from this area have low or no selection pressure, making them a good option to be used as the reference strain. Furthermore, Galician populations of *S. nonagrioides* had not shown differences in susceptibility to the Cry1Ab protein with respect to those of other Spanish populations in bioassays carried out in 1998 (González-Núñez et al., 2000) and in 2011 (unpublished results).

Reference populations of *S. nonagrioides* and *O. nubilalis* for this season originated collecting field larvae in three locations of Pontevedra province (Galicia) in 2020 and 2015, respectively. The collections were carried out with the assistance of La Misión Biológica de Galicia (MBG, CSIC) staff. The field strain of *O. nubilalis* collected in 2015 has been maintained by the BTL GmbH Sagerheide laboratory (Germany) since then, and a part of it was sent to the CIB-CSIC laboratory (Spain) in 2022. Individuals from both strains were incorporated 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. This is particularly critical in the case of *S. nonagrioides* (personal observation), so, as far as possible, it is advisable to add new individuals periodically to refresh them.

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 has been 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 was sent in October 2011, B2-2 in February 2014, B2-3 in April 2016, B2-4 in July 2017, B2-6 in July 2018, B2-7 in September 2019, B2-8 in September 2020, B2-9 in July 2021 and B2-10 in August 2022. 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. The lot of Cry1Ab protein B2-10 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 (DR) 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 2nd 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 32 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 32 larvae per concentration (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 dose-response bioassays. This DC has been used from the 2016 campaign onwards, as no new dose-response bioassay data of field populations have been available 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 2022 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 two means: 1) use of ImmunoStrip® for Bt-Cry1Ab/1Ac (Agdia Inc., Elkhart, IN); 2) testing each plant with susceptible neonates of *O. nubilalis*: 10-15 neonates per plant were fed *ad libitum* on maize tissue, and for a plant to be used in bioassays, mortality after one

week should be 100%. This experiment was performed at the same conditions of insect culture: 25 ± 1ºC, 70 ± 10% relative humidity and a photoperiod of 16:8 hours (L:D).

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 during 10 days were able to moult to the 3rd larval instar and they were alive at the end of this period, 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 during 10 days were able to moult to the 2nd larval instar and they were alive at the end of this period, 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 (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, 2002-2023). 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 generic value of 99%. Values were compared by a one-sample t-test and a one-tailed probability distribution (IBM SPSS Statistics 29). Moulting inhibition values of each zone were corrected with Abbott's formula (Abbott, 1925) and logit transformed before analyses.

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 217 hours of fieldwork, travelled over 3380 km and made a total of three round trips, one for each different field zone.

A total of 1967 last-instars larvae of *S. nonagrioides* were collected between September and October 2022 from three different Zones in NE Spain (630, 621 and 716 larvae from Zones 1, 2 and 3, respectively; **Table 3**). A map showing the sampling points for *S. nonagrioides* is displayed in **Annex IIa.** Fifteen, six and fourteen fields in Zones 1, 2 and 3, were searched, respectively, but larvae were successfully collected in four fields in each zone **(Figure 1, Annex IIb**). The maximum distance between successfully sampled fields was about 39, 26 and 4 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 2022 from the same three Zones in the North-eastern Spain, yielding a total of 780 larvae (445, 334

and 1 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 one in Zone 2 (**Figure 1, Annex IIIb**), although the number of fields surveyed was higher (fifteen in Zone 1, six in Zone 2 and fourteen in Zone 3). The maximum distance between successfully sampled fields was about 39 Km within Zone 1 (**Annex IIIb**). Even though 35 maize fields have been sampled in all three Zones, 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, either due to lack of larvae in the prospected fields 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 dose-response bioassays

The susceptibility to Cry1Ab protein of the laboratory population of *S. nonagrioides* was

performed with 720 neonates using a dose-response bioassay, resulting in a MIC_{50} value of 28 (20-38) 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 1117 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 2.1 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 1967 *S. nonagrioides* last-instar larvae collected, 727 (37.0%), combining larvae and pupae, died in the process of rearing in the laboratory, mainly during the diapause period. In addition, 41 adults (2.1%) did not emerge in the date range for oviposition cages (between 3rd October 2022 and 9th February 2023) or had some malformation upon emergence, so they were not used in the bioassays (**Table 7**)**.** Thus, of the 1240 adults that emerged, 1199 were placed in 117 oviposition cages for mating. The offspring of 1182 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 2022 is 0.029 (2.9%), calculated considering the model developed by Andow and Ives (2002).

Of the total F1 neonates originated from the field collected larvae, 3358 were used in the bioassays. The DC (1091 ng Cry1Ab/cm^2) caused a corrected moulting inhibition (MI) of 96.14%, 95.67% and 89.16% in Zones 1, 2 and 3, respectively (**Table 8**).

Statistically significant differences were observed between field-collected populations MI value (93.65 ± 2.25%) and the expected MI value of 99% (t = 5.10, df = 2, *p* = 0.018), but no statistically significant differences were found between field-collected populations and the laboratory strain (96.77%) MI values (t = 1.69, df = 2, *p* = 0.116) (**Table 9**). Likewise, significant differences between the MI value of the field populations and the expected MI value were obtained in 2017 and 2019, but only in 2017 the MI value of the field populations was significantly lower than that of the laboratory strain (**Table 9**). Therefore, no trend over time is observed in terms of changes in the susceptibility of populations from NE Spain to the Cry1Ab protein **(Figure 4)**.

The MI values obtained in the 2022 campaign have been the lowest since 2016 for both field (93.65%) and laboratory (96.77%) populations of *S. nonagrioides*. In fact, it is noteworthy that only two years (2016 and 2021) out of seven (from 2016 to 2022), and only with the laboratory strain, the obtained MI values were over the expected 99% value (**Table 9; Figure 4**). The annual fluctuations of these values could be due to the great natural variability in susceptibility to the toxin shown by this species. These variations are also evident in control strains (such as the one from Galicia, where MON 810 maize has never been grown), 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 from areas where Bt maize is not grown, which enables a correct interpretation of the results.

3.4. Larval development on MON 810 tissue: plant bioassays

21,697 F1 first-instar larvae of *S. nonagrioides*, from the populations collected in three zones in NE Spain in 2022, and 5200 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, 1120 neonates of these field-collected populations and 260 neonates of the laboratory strain were reared on conventional maize. 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 Bt maize. Most larvae fed on conventional maize from the field and the laboratory populations (96.1% and 96.9%, respectively) moulted to 2nd or 3rd larval instar **(Table 10)**.

3.5 Confirmatory experiments

A total of 198 (5.90%) larvae from 47 oviposition cages reached the 2nd larval instar in the F1 DC bioassay. Consequently, the following confirmatory experiments were conducted (**Table 11a**, **Annex IV**).

The 198 surviving L2 larvae from the DC bioassays were collected and grouped into boxes, according to the oviposition cage of origin, and then fed on MON 810 leaves. Six of these larvae, from four oviposition cages (two from Zone 1 and one each from Zones 2 and 3), moulted to the 3rd larval instar and survived 10 days feeding on Bt maize leaves (two larvae reached the fourth and one larva the fifth larval instar continuously feeding on MON 810 leaves) (**Table 11a, Annex IV**). Thus, the siblings of these six larvae (about 125 larvae per original oviposition cage) were raised on artificial diet up to the next generation (F2). As a result, 416 F2 neonates were treated with the diagnostic concentration (1091 ng/cm^2). Eighteen of the treated larvae moulted to the 2nd larval instar after 7 days, but they did not survive for ten days when they were subsequently fed on MON 810 maize (**Annex IV**). In addition, 2260 F2 neonates, siblings of the above, were fed on MON 810 maize and 120 neonates, used as controls, on conventional maize. After 10 days, none of the larvae fed on Bt tissue were able to moult, whereas 119 larvae (99%) fed on conventional maize had moulted to 2nd or 3rd larval instar (**Table 11b, Annex V**).

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 2022 has been focused for the seventh time in North-eastern (NE) Spain, where the adoption rate of MON 810 maize is highest of Europe. In 2021 adoption rate of MON 810 maize on the three NE Spanish regions was 64% in Catalonia, 43% in Aragon and 43% in Navarre (MAPA, 2023a, 2023b). A total of 1967 larvae of *S. nonagrioides* and 780 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 1967 larvae of *S. nonagrioides* collected, 1240 adults emerged, of whom 1199 mated. The offspring of 95% of the emerged adults (1182) was used in the

bioassays and treated with the diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2, intended to cause moulting inhibition ≥ 99% to first-instar larvae of *S. nonagrioides*. The implementation of best practices in larvae rearing therefore allowed 60% 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 2022 is 0.029 (2.9%).

3. The values of the detection limit for resistance allele frequency in field populations of *S. nonagrioides* in the seventh last seasons, (3.3, 3.7, 4.2, 3.4, 3.6, 3.0 and 2.9 in 2016, 2017, 2018, 2019, 2020, 2021 and 2022, respectively) *vs.* the number of larvae collected in the field each year (1364, 1452, 1490, 1644, 1569, 1699 and 1967), 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 93.65% (S.E. 2.25%) to F1 neonates from the field collected larvae of the NE Spain. This outcome was significantly different from the expected value of 99% ($t = 5.10$, $df = 2$, $p = 0.018$) but not from the laboratory strain moulting inhibition value (96.77%) ($t = 1.69$, $df = 2$, $p = 0.116$).

5. None of the 21,697 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.

6. Laboratory *S. nonagrioides* and *O. nubilalis* strains showed susceptibility levels to the batch B2-10 of the Cry1Ab protein (MIC_{50} values of 28 and 2.1 ng Cry1Ab/cm^2, respectively) comparable with those obtained from laboratory strains in previous years.

5. Concluding remarks

Considerable effort has been made over the past seven seasons to collect increasing numbers of last instar larvae of *S. nonagrioides*. This is the second time that the required limit of detection of resistance allele frequency of 3% could be reached, highlighting the technical difficulties encountered in achieving this goal (García et al., 2023). In addition, it should be mentioned that the number of larvae that can be kept in the laboratory after being collected in the field is limited, for reasons of space, facilities and handling.

The moult inhibition (93.65%) of *S. nonagrioides* F1 neonates from NE Spain in 2022, treated with a diagnostic concentration (DC), was significantly different than the hypothetical value of 99%, but not from the moult inhibition value (96.77%) 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, underscore the importance of maintaining a susceptible laboratory strain against which the field populations should be compared, enabling correct interpretation of the results.

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 obtained 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). Since 2016 the percentage of Bt maize in the province of Girona has been around 60% of the total maize cultivated (MAPA, 2023a; 2023b). It is therefore essential to keep this point in the resistance monitoring plan since it should be considered as a hotspot for the evolution of corn borers' resistance to Bt maize (CropLife Europe, 2023; EFSA, 2018).

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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-Aldrich

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 2022 season in NE Spain.

Zone	Field	Province [a]	Postal Code	Date	Surface (hectares) [b]	Distance to the nearest MON810 field (m) [c]	Nº of larvae collected
1	**2022-Candasnos 1**	**HU**	**22591**	**26-29/09/2022**	**24.1**	**n.d.**	**199**
	2022-Candasnos 2	HU	22591	26-29/09/2022	41.75	n.d.	40
	2022-Candasnos 3	HU	22591	26-29/09/2022	41.75	n.d.	0
	2022-Sariñena 1	HU	22200	26-29/09/2022	19.8	n.d.	0
	2022-Sariñena 2	**HU**	**22200**	**26-29/09/2022**	**9.0**	**n.d.**	**138**
	2022-Sariñena 3	HU	22200	26-29/09/2022	7.0	n.d.	0
	2022-Sariñena 1	HU	22200	26-29/09/2022	19.0	n.d.	0
	2022-Sariñena 5	HU	22200	26-29/09/2022	17.0	n.d.	0
	2022-Sariñena 6	HU	22200	26-29/09/2022	47.0	n.d.	0
	2022-Sariñena 7	**HU**	**22200**	**26-29/09/2022**	**8.4**	**n.d.**	**144**
	2022-Sariñena 8	**HU**	**22200**	**26-29/09/2022**	**31.0**	**n.d.**	**109**
	2022-Sariñena 9	HU	22200	26-29/09/2022	40.0	n.d.	0
	2022-Sariñena 10	HU	22200	26-29/09/2022	33.0	n.d.	0
	2022-Sariñena 11	HU	22200	26-29/09/2022	28.0	n.d.	0
	2022-Sariñena 12	HU	22200	26-29/09/2022	105.2	n.d.	0
	Total Zone 1						**630**
2	2022-Girona 1	GI	17142	20-22/09/2022	2.5	10	0
	2022-Girona 2	**GI**	**17142**	**20-22/09/2022**	**3.75**	**0**	**161**
	2022-Girona 3	**GI**	**17142**	**20-22/09/2022**	**0.8**	**0**	**224**
	2022-Girona 4	**GI**	**17142**	**20-22/09/2022**	**2.0**	**0**	**94**
	2022-Girona 5	**GI**	**17142**	**20-22/09/2022**	**3.0**	**0**	**120**
	2022-Girona 6	GI	17142	20-22/09/2022	1.35	0	22
	Total Zone 2						**621**
3	2022-Mendigorria 1	NA	31140	17-19/10/2022	3.1	0	0
	2022-Mendigorria 2	**NA**	**31140**	**17-19/10/2022**	**10.9**	**0**	**255**
	2022-Mendigorria 3	**NA**	**31140**	**17-19/10/2022**	**2.16**	**0**	**90**
	2022-Mendigorria 4	NA	31140	17-19/10/2022	1.98	0	1
	2022-Mendigorria 5	**NA**	**31140**	**17-19/10/2022**	**5.5**	**0**	**248**
	2022-Mendigorria 6	**NA**	**31140**	**17-19/10/2022**	**2.7**	**0**	**63**
	2022-Mendigorria 7	NA	31140	17-19/10/2022	37.9	0	35
	2022-Mendigorria 8	NA	31140	17-19/10/2022	7.5	0	17
	2022-Mendigorria 9	NA	31140	17-19/10/2022	6.95	0	0
	2022-Mendigorria 10	NA	31140	17-19/10/2022	22.2	0	0
	2022-Mendigorria 11	NA	31140	17-19/10/2022	25.0	0	3
	2022-Mendigorria 12	NA	31140	17-19/10/2022	6.1	0	2
	2022-Mendigorria 13	NA	31140	17-19/10/2022	4.5	0	2
	2022-Mendigorria 14	NA	31140	17-19/10/2022	6.0	0	0
	Total Zone 3						**716**
	GRAND TOTAL						**1967**

[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 closer Bt fields, 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 2022 season in NE Spain.

Zone	Field	Province [a]	Postal Code	Date	Surface (hectares) [b]	Distance to the nearest MON810 field (m) [c]	Nº of larvae collected
1	**2022-Candasnos 1**	**HU**	**22591**	**26-29/09/2022**	**24.1**	**n.d.**	**205**
	2022-Candasnos 2	HU	22591	26-29/09/2022	41.75	n.d.	19
	2022-Candasnos 3	HU	22591	26-29/09/2022	41.75	n.d.	0
	2022-Sariñena 1	HU	22200	26-29/09/2022	19.8	n.d.	0
	2022-Sariñena 2	**HU**	**22200**	**26-29/09/2022**	**9**	**n.d.**	**183**
	2022-Sariñena 3	HU	22200	26-29/09/2022	7	n.d.	0
	2022-Sariñena 4	HU	22200	26-29/09/2022	19	n.d.	0
	2022-Sariñena 5	HU	22200	26-29/09/2022	17	n.d.	0
	2022-Sariñena 6	HU	22200	26-29/09/2022	47	n.d.	0
	2022-Sariñena 7	HU	22200	26-29/09/2022	8.4	n.d.	26
	2022-Sariñena 8	HU	22200	26-29/09/2022	31	n.d.	10
	2022-Sariñena 9	HU	22200	26-29/09/2022	40	n.d.	0
	2022-Sariñena 10	HU	22200	26-29/09/2022	33	n.d.	0
	2022-Sariñena 11	HU	22200	26-29/09/2022	28	n.d.	2
	2022-Sariñena 12	HU	22200	26-29/09/2022	105.2		0
	Total Zone 1						**445**
2	2022-Girona 1	GI	17142	20-22/09/2022	2.5	10	8
	2022-Girona 2	GI	17142	20-22/09/2022	3.75	0	0
	2022-Girona 3	GI	17142	20-22/09/2022	0.8	0	0
	2022-Girona 4	GI	17142	20-22/09/2022	2	0	0
	2022-Girona 5	GI	17142	20-22/09/2022	3	0	0
	2022-Girona 6	**GI**	**17142**	**20-22/09/2022**	**1.35**	**0**	**326**
	Total Zone 2						**334**
3	2022-Mendigorria 1	NA	31140	17-19/10/2022	3.1	0	0
	2022-Mendigorria 2	NA	31140	17-19/10/2022	10.9	0	0
	2022-Mendigorria 3	NA	31140	17-19/10/2022	2.2	0	0
	2022-Mendigorria 4	NA	31140	17-19/10/2022	2.0	0	0
	2022-Mendigorria 5	NA	31140	17-19/10/2022	5.5	0	0
	2022-Mendigorria 6	NA	31140	17-19/10/2022	2.7	0	1
	2022-Mendigorria 7	NA	31140	17-19/10/2022	37.9	0	0
	2022-Mendigorria 8	NA	31140	17-19/10/2022	7.5	0	0
	2022-Mendigorria 9	NA	31140	17-19/10/2022	7.0	0	0
	2022-Mendigorria 10	NA	31140	17-19/10/2022	22.2	0	0
	2022-Mendigorria 11	NA	31140	17-19/10/2022	25.0	0	0
	2022-Mendigorria 12	NA	31140	17-19/10/2022	6.1	0	0
	2022-Mendigorria 13	NA	31140	17-19/10/2022	4.5	0	0
	2022-Mendigorria 14	NA	31140	17-19/10/2022	6.0	0	0
	Total Zone 3						**1**
	GRAND TOTAL						**780**

[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 closer Bt fields, 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	B2-10	720	1.7 ± 0.2	34.5	19	28 (20-38)	158 (103-321)
O. nubilalis	B2-10	1005	3.0 ± 0.3	98.2	26	2.1 (1.3-2.7)	5.5 (4.1-9.6)

[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 2022. The bioassay performed during the present campaign is shaded.

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)

[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	630	349 (55.4%)	12 (1.9%)
Zone 2	621	146 (23.5%)	7 (1.1%)
Zone 3	716	232 (32.4%)	22 (3.1%)
Total	1967	727 (37.0%)	41 (2.1%)

[a] Adults that did not emerge between 3rd October 2022 and 9th February 2023, and adults having some malformation upon emergence.

Table 8. Tracking of *S. nonagrioides* populations from 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	630	281 (45%)	259 (43%)	29	26	263 (42%) (94%)	1128	96.63	148	12.84	96.14
	Zone 2	621	475 (76%)	468 (75%)	44	42	457 (74%) (96%)	1112	95.95	167	6.59	95.67
	Zone 3	716	484 (68%)	462 (65%)	44	44	462 (65%) (95%)	1118	89.71	255	5.10	89.16
	All zones [g]	1967	1240 (63%)	1199 (61%)	117	112	1182 (60%) (95%)	3358	94.10	570	7.54	93.62
Laboratory	-	-	-	416	26	24	382	1048	97.33	104	17.31	96.77

[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 3rd October 2022 and 9th February 2023, 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 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 (%)			*p*-values [a]	
	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***

[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 *S. nonagrioides* 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	26	MON 810	4980	0	0.00
			Conventional	260	244	93.85
	Zone 2	42	MON 810	8318	0	0.00
			Conventional	420	402	95.71
	Zone 3	44	MON 810	8399	0	0.00
			Conventional	440	430	97.73
	All zones	112	MON 810	21697	0	0.00
			Conventional	1120	1076	96.07
Laboratory	-	26	MON 810	5200	0	0.00
			Conventional	260	252	96.92

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

Table 11. *Sesamia nonagrioides* confirmatory bioassays

11a. Larvae 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] (%)	L4 [b] (%)	L5 [b] (%)	L6 [b] (%)
NE Spain	Zone 1	1128	38 (3.37)	4 (0.35)	2 (0.18)	1 (0.09)	0 (0.00)
	Zone 2	1112	45 (4.05)	1 (0.09)	0 (0.00)	0 (0.00)	0 (0.00)
	Zone 3	1118	115 (10.29)	1 (0.09)	0 (0.00)	0 (0.00)	0 (0.00)
	All zones	3358	198 (5.90)	6 (0.18)	2 (0.06)	1 (0.03)	0 (0.00)
Laboratory	-	1048	28 (2.67)	0 (0.00)	0 (0.00)	0 (0.00)	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. Percentages with respect to the number of treated larvae.

11b. Larval growth of neonates of the F2 generation after 10 days feeding on Bt (MON 810) or non-Bt (conventional) maize tissue.

Population	Field	Nº of F1 oviposition cages used [a]	Maize leaves	Nº of F2 neonates exposed [b]	Nº of moulted larvae (≥ L2)	% moulting
NE Spain	Zone 1	6	MON 810	1200	0	0.00
			Conventional	60	59	98.33
	Zone 2	5	MON 810	1000	0	0.00
			Conventional	50	50	100.00
	Zone 3	1	MON 810	60	0	0.00
			Conventional	10	10	100.00
	All zones	12	MON 810	2260	0	0.00
			Conventional	120	119	99.17

[a] 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 2022. A collection at a field within a zone was considered successful if at least 50 larvae were gathered.

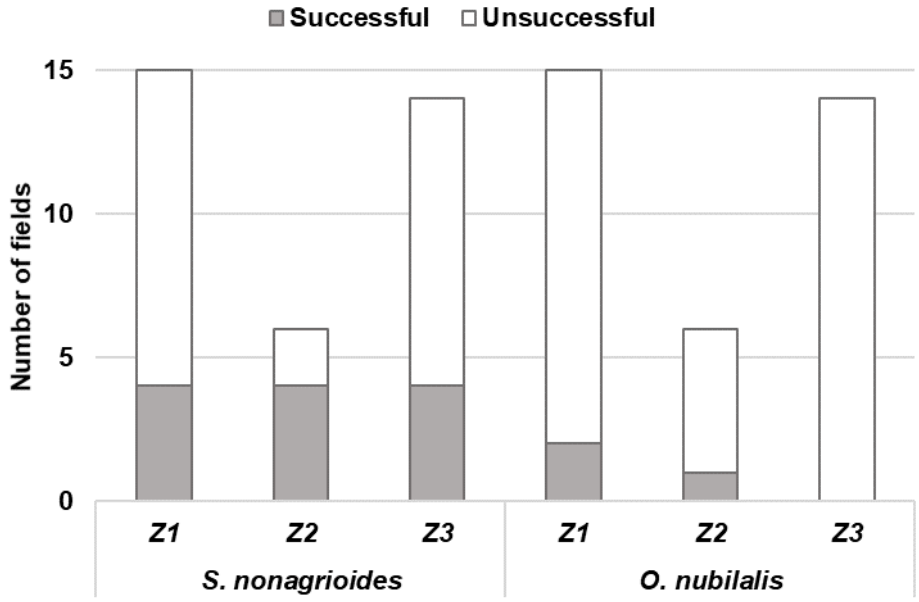


Figure 2. Fitted curves of susceptibility to the Cry1Ab protein of the laboratory populations of *S. nonagrioides* and *O. nubilalis* (PoloPlus 1.0, LeOra Software 2002-2023). Response is moulting inhibition after seven days feeding on treated diet. a: *S. nonagrioides*. b: *O. nubilalis*.

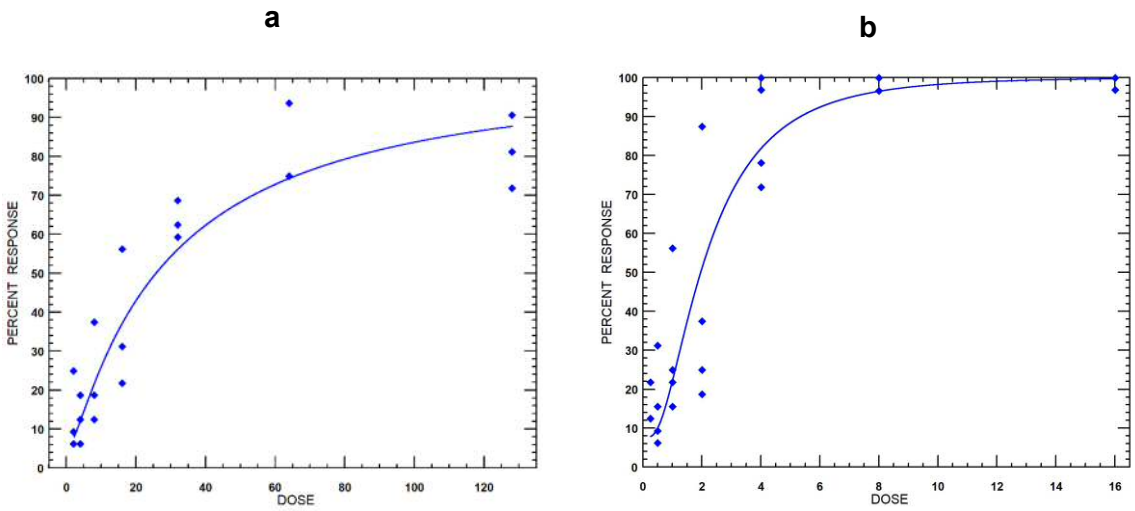


Figure 3. Susceptibility to Cry1Ab toxin measured by MIC_{50} values of a laboratory population of *O. nubilalis.* Colours indicate the B1 (blue) and B2 (pink) toxin batches.

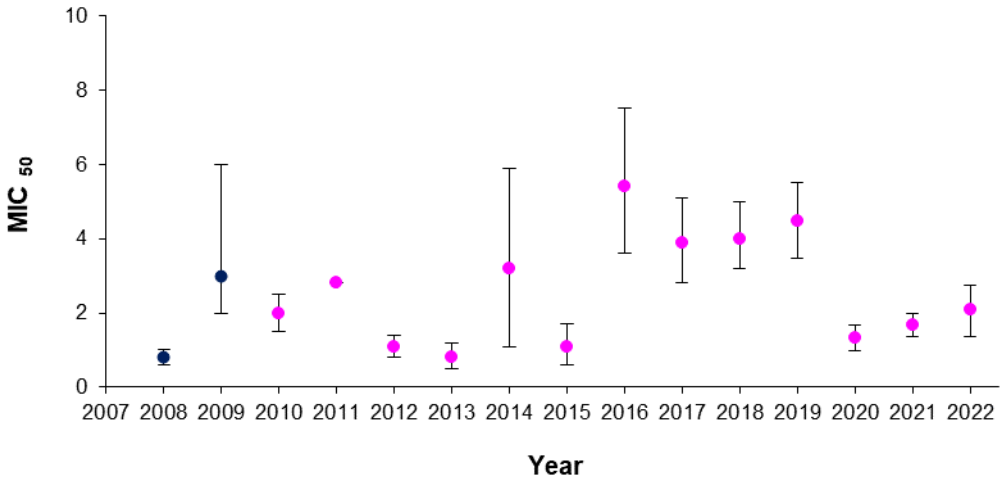
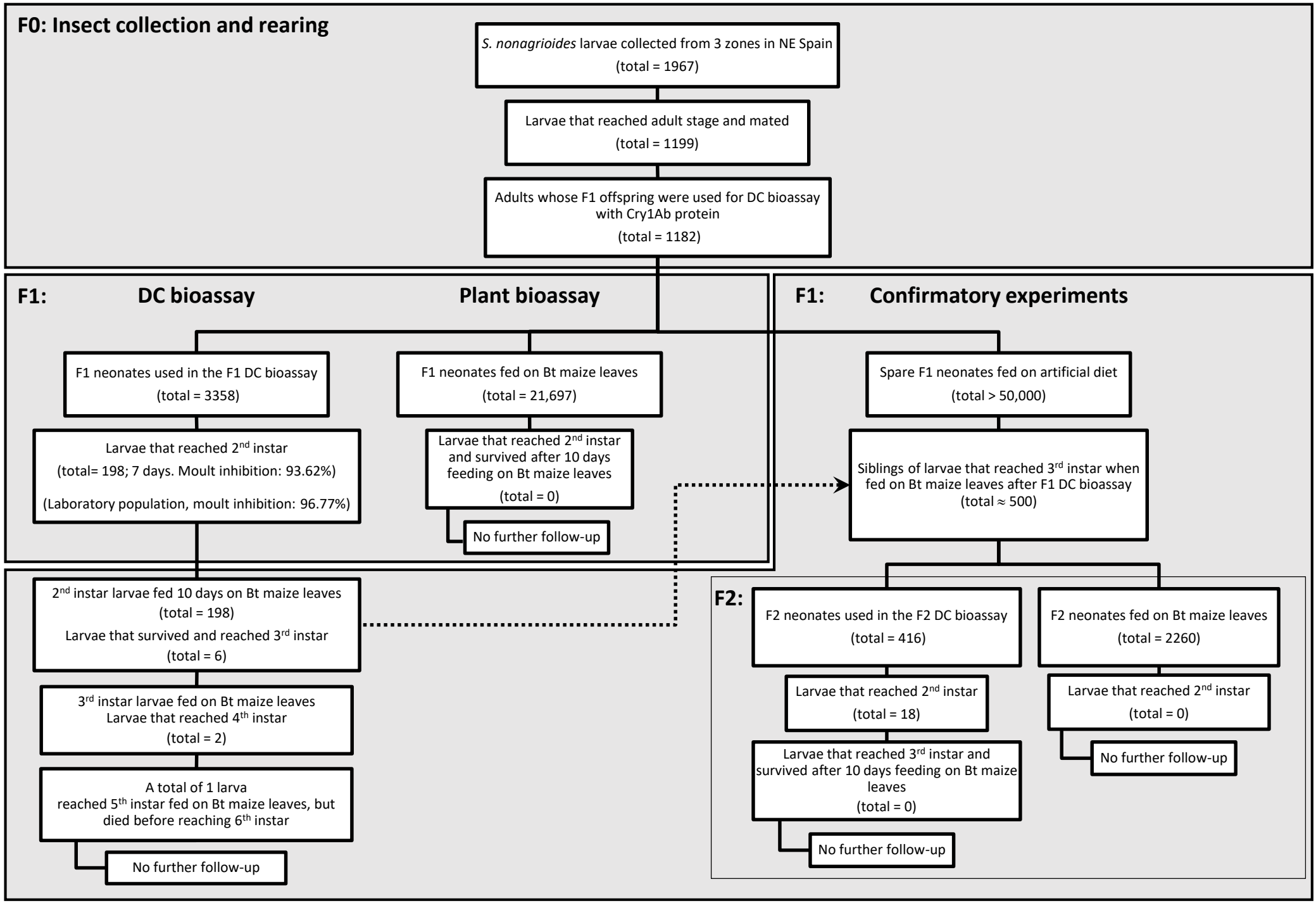


Figure 4. Moulting inhibition of neonates of *S. nonagrioides* from three zones of NE Spain (mean ± SE) and from the laboratory population, treated with a diagnostic concentration (DC) of 1091 ng/cm^2 bioassays. The dotted black line represents the expected 99% moult inhibition (MI) value.

Annex I. Stepwise approach followed to do bioassays with *Sesamia nonagrioides* (season 2022)

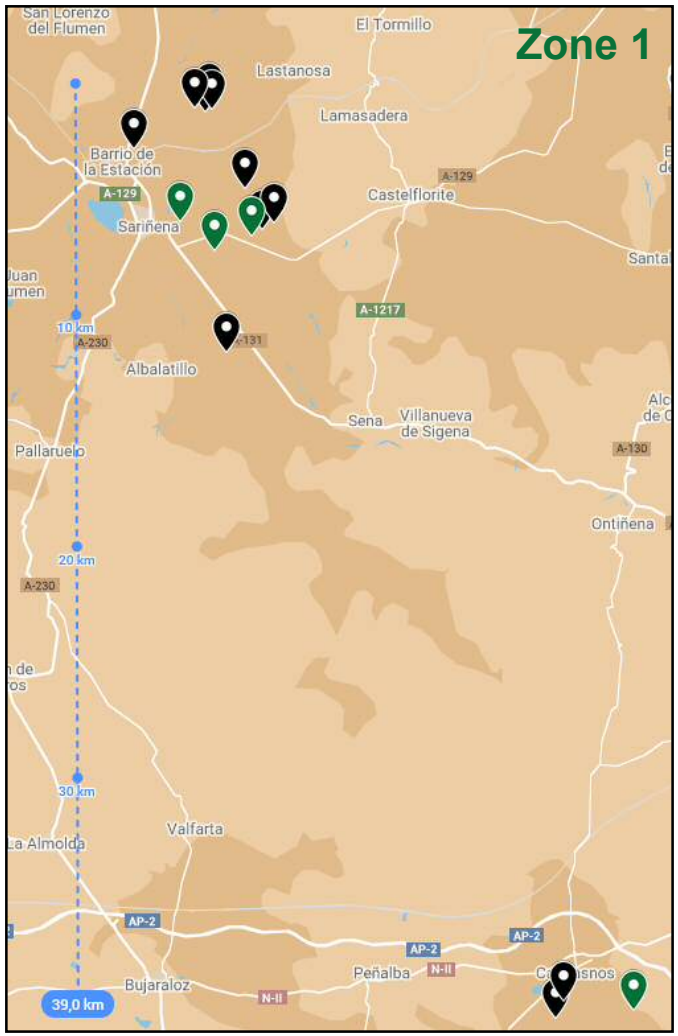


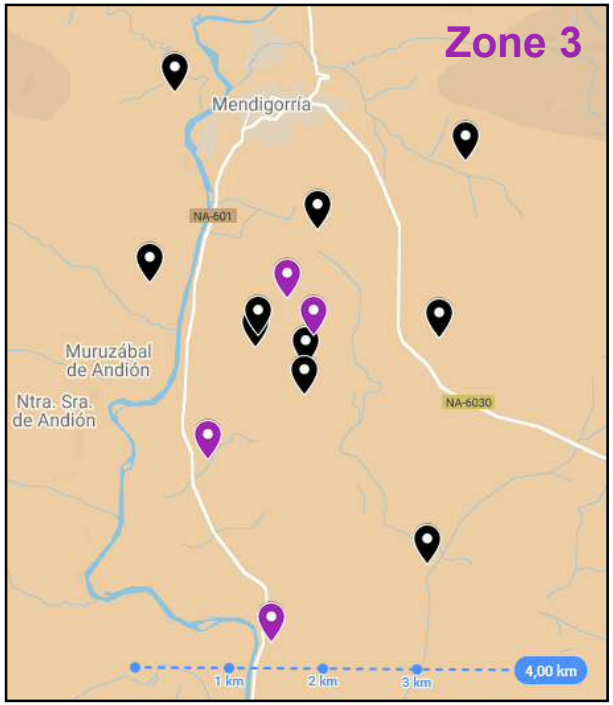
ANNEX IIa. Collection of *S. nonagrioides* larvae in NE Spain in 2022

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 2022





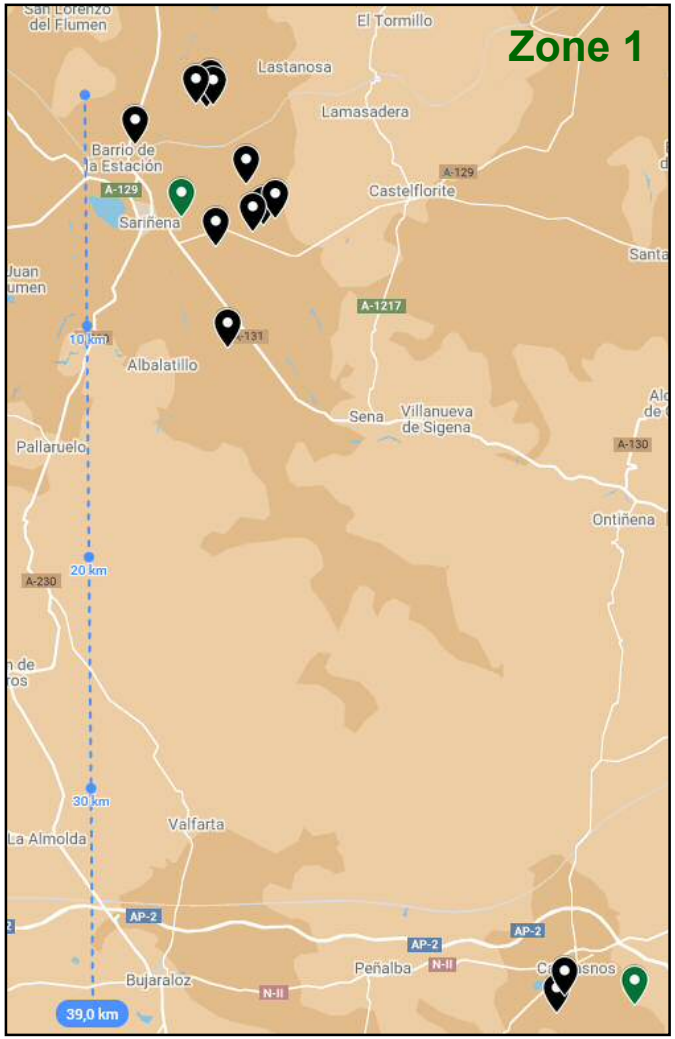
- Successful sampling sites-Zone 1
- Successful sampling sites-Zone 2
- Successful sampling sites-Zone 3
- Unsuccessful sampling sites

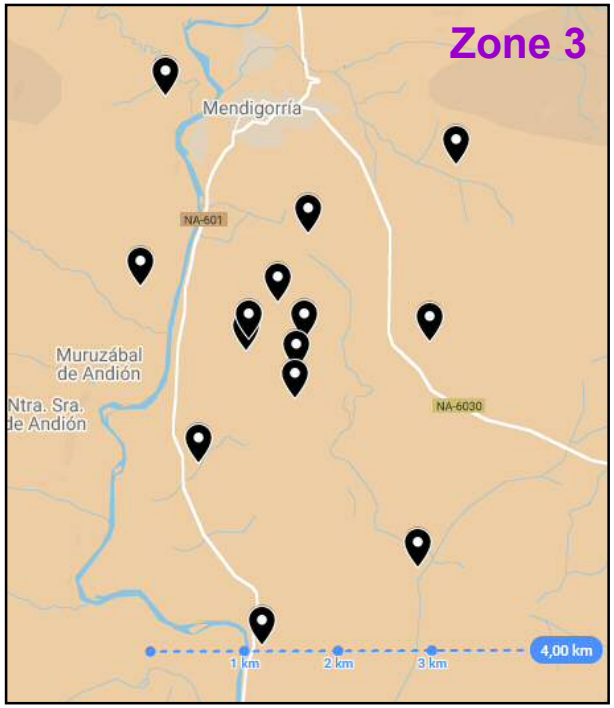
ANNEX IIIa. Collection of *O. nubilalis* larvae in NE Spain in 2022

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 2022





- Successful sampling sites-Zone 1
- Successful sampling sites-Zone 2
- Successful sampling sites-Zone 3
- Unsuccessful sampling sites