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

Season 2019

CONTENTS

1. Introduction

Maize containing event MON 810 is transgenic improved maize expressing the Cry1Ab protein derived from *Bacillus thuringiensis* subsp. *kurstaki*, and conferring protection against certain lepidopteran insect pests such as *Ostrinia nubilalis* and *Sesamia nonagrioides*. Resistance development in targeted lepidopteran pests is a potential concern arising from the widespread cultivation of MON 810 maize varieties. In order to maintain the benefits obtained from growing MON 810 maize varieties, Bayer, following directions described in the industry IRM (Insect Resistance Management) working group guidelines proposed to the competent authority (EU Commission), available since 2003 but published in 2007 (Alcalde et al., 2007) and subsequently updated as the EuropaBio harmonised IRM plan (EuropaBio, 2012; 2017; 2019), established an insect resistance monitoring program across Europe and in particular, in areas where commercial activity of MON 810 genetically improved maize is occurring or planned for the European targeted pests *O. nubilalis* and *S. nonagrioides*. The objective is to detect, in a timely manner, the potential development of resistance that could result in inadequate protection against the target species. This report focuses on the monitoring plan for *S. nonagrioides* in the 2019 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; Farinós et al., 2012). This species completes a variable number of generations per year depending on latitude, ranging from two in southern France to up to four in Morocco (Anglade, 1972, Eizaguirre and Fantinou, 2012). Larvae of the first generation are particularly destructive because they tunnel throughout the maize stem during the whole larval stage, causing great damage to maize seedlings and making their control particularly difficult.

Routine monitoring for changes in the susceptibility of EU field populations of *S. nonagrioides* to the Cry1Ab protein has been carried out in the period 2004-2018[1]. During the period 2004-2015, the plan covered the three maize-growing areas in the EU where MON 810 hybrids have been grown and *S. nonagrioides* is present: Northeast Iberia, Central Iberia and Southwest Iberia. Baselines were also gathered for other areas in Europe but no further samplings were performed in these areas because

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

the adoption rate of Bt maize is less than 20% and monitoring resistance is not necessary according to the EuropaBio Harmonized IRM plan (EuropaBio, 2012). In Iberia, each target field population was initially monitored every two years, but for practical reasons they were divided into two groups so that each year sampling was carried out in one of the groups. The susceptibility of *S. nonagrioides* field populations to the Cry1Ab protein expressed in MON 810 maize varieties was estimated by means of dose-response bioassays. Measured endpoints of the tests were mortality (lethal concentration, LC) and moulting inhibition (moulting inhibition concentration, MIC). From 2010 onward, it was decided to switch from LC to MIC values because it was observed that variations in the susceptibility were better reflected in MIC_{50} values than in LC_{50} values, given the characteristics of the bioassay and the biology of the species. In both cases, MIC_{50} and LC_{50} values of field populations were compared with previous baseline susceptibility data established for this species or with a susceptible laboratory strain assayed with the same batch of toxin (González-Núñez et al., 2000; Farinós et al., 2004, 2018).

The harmonised IRM plan was subsequently updated to accommodate the upgrades in the regulatory framework, and to incorporate the available scientific information and new learnings gained from this and other IRM plans (EuropaBio, 2017; Farinós et al., 2018; Thieme et al., 2018; Bertho et al., 2020). The revised plan establishes that sampling for resistance monitoring will take place in areas where the Bt maize adoption is over 60% and where the target pest is present. Currently, this situation only occurs in the area of the Ebro valley (Northeast of Spain) within the EU. Since *S. nonagrioides* and *O. nubilalis* are multivoltine species, the revised plan proposes that monitoring for these corn borers in this area should be carried out on an annual basis. This revised plan was put in practice for the first time during the season of 2016, being this season (2019) the fourth time.

In addition, the EFSA Scientific Opinion (EFSA GMO Panel, 2017) and Statements (EFSA, 2018, 2019) on the annual post-market environmental monitoring (PMEM) reports on the cultivation of genetically modified maize MON 810 in 2015, 2016 and 2017, respectively, included two main recommendations: *(1) annual sampling of target pests in the Ebro Valley, the area where deployment of Bt-maize is the highest and where resistance is likely to evolve more quickly; and (2) setting a maximum detection threshold for resistance allele frequency at 3% to enable the early detection of resistance so that alternative management measures can be implemented in time to delay the development of resistance.*

In accordance with these recommendations and following the revised harmonized IRM plan (EuropaBio, 2017), from the 2016 season onwards the collection of field larvae has focused on the Ebro valley (including the Autonomous Communities of Aragón, Cataluña and Navarra), where the adoption rate of Bt maize is over 60%. Moreover, a diagnostic concentration bioassay (Sims et al., 1997; Marçon et al., 2000) has been used to monitor for changes in susceptibility to the Cry1Ab protein in *S. nonagrioides* field populations from Northeast Spain. The aim of using this methodology is that a high number of field-collected individuals are represented in the laboratory bioassays as F1 larvae, thereby helping to decrease the detection limit for resistance allele frequency.

The tasks carried out in the 2019 maize growing season were the following:

1. Collection of larvae of *S. nonagrioides* in three different zones from Northeast of Spain (Ebro valley) to be used in diagnostic concentration bioassays, and comparison of the susceptibility value obtained with that of the susceptible laboratory strain and with the hypothetical value of 99%.

2. Collection of larvae of *O. nubilalis* in three different zones from Northeast of Spain (Ebro valley) to be sent to the laboratory BTL GmbH Sagerheide (Germany), which is carrying out the European resistance monitoring programme of *O. nubilalis* for MON 810 maize.

3. Analysis of the susceptibility to Cry1Ab of laboratory strains of *S. nonagrioides and O. nubilalis* by means of moulting inhibition concentration (MIC) values, aiming at verifying the activity of the batch of protein used in the bioassays.

2. Materials and Methods

2.1. Insect collection

Last-instar larvae of *S. nonagrioides* and *O. nubilalis* were collected from three sampling zones for each species within the Ebro valley area, in the Northeast of Spain (NE Spain), each zone comprising at least three maize fields in the smallest possible surface. A minimum of 1000 larvae were 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 habitual high mortality rates of field larvae when they are brought to 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%. There are different reasons that could explain the high mortality of both lepidopteran species during the diapause period (about 3 months). 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).

The process of identifying fields for insect collection starts by contacting local field technicians of seed companies that commercialize MON 810 in Spain. They are asked if they are aware of the presence of corn borers (*Sesamia* and/or *Ostrinia*) in the area to be sampled, for which they may consult a number of growers about the detection of corn borer damage in their fields. When that is the case, the growers are asked for permission to enter their fields during one to three days to collect larvae, which requires destructive sampling of the maize stalk. The amount of maize plants used depends on the presence of one or two species, the severity of the attack, etc.

Last-instar larvae of both corn borers were collected following standard operative procedures (SOP) of each species (EuropaBio, 2017). The samples were collected at the end of the maize-growing season, during September and October 2019, from refuges and fields of conventional maize adjacent to MON 810 maize, by cutting the stalk of the maize plants and taking only one larvae of each species per plant to avoid collecting siblings.

2.2. Insect culture

Field collected larvae were brought to the laboratory, dipped in a solution containing 1% bleach to avoid contamination by pathogens and placed in 21x16x4 cm plastic boxes (50 larvae of *S. nonagrioides* or 100 larvae of *O. nubilalis*). Both species were fed on an artificial diet established from that described by Poitout and Buès (1970) with some modifications (**Tables 1** 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* were in diapause at the time of collection, so they 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. The sex was determined at the pupal stage and a variable number of couples from the same zone (from 3 to 8), in function of the day of adult emergence, were confined in ventilated plastic cylinders (12 cm diameter x 30 cm high) containing 5-7 maize seedlings for oviposition at standard rearing conditions (25 ± 1ºC, 70 ± 10% relative humidity and a photoperiod of 16:8 hours (L:D). After 7 days the eggs were collected and placed into ventilated plastic boxes containing wet filter paper. The eggs were incubated under the same conditions and neonate larvae (< 24 h old) were utilized in the bioassays.

2.3. Quality of the laboratory strains

Reference susceptible strains of *S. nonagrioides* and *O. nubilalis* have been maintained under laboratory conditions to serve as control in this study. As a general rule, these populations are formed from individuals collected in non-Bt fields from Galicia (northwest of Spain), where Bt maize has never been commercially grown, so corn borers have low or no selection pressure, making them a good option to be used as the reference strain. Formerly, Galician populations of *S. nonagrioides* had not shown differences in susceptibility to the Cry1Ab toxin with respect to those of other Spanish populations in bioassays carried out in 1998 (González-Núñez et al., 2000) and in 2011 (unpublished results). Laboratory populations of *O. nubilalis* and *S. nonagrioides* coming from Galicia have been used since 2016 and 2018, respectively, as reference strains for insect resistance monitoring at the CIB in Madrid. A new collection of *S. nonagrioides* larvae was made in Galicia in 2019, with the assistance of La Misión Biológica de Galicia (MBG, CSIC) staff, to be incorporated as reference population after adaptation to the artificial diet and to laboratory conditions (Hoffmann & Ross, 2018).

In the laboratory, a minimum of 300 adults are crossed every generation. Neonate larvae are collected from all the oviposition cages formed with the adults of the previous generation, unless some cage has evidences of some disease, in which case it is removed. Populations maintained for many years in the laboratory typically suffer excessive inbreeding. To preserve the vigour of the laboratory colonies of *S.*

nonagrioides and *O. nubilalis* and to ensure that the populations do not collapse, they need to be periodically refreshed with new individuals collected in non-Bt fields, after taking some precautions: i) comparison of the LC_{50} values of both the laboratory and field populations by susceptibility bioassays to guarantee that there are no significant differences between them; ii) check of pathogens (namely *Nosema* sp.) by inspecting a number of larvae in slides under the microscope and by molecular methods (PCR); and iii) adaptation of the population to laboratory conditions. To the best of our knowledge, this has been proved to be the best practice to avoid the collapse of populations that we have experienced in the past working with these species.

2.4. Cry1Ab protein

Two batches of Cry1Ab protein have been used since the start of the MON 810 monitoring plan (2004). The first batch (B1) was provided by Bayer in 2003 (concentration 2.03 mg/ml in sodium bicarbonate buffer, pH 10.5; purity 95%). The second batch (B2) (concentration 1.8 mg/ml in 50 mM sodium bicarbonate buffer, pH 10.25; purity 91%) has been provided at different times: B2-1 was sent in October 2011, B2-2 in February 2014, B2-3 in April 2016, B2-4 in July 2017, B2-6 in July 2018 and B2-7 in September 2019. 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 toxin sent in September 2019 (B2-7) has been used for the bioassays of this season.

2.5. Bioassays

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

The bioassays were carried out in accordance with the methods described by Farinós et al. (2004), using “Bio-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 larva (<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 (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 second larval instar after 7 days.

The concentration ranges were comprised between 2 and 128 ng Cry1Ab/cm^2 for *S. nonagrioides* and between 1 and 64 ng Cry1Ab/cm^2 for *O. nubilalis*. To determine the susceptibility of each population, 6 to 8 different concentrations resulting in mortality or moulting inhibition higher than 0% and below 100% were used. Three replicates were prepared for each concentration and the control in the case of *O. nubilalis*, whereas two replicates were used for *S. nonagrioides*, due to causes related to COVID19 **(Annex IV)**. Each replicate consisted of 32 larvae per concentration (64 for controls), giving 96 larvae for each concentration tested (192 for controls). For each replicate neonate larvae from different oviposition cages were used. The susceptibility of the laboratory strains of *S. nonagrioides* and *O. nubilalis* to Cry1Ab was assessed using the batch B2-7 of protein. The MIC_{50} value obtained for *S. nonagrioides* was compared with those obtained with the reference population in previous years. The MIC_{50} value was determined for the fourth time for the reference strain of *O. nubilalis* established in the laboratory in the 2016 season.

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

A diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2, intended to cause moulting inhibition between 99 and 100% to first-instar larvae of *S. nonagrioides*, was used for DC bioassays to measure susceptibility to the Cry1Ab protein. This DC was calculated with all the available data of MIC bioassays performed with larvae from the Ebro valley, that is to say, with larvae collected in NE Spain over the seasons 2009, 2011, 2013 and 2015, and the resulting value represents the response of more than 4300 larvae in four dose-response bioassays with these populations. It was decided that this DC would be used from the 2016 campaign onwards.

The susceptibility to the protein Cry1Ab by the use of DC bioassays was tested on F1 progeny of the field populations collected in NE Spain in 2019 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.

To ensure that as many field collected individuals as possible were represented in the bioassay, and to calculate the detection limit for resistance allele frequency in this study, the following parameters were quantified: number of field collected larvae, number of emerged adults, number of oviposition cages settled and used in bioassays and number of neonates used in the bioassays from each oviposition cage (treated and controls). The number of egg masses used for the bioassays was maximized by doing the bioassay at a daily basis, whenever neonates of an oviposition cage emerged (larvae emerged during the weekend and 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. For all that, the DC bioassay with the F1 generation extended for about twelve 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 second larval instar.

About two-hundred neonates (not used in the DC bioassays) of each oviposition cage of the F1 generation coming from the three Ebro valley 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 second larval instar was recorded during 10 days.

It was ensured that all the Bt plants used in the bioassay were transgenic 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 (light: dark).

2.5.4. Confirmatory experiments

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

Firstly, all second-instar larvae recovered alive after 7 days in the DC bioassay were placed in plastic boxes of 9 cm diameter and 3 cm height, with those coming from the same oviposition cage grouped together. Then, they were fed *ad libitum* on MON 810 leaves, following the same procedure of section 2.5.3. If some of these larvae were able to moult to the third larval instar, 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. Due to COVID19, the number of oviposition cages tested with a DC in the F2 generation was reduced (see **Annex IV** for details).

In the case of plant bioassays, if some neonate fed on MON 810 during 10 days was able to moult to the second larval instar, 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, 2002-2019). 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 26). 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 showed in **Annex I**.

3.1. Collection of larvae

This campaign, the technicians involved in the collection of field larvae for *S. nonagrioides* and *O. nubilalis* carried out about 300 hours of fieldwork, travelled over 2800 km and made a total of three round trips, one for each different field zone.

A total of 1644 last-instars larvae of *S. nonagrioides* were collected between September and October 2019 from three different Zones in NE Spain (655, 560 and 429 larvae from Zones 1, 2 and 3, respectively; **Table 3**). A map showing the sampling points for *S. nonagrioides* is displayed in **Annex IIa.** Five, ten and thirteen fields in Zones 1, 2 and 3, were searched respectively, although larvae were successfully collected in three fields in Zones 1 and 3 and four fields in Zone 2 **(Figure 1, Annex IIb**). The maximum distance between successfully sampled fields was about 10, 3 and 3 Km within Zones 1, 2 and 3, respectively (**Annex IIb**).

Larvae of *O. nubilalis* were collected between September and October 2019 from three Zones in the Northeast of Spain, yielding a total of 1110 larvae (368, 547 and 195 larvae from Zones 1, 2 and 3, respectively; **Table 4**). A map showing the sampling points for *O. nubilalis* is displayed in **Annex IIIa.** Despite the number of fields sampled (five in Zone 1, ten in Zone 2 and thirteen in Zone 3), larvae were mainly gathered in three fields in Zone 1, four fields in Zone 2 and one field in Zone 3 (**Figure 1, Annex IIIb**). The maximum distance between successfully sampled fields was about 3 and 2 Km within Zones 1 and 2 respectively (**Annex IIIb**).

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

The susceptibility to Cry1Ab toxin of the laboratory population of *S. nonagrioides* was performed with 533 neonates using a dose-response bioassay, resulting in a MIC_{50} value of 27 (16-40) 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**). The MIC_{90} value was higher than in previous years, although when compared with data for 2018 there were no significant differences: LC ratio at LC_{90} (95% CI) = 2.01 (0.86-4.70).

A number of 854 neonates of the *O. nubilalis* laboratory strain were used for the Cry1Ab susceptibility assessment bioassay. The MIC_{50} value obtained was 4.5 (3.5-5.5) ng Cry1Ab/cm^2 (**Table 5**, **Figure 2b**), similar to the MIC_{50} values observed in the last years and within the range of values obtained since 2010 with the previous reference strain and the same batch of toxin (0.8-5.4 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). 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 1644 *S. nonagrioides* last-instars larvae collected, 662 (40.3%), combining larvae and pupae, died in the process of rearing in the laboratory, mainly during the diapause period. In addition, 60 adults (3.6%) did not emerge in the date range for oviposition cages or had some malformation upon emergence, so they were not used in the bioassays (**Table 7**)**.** Thus, of the 982 adults that emerged, 922 (56.1%) did so between 4th November 2019 and 12^{tht} February 2020 and were placed in 99 oviposition cages for mating. The offspring of 868 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 2019 is 0.034 (3.4%),

calculated considering the model developed by Andow and Ives (2002).

Of the total F1 neonates originated from the field collected larvae, 3551 were used in the bioassays. The DC (1091 ng Cry1Ab/cm^2) caused a mean (± S.E.) corrected moulting inhibition (MI) of 97.97% ± 0.36% (98.20%, 97.26% and 98.44% in Zones 1, 2 and 3, respectively; **Tables 8** and **9**).

Statistically significant differences were observed between field populations MI value (97.97%) and the expected MI value of 99% (t = 4.000, df = 2, *p* = 0.029). However, no significant differences were found between field populations (97.97%) and laboratory strain (97.02%) MI values (t = 2.440, df = 2, *p* = 0.067) (**Table 9**).

In 2016 and 2018 no significant differences were observed between field populations MI value and neither laboratory population nor expected MI values. The opposite happened in 2017, when significant differences were detected between field population MI value and both laboratory population and expected MI values. This year significant differences have been observed only between field population and expected MI values (**Table 9**). Therefore, no trend is observed in terms of changes in the susceptibility of populations from the Ebro valley to the Cry1Ab toxin **(Figure 4)**.

It is noteworthy that in 2017, 2018 and 2019 campaigns, the percentage of moulting inhibition of *S. nonagrioides* obtained with the laboratory susceptible strain was below the expected value of 99% (97.69%, 97.75% and 97.02%, respectively), and only in 2016 was this value (99.20%) over the expected value (**Table 9**). Fluctuations of about 6-fold for both LC_{50} and MIC_{50} were also found in the laboratory strain during the period in which monitoring was performed by means of dose-response bioassays (2004–2015), although no trends were observed over time (**Table 6**). This underlines the importance of testing the field populations against a reference population from areas where Bt maize is not grown, enabling the correct interpretation of the results.

3.4. Larval development on MON 810 tissue: plant bioassays

17,300 F1 first-instar larvae of *S. nonagrioides*, from the populations collected in three zones in the Ebro valley in 2019, and 2675 from the laboratory strain were fed *ad libitum* on MON 810 tissue to test if they were able to moult to the second larval instar within ten days. As a control, 881 neonates of these field populations and 140 neonates of the laboratory strain were reared on conventional maize. During the assay only 1 larva from Zone 2 fed on Bt maize was able to moult to the second larval instar

after 5 days. This larva was transferred to artificial diet without Cry1Ab toxin and it died 2 days later. Most larvae fed on conventional maize from the field and the laboratory populations (98.2% and 95.7%, respectively) moulted to second or third larval instar **(Table 10)**.

3.5 Confirmatory experiments

A total of 67 (1.89%) larvae from 32 oviposition cages and one larva from one oviposition cage of Zone 2 reached the second larval instar in the F1 DC and plant bioassays, respectively. Consequently, the following confirmatory experiments were conducted.

The 67 surviving second-instar larvae from the DC bioassays were individualized in boxes and fed on Bt leaves. Eight of these larvae, each from a different oviposition cage (one from Zone 1, five from Zone 2 and two from Zone 3) moulted to the third larval instar, but died after a few days without reaching the fourth instar (**Table 11a**). The surviving second-instar larvae from the plant bioassays also died after a few days feeding on Bt maize.

The siblings of the larvae that reached the third instar larvae from the DC and plant bioassays (about 125 larvae from each of the nine oviposition cages) were raised on artificial diet up to the next generation (F2). In two of the oviposition cages the eggs were not fertile, and other four oviposition cages could not be used for DC bioassays due to COVID19 **(Annex IV)**.

As a result, 287 F2 neonates (96 from Zone 2 and 191 from Zone 3) from three oviposition cages could be treated with the diagnostic concentration (1091 ng/cm^2). Two of the treated larvae moulted to the second larval instar after 7 days, but when they were subsequently fed on MON 810 maize, none of them moulted to the third larval instar.

In addition, 3680 neonates from the seven fertile oviposition cages were fed on MON 810 maize and 190 neonates, used as controls, on conventional maize. After 10 days, none of the larvae fed on Bt tissue were able to moult, whereas 179 larvae (94%) fed on conventional maize had moulted to second or third larval instar (**Table 11b**).

4. Summary of results

1. Monitoring for changes in the susceptibility of EU field populations of *S. nonagrioides* and *O. nubilalis* to the Bt Cry1Ab in 2019 has been focused for the fourth time in the Ebro valley, in the Northeast (NE) of Spain, where the adoption rate of Bt maize in 2019 was over 60%. A total of 1644 larvae of *S. nonagriodes* and 1110 larvae of *O. nubilalis* were collected in three sampling zones for each species. Larvae of *O. nubilalis* were sent to the laboratory BTL GmbH Sagerheide (Germany) for testing their susceptibility to the Cry1Ab protein.

2. The susceptibility to the Cry1Ab toxin of the field populations of *S. nonagrioides* from NE Spain has been determined in bioassays by the use of a diagnostic-concentration (DC) of 1091 ng Cry1Ab/cm^2, intended to cause moulting inhibition ≥ 99% to first-instar larvae of *S. nonagrioides*. This DC was estimated with data from larvae collected from NE Spain in four previous seasons: 2009, 2011, 2013 and 2015.

3. From the 1644 larvae of *S. nonagrioides* collected, 982 adults (60%) emerged, of whom 922 mated. The offspring of 94% of these adults (868) was used in the bioassays and treated with the diagnostic concentration (DC) of 1091 ng Cry1Ab/cm^2. These values indicate that despite the application of best practices in larvae rearing only 53% of the field collected larvae were represented in the DC bioassays. Thus, the detection limit for resistance allele frequency in field populations of *S. nonagrioides* in 2019 is 0.034 (3.4%).

4. The values of the detection limit for resistance allele frequency in field populations of *S. nonagrioides* in the four last seasons, (3.3, 3.7, 4.2 and 3.4 in 2016, 2017, 2018 and 2019, respectively) *vs.* the number of larvae collected in the field each year, which has gradually increased (1364, 1452, 1490 and 1644), highlight the technical difficulties that can be encountered, depending on different factors, in each campaign, regardless of the number of larvae collected.

5. The treatment with the DC caused moulting inhibition of 97.97% (S.E. 0.36%) to F1 neonates from the field collected larvae of the NE Spain. This outcome was significantly different from the expected value of 99% (t = 4.000, df = 2, p = 0.029) but not from the laboratory population moulting inhibition value (97.02%) (t = 2.400, df = 2, p = 0.067).

6. Only one of the 17,300 neonates of the F1 generation of the field collected

populations reared on MON 810 leaves was able to moult to the second larval instar, but it died 2 days after being put on an artificial diet, without having moulted to the third larval instar.

7. Laboratory *S. nonagrioides* and *O. nubilalis* strains showed susceptibility levels to the batch B2-7 of the Cry1Ab toxin (MIC_{50} values of 27 and 4.5 ng Cry1Ab/cm^2, respectively) comparable with those obtained from laboratory strains in previous years.

5. Concluding remarks

In the last four seasons, a considerable effort to collect a rising number of last-instars larvae of *S. nonagrioides* has been made. However, it has not resulted in an improvement in the detection limit for resistance allele frequency, highlighting the technical difficulties encountered to achieve the objective of 3%. 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 (97.97%) of *S. nonagrioides* F1 neonates from the Ebro Valley in 2019, treated with a diagnostic concentration (DC), was significantly lower than the hypothetical value of 99%, but not different from the moult inhibition value (97.02%) 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 indicate that there are no evidences of resistance development of *S. nonagrioides* to MON 810 maize. Our results are in line with those revealed in ten years of surveys of farmers, in which no evidence of any unexpected adverse effect associated with the cultivation of MON 810 was found (Bertho et al., 2020).

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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 2019 season in the Ebro valley (NE Spain).

Zone	Field	Province [a]	Postal Code	Date	Surface (Ha) [b, c]	Distance to the nearest MON810 field (m) [d]	No of larvae collected
1	2019-Lanaja 1	HU	22250	16-19/09/2019	115	20	261
	2019-Lanaja 2	HU	22250	16-19/09/2019	1.2	1000	37
	2019-Lanaja 3	HU	22250	16-19/09/2019	110.8	1000	161
	2019-Lanaja 4	HU	22250	16-19/09/2019	5.5	1000	0
	2019-Cantalobos	HU	22251	16-19/09/2019	8.8	10	196
	Total						**655**
2	2019-Candasnos 1	HU	22591	30/09-03/10/2019	7.8	250	206
	2019-Candasnos 2	HU	22591	30/09-03/10/2019	24.3	10	0
	2019-Candasnos 3	HU	22591	30/09-03/10/2019	16.4	10	0
	2019-Candasnos 4	HU	22591	30/09-03/10/2019	36.3	590	0
	2019-Candasnos 5	HU	22591	30/09-03/10/2019	84.5	10	53
	2019-Candasnos 6	HU	22592	30/09-03/10/2019	15.5	1290	204
	2019-Candasnos 7	HU	22591	30/09-03/10/2019	9.7	1290	0
	2019-Candasnos 8	HU	22591	30/09-03/10/2019	31.4	160	0
	2019-Candasnos 9	HU	22591	30/09-03/10/2019	50.2	1160	79
	2019-Candasnos 10	HU	22591	30/09-03/10/2019	16.2	590	18
	Total						**560**
3	2019-Mendigorria 1	NA	31150	14-17/10/2019	6.3	1000	0
	2019-Mendigorria 2	NA	31150	14-17/10/2019	7.1	1000	165
	2019-Mendigorria 3	NA	31150	14-17/10/2019	14.0	1000	0
	2019-Mendigorria 4	NA	31150	14-17/10/2019	6.3	0	0
	2019-Mendigorria 5	NA	31150	14-17/10/2019	1.4	0	13
	2019-Mendigorria 6	NA	31150	14-17/10/2019	5.2	0	63
	2019-Mendigorria 7	NA	31150	14-17/10/2019	7.3	650	0
	2019-Mendigorria 8	NA	31150	14-17/10/2019	11.1	20	188
	2019-Mendigorria 9	NA	31150	14-17/10/2019	6.1	0	0
	2019-Mendigorria 10	NA	31150	14-17/10/2019	2.4	0	0
	2019-Mendigorria 11	NA	31150	14-17/10/2019	3.3	400	0
	2019-Mendigorria 12	NA	31150	14-17/10/2019	25.6	400	0
	2019-Mendigorria 13	NA	31150	14-17/10/2019	1.0	300	0
	Total						**429**
	GRAND TOTAL						**1644**

[a] Provinces: HU = Huesca; NA = Navarra.
[b] Data are approximate.
[c] The area of the whole field, even though larvae were collected on the refuge area of the field.
[d] There could be other nearer fields that are not known by the technician and/or the farmer. "0" means that it is adjacent to a MON 810 field.

Table 4. *Ostrinia nubilalis* larvae collection details for the 2019 season in the Ebro valley (NE Spain).

Zone	Field	Province [a]	Postal Code	Date	Surface (Ha) [b, c]	Distance to the nearest MON810 field (m) [d]	No of larvae collected
1	2019-Lanaja 1	HU	22250	16-19/09/2019	115	20	0
	2019-Lanaja 2	HU	22250	16-19/09/2019	1.2	1000	79
	2019-Lanaja 3	HU	22250	16-19/09/2019	110.8	1000	0
	2019-Lanaja 4	HU	22250	16-19/09/2019	5.5	1000	66
	2019-Cantalobos	HU	22251	16-19/09/2019	8.8	10	223
	Total						**368**
2	2019-Candasnos 1	HU	22591	30/09-03/10/2019	7.8	250	247
	2019-Candasnos 2	HU	22591	30/09-03/10/2019	24.3	10	0
	2019-Candasnos 3	HU	22591	30/09-03/10/2019	16.4	10	0
	2019-Candasnos 4	HU	22591	30/09-03/10/2019	36.3	590	0
	2019-Candasnos 5	HU	22591	30/09-03/10/2019	84.5	10	0
	2019-Candasnos 6	HU	22592	30/09-03/10/2019	15.5	1290	102
	2019-Candasnos 7	HU	22591	30/09-03/10/2019	9.7	1290	0
	2019-Candasnos 8	HU	22591	30/09-03/10/2019	31.4	160	0
	2019-Candasnos 9	HU	22591	30/09-03/10/2019	50.2	1160	72
	2019-Candasnos 10	HU	22591	30/09-03/10/2019	16.2	590	126
	Total						**547**
3	2019-Mendigorria 1	NA	31150	14-17/10/2019	6.3	1000	0
	2019-Mendigorria 2	NA	31150	14-17/10/2019	7.1	1000	6
	2019-Mendigorria 3	NA	31150	14-17/10/2019	14.0	1000	1
	2019-Mendigorria 4	NA	31150	14-17/10/2019	6.3	0	0
	2019-Mendigorria 5	NA	31150	14-17/10/2019	1.4	0	1
	2019-Mendigorria 6	NA	31150	14-17/10/2019	5.2	0	2
	2019-Mendigorria 7	NA	31150	14-17/10/2019	7.3	650	0
	2019-Mendigorria 8	NA	31150	14-17/10/2019	11.1	20	182
	2019-Mendigorria 9	NA	31150	14-17/10/2019	6.1	0	2
	2019-Mendigorria 10	NA	31150	14-17/10/2019	2.4	0	1
	2019-Mendigorria 11	NA	31150	14-17/10/2019	3.3	400	0
	2019-Mendigorria 12	NA	31150	14-17/10/2019	25.6	400	0
	2019-Mendigorria 13	NA	31150	14-17/10/2019	1.0	300	0
	Total						**195**
	GRAND TOTAL						**1110**

[a] Provinces: HU = Huesca; NA = Navarra
[b] Data are approximate.
[c] The area of the whole field, even though larvae were collected on the refuge area of the field.
[d] There could be other nearer fields that are not known by the technician and/or the farmer. "0" means that it is adjacent to a MON 810 field.

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

Species	Toxin batch	n	Slope ± SE	χ^2	d.f.	MIC_{50} [a] (FL 95%)	MIC_{90} [a] (FL 95%)
S. nonagrioides	B2-7	533	1.4 ± 0.2	4.9	11	27 (16-40)	233 (133-656)
O. nubilalis	B2-7	854	2.7 ± 0.2	28.8	16	4.5 (3.5-5.5)	13.4 (10.5-19.1)

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

Table 6. Susceptibility to Cry1Ab toxin of laboratory populations of *S. nonagrioides* between 2004 and 2019. 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)

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

Table 7. Individuals of *S. nonagrioides* lost in the process of rearing or discarded for susceptibility bioassays. Percentages are shown with respect to the number of field larvae collected in each zone.

Fields	Field larvae collected	Dead larvae and pupae	Adults not used for mating[a]
Zone 1	655	251 (38.3%)	24 (3.7%)
Zone 2	560	214 (38.2%)	19 (3.4%)
Zone 3	429	197 (45.9%)	17 (4.0%)
All zones	1644	662 (40.3%)	60 (3.6%)

[a] Adults that did not emerge between 4th November 2019 and 12th February 2020, and adults having some malformation upon emergence

Table 8. Tracking of *S. nonagrioides* from the NE Spain populations and from the laboratory population used in the diagnostic concentration (DC) bioassays, and outcomes of the bioassays.

	Tracking of the larvae used in the diagnostic concentration bioassays							Diagnostic concentration bioassays				
Population	**Fields**	**Last instar larvae collected**	**Adults emerged[a]**	**Oviposition cages**	**Oviposition cages used in bioassays[b]**	**Adults used in bioassays (M-F)[c]**	**Total adults whose offspring was used[d]**	**Nº larvae treated in bioassays**	**MI (%)[e]**	**Nº larvae control**	**MI in control (%)[e]**	**Corrected MI (%)[f]**
NE Spain	Zone 1	655	404 (62%)	39	35	164-188	352 (54%) (87%)	1162	98.28	217	4.15	98.20
	Zone 2	560	346 (62%)	35	33	143-166	309 (55%) (89%)	1195	97.57	191	11.52	97.26
	Zone 3	429	232 (54%)	25	24	86-121	207 (48%) (89%)	1194	98.49	167	3.59	98.44
	All zones[g]	1644	982 (60%)	99	92	393-475	868 (53%) (88%)	3551	98.11	575	6.43	97.98
Laboratory	-	-	361	19	14	121-143	264	679	97.35	108	11.11	97.02

[a] Total adults emerged. Of these, we excluded for mating those that did not emerge between 4th November 2019 and 12th February 2020, and those that presented malformations at the time of their emergence. The percentage with respect to the number of larvae collected is in brackets.
[b] Oviposition cages were discarded when eggs hatched on non-working days or when the fecundity and/or fertility was too low.
[c] M, males; F, females.
[d] For field populations, percentages with respect to the number of collected larvae and with respect to the number of emerged adults are in brackets (in this order).
[e] MI, moulting inhibition: larvae that have not reached the second larval instar.
[f] Calculated using Abbot's formula (Abbot, 1925).
[g] Results obtained pooling the data of the three zones.

Table 9. Moulting inhibition values of F1 neonates of the Northeast (NE) population of Spain compared with those of the laboratory population and with the expected value of 99%.

Year	Moulting inhibition at DC (%)			*p*-values [b]	
	NE Spain[a]	Lab strain[a]	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***

[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 not-Bt (conventional) maize tissue.

Population	Fields	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	35	MON 810	6850	0	0.00
			Conventional	342	338	98.83
	Zone 2	33	MON 810	6400	1	0.02
			Conventional	333	326	97.90
	Zone 3	21	MON 810	4050	0	0.00
			Conventional	206	201	97.57
	All zones	89	MON 810	17300	1	0.01
			Conventional	881	865	98.18
Laboratory	-	14	MON 810	2675	0	0.00
			Conventional	140	134	95.71

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

Table 11. Confirmatory bioassays

11a. Larvae that were able to moult to the second larval instar (L2) in the DC bioassay and then moulted again to the third larval instar (L3) when fed MON 810 maize leaves.

Population	Fields	Nº larvae treated in DC bioassays	L2 (%)[a]	L3 (%)[b]	L4 (%)[b]
NE Spain	Zone 1	1162	20 (1.72)	1 (0.09)	0 (0.00)
	Zone 2	1195	29 (2.43)	5 (0.42)	0 (0.00)
	Zone 3	1194	18 (1.51)	2 (0.17)	0 (0.00)
	All zones	3551	67 (1.89)	8 (0.23)	0 (0.00)
Laboratory	-	679	18 (2.65)	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 and L4 after feeding on MON 810. Percentages with respect to the number of treated larvae.

11b. Larval growth of neonates of the F2 generation of *S. nonagrioides* after 10 days feeding on Bt (MON 810) or not-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	2	MON 810	400	0	0.00
			Conventional	20	20	100.00
	Zone 2	9	MON 810	1680	0	0.00
			Conventional	90	81	90.00
	Zone 3	8	MON 810	1600	0	0.00
			Conventional	80	78	97.50
	All zones	19	MON 810	3680	0	0.00
			Conventional	190	179	94.21

[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 2019. A collection at a field within a zone was considered successful if at least 50 larvae were gathered.

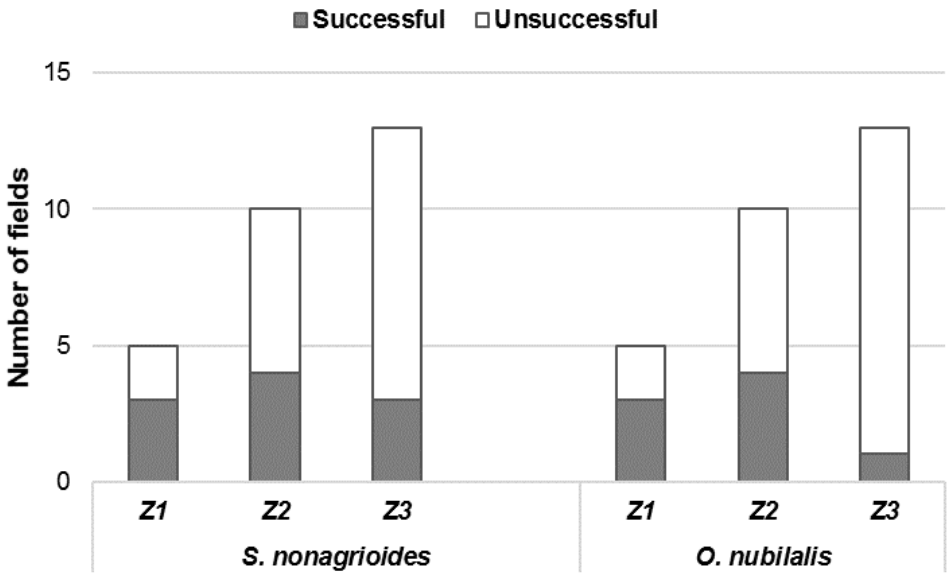


Figure 2. Fitted curves of susceptibility to the toxin Cry1Ab of the laboratory populations of *S. nonagrioides* and *O. nubilalis* (PoloPlus 1.0, LeOra Software 2002-2019). Response is moulting inhibition after seven days feeding on treated diet. a: *S. nonagrioides*. b: *O. nubilalis*.

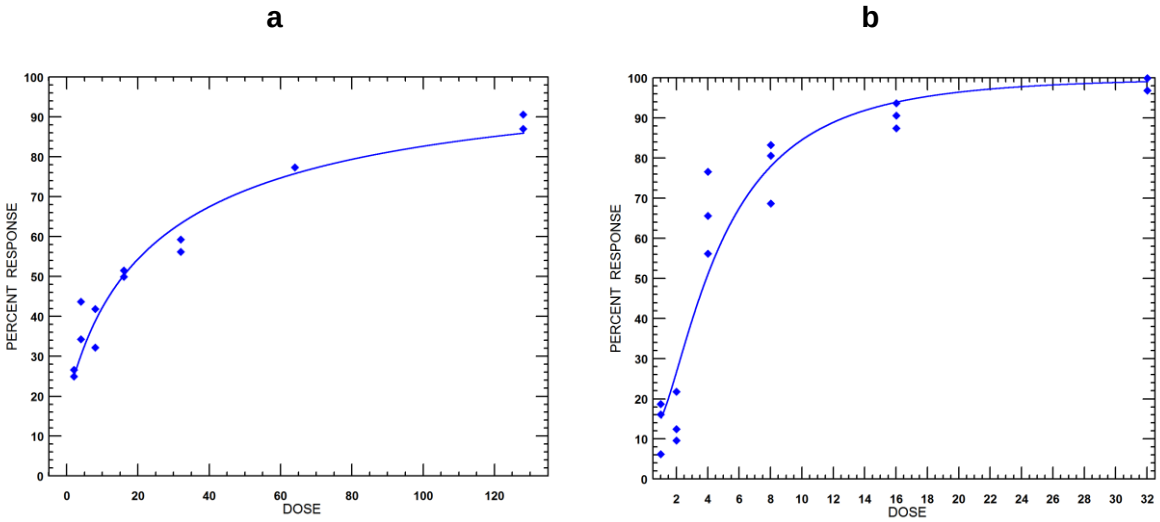


Figure 3. Susceptibility to Cry1Ab toxin measured by MIC_{50} values of a laboratory population of *O. nubilalis.* Colours indicate the B1 (blue) and B2 (pink) toxin batches.

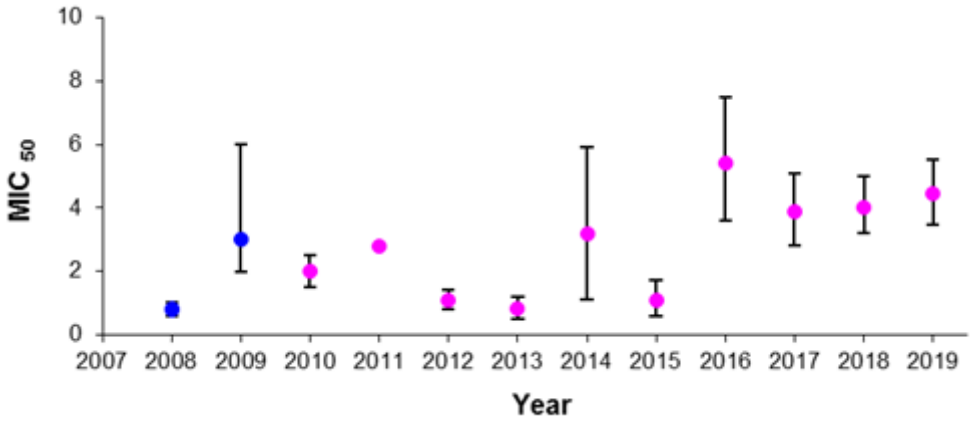
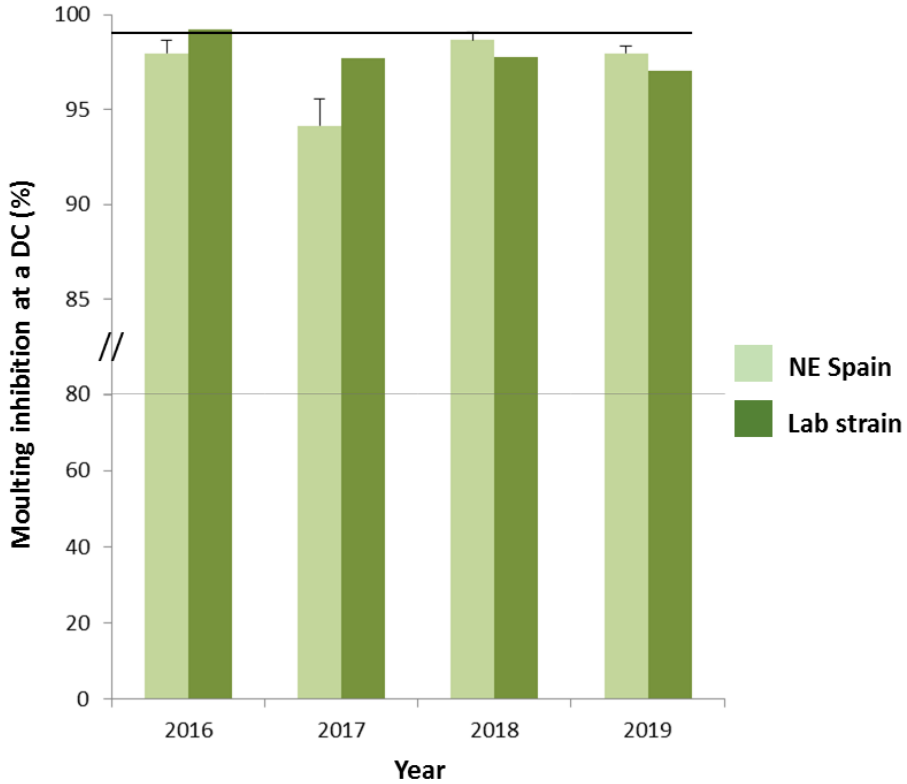
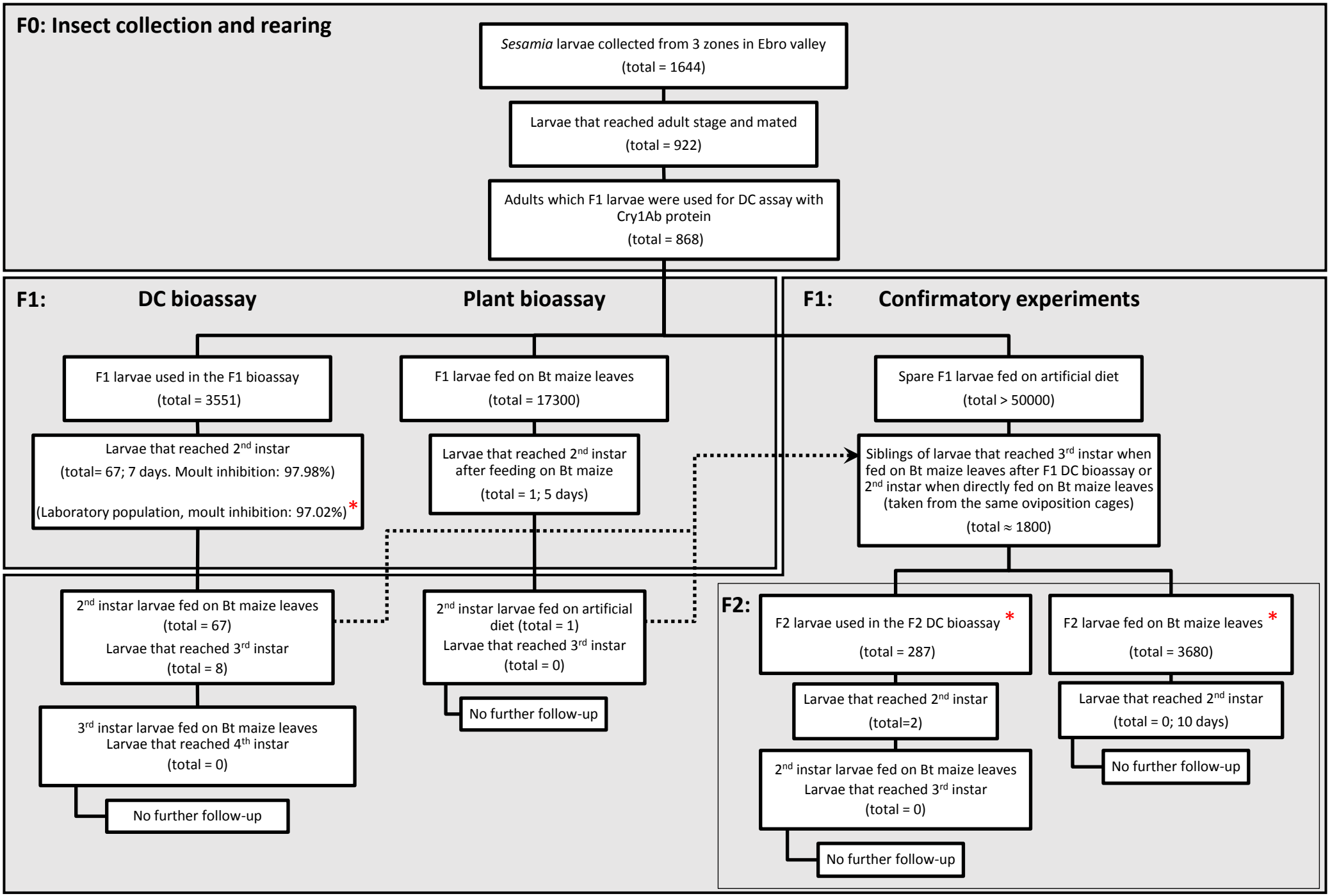


Figure 4. Moulting inhibition of neonates of *S. nonagrioides* from three zones of NE Spain (mean ± SE) and from the laboratory population, treated with a diagnostic concentration (DC) of 1091 ng/cm^2 bioassays. The solid black line represents the expected 99% moult inhibition (MI) value.



Annex I. Stepwise approach followed to do the bioassays



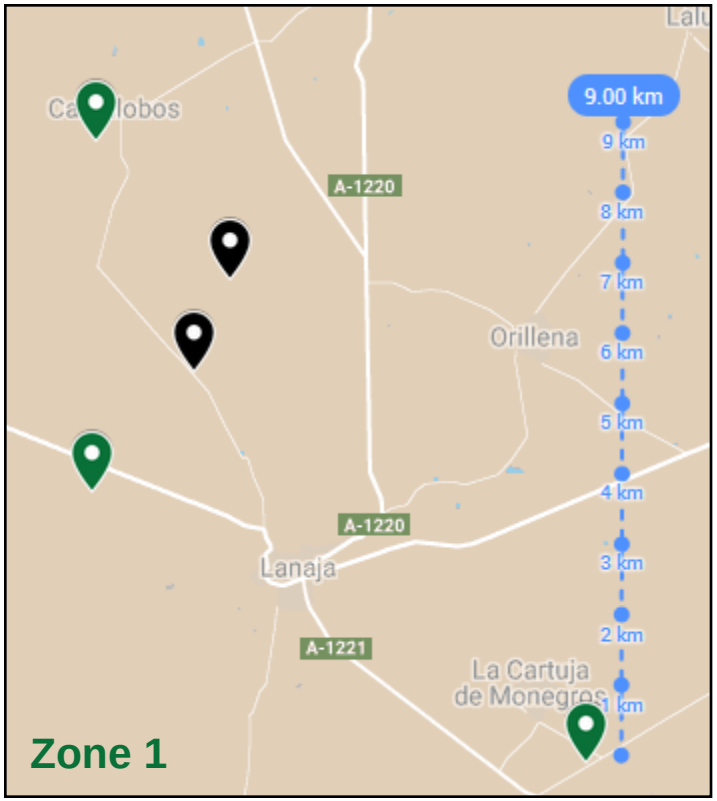
* The number of larvae used in these experiments was affected by COVID19 (Annex IV)

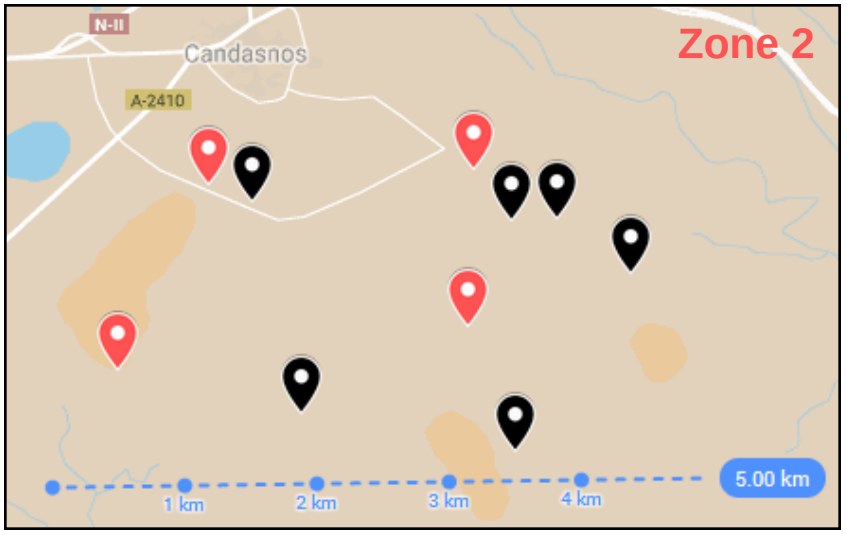
ANNEX IIa. Collection of *S. nonagrioides* larvae in the Ebro valley in 2019

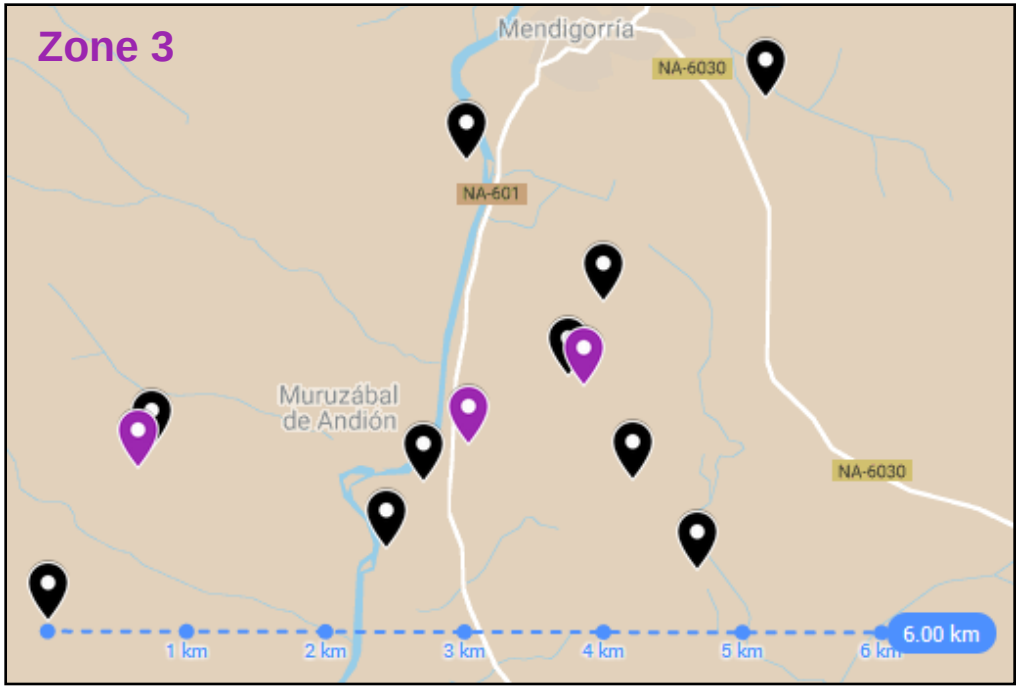
Major sampling site: at least 50 larvae were gathered.

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

ANNEX IIb. Collection of *S. nonagrioides* larvae in the Ebro valley in 2019







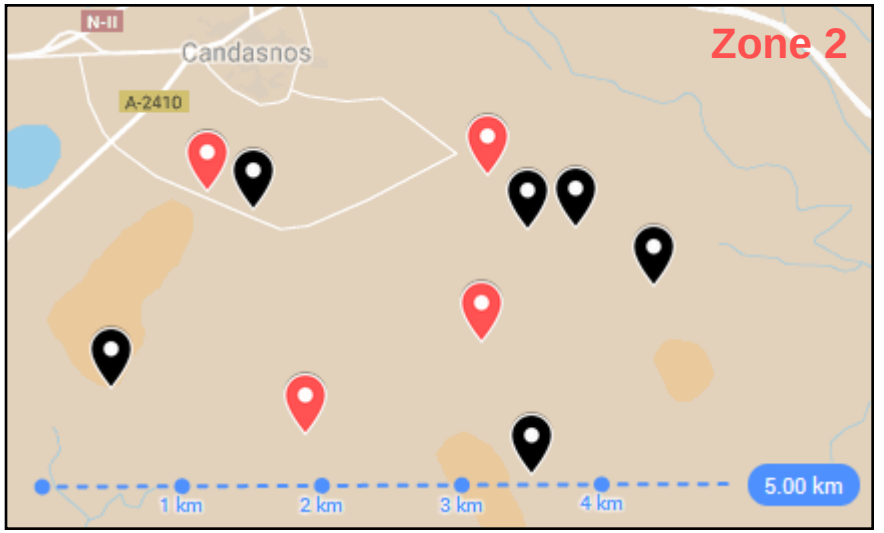
Major sampling site-Zone 1
Major sampling site-Zone 2
Major sampling site-Zone 3
Minor sampling sites

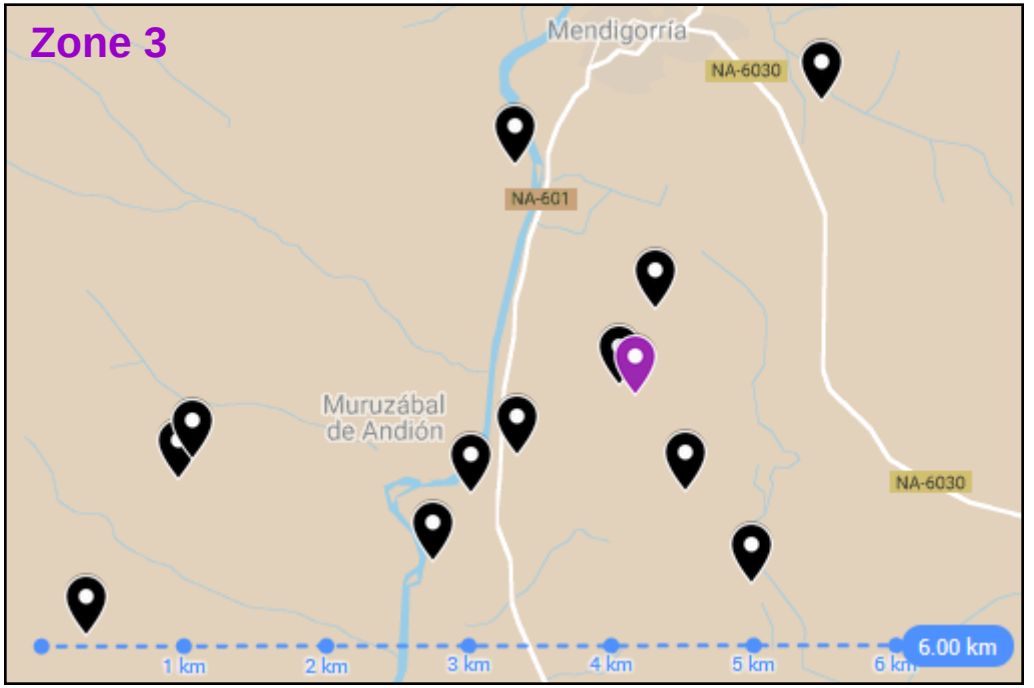
ANNEX IIIa. Collection of *O. nubilalis* larvae in the Ebro valley in 2019

Major sampling site: at least 50 larvae were gathered.

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

ANNEX IIIb. Collection of *O. nubilalis* larvae in the Ebro valley in 2019





Major sampling site-Zone 1
Major sampling site-Zone 2
Major sampling site-Zone 3
Minor sampling sites

ANNEX IV

Impact of the COVID19 pandemic on this study:

The outbreak of the global pandemic COVID19 has affected, but not compromised, the development of some of the experiments carried out for this report. This has been due to: 1) the restrictions on access to the CIB at the time of total confinement of the population in Madrid, only with permission to carry out, in one person's turns, activities considered essential (as the maintenance of animal populations) that required the shortest possible stay in the Centre; and 2) the mandatory quarantine of some of our staff, after exposure to COVID19.

The specific tasks that were affected are the following:

1) Confirmatory experiments, on siblings of larvae that were able to moult to the 3rd larval instar when fed on Bt maize leaves after the F1 DC bioassay, or to the 2nd larval instar in the plant bioassay (Annex I).

These experiments were being carried out at the time of the population's confinement and the restriction of access to the CIB. Therefore they were the most affected experiments, although none of the 7 fertile oviposition cages concerned (1 from Zone 1; 4 from Zone 2; 2 from Zone 3) were left without data (either from DC or plant bioassays), as explained below:

- Diagnostic concentration (DC) confirmatory experiments were performed with the F2 larvae of 3 (1 from Zone 2; 2 from Zone 3) of the 7 egg-laying boxes that were planned

- Plant confirmatory experiments could be performed with all the seven aforementioned oviposition cages.

2) Dose-response (DR) and diagnostic-concentration (DC) bioassays with the lab strain of *S. nonagrioides:*

The number of individuals of the reference strains had to be reduced to simplify the management of the populations in order to shorten the duration of the staff's stay at the CIB. This affected the number of oviposition cages used in the bioassays with the lab strain of *S. nonagrioides*, which meant making two full replicates instead of three. Furthermore, these bioassays were conducted in June and July and not in March as it was planned.