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

Season 2018

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*.

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 2004-2017[1]. During the period 2004-2015 the plan covered the three maize-growing areas in the EU where MON 810 hybrids have been grown and *S. nonagrioides* is present: Northeast Iberia, Central Iberia and Southwest Iberia. Baselines were also gathered for other areas in Europe but no further samplings were performed in these areas because the adoption rate of Bt maize is less than 20% and monitoring resistance is not necessary according to the EuropaBio Harmonized IRM plan (EuropaBio, 2012). In Iberia, each target field

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

population was monitored every two years, but for practical reasons they were divided into two groups so that each year sampling was carried out in one of the groups. The susceptibility of *S. nonagrioides* field populations to the Cry1Ab protein expressed in MON 810 maize varieties was estimated by means of dose-response bioassays. Measured endpoints of the tests were mortality (lethal concentration, LC) and moulting inhibition (moulting inhibition concentration, MIC). From 2010 onward, it was decided to switch from LC to MIC values because it was observed that variations in the susceptibility were better reflected in MIC_{50} values than in LC_{50} values, given the characteristics of the bioassay and the biology of the species. In both cases, MIC_{50} and LC_{50} values of field populations were compared with previous baseline susceptibility data established for this species or with a susceptible laboratory strain assayed with the same batch of toxin (González-Núñez et al., 2000; Farinós et al., 2004, 2018)

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

Accordingly with these recommendations and following the revised harmonized IRM plan (EuropaBio, 2017), in the seasons 2016, 2017 and 2018 the collection of field larvae has been concentrated in the Ebro valley (including the Autonomous Communities of Aragón, Cataluña and Navarra), where the adoption rate of Bt maize is over 60%. Moreover, a

diagnostic concentration bioassay (Sims et al., 1997; Marçon et al., 2000) has been used to monitor for changes in susceptibility to the Cry1Ab protein in *S. nonagrioides* field populations from Northeast Spain. 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 2018 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. 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 nature of 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 time of the diapause may increase due to the transmission of diseases or to the emergence of

parasitoids that part of the larvae bring 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 damages in their fields. If yes, the growers are asked for permission to enter their fields during one to three days to collect larvae, which requires destructive sampling of the maize stalk. The amount of maize plants used depends on the presence of one or two species, the severity of the attack, etc.

Last-instar larvae of both corn borers were collected following standard operative procedures (SOP) of each species (EuropaBio, 2017). The samples were collected at the end of the maize-growing season, during September and October 2018, from refuges and fields of conventional maize adjacent to MON 810 maize, by cutting the stalk of the maize plants and taking only one larvae of each species per plant to avoid collecting siblings.

2.2. Insect culture

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

Larvae of *S. nonagrioides* were in diapause at the time of collection, so they were placed on a rearing chamber (Sanyo MLR-350 H, Sanyo, Osaka, Japan) at 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 4 to 10), in function of the day of adult emergence, were confined in

ventilated plastic cylinders (12 cm diameter x 30 cm high) containing 5-7 maize seedlings for oviposition at standard rearing conditions (25 ± 1ºC, 70 ± 10% relative humidity and a photoperiod of 16:8 hours (L:D). After 7 days the eggs were collected and placed into ventilated plastic boxes containing wet filter paper. The eggs were incubated under the same conditions and neonate larvae (< 24 h old) were utilized in the bioassays.

2.3. Quality of the laboratory strains

Reference susceptible strains of *S. nonagrioides* and *O. nubilalis* have been maintained under laboratory conditions to serve as control in this study. As a general rule, these populations are formed from individuals collected in non-Bt fields from Galicia, where larvae have low or no selection pressure. In the laboratory, a minimum of 300 adults are crossed every generation. Neonate larvae are collected from all the ovipositional cages formed with the adults of the previous generation, unless some cage has evidences of some disease, in which case it is removed.

Populations that are maintained for many years in the laboratory typically suffer excessive inbreeding. To preserve the vigour of the laboratory colonies of *S. nonagrioides* and *O. nubilalis* and to ensure that the populations do not collapse, they 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 that we have experienced in the past working with these species.

Bt maize has never been commercially grown in the region of Galicia (northwest of Spain), so corn borers are not subject to the selection pressure caused by Bt maize cultivation, making them a good option to be used as reference strain. Thus, a laboratory population of *O. nubilalis* coming from Galicia has been used since 2016 as a reference strain. Similarly, through 2018, different lots of larvae of *S. nonagrioides* were kindly provided by the Misión Biológica de Galicia (MBG, CSIC) in Galicia, Spain, and they are now adapted to feed on an artificial diet and to laboratory conditions (Hoffmann & Ross, 2018). The susceptibility (MIC_{50}) to the Cry1Ab toxin of this population was 19 (13-26) ng Cry1Ab/cm^2 whereas the MIC_{50} value of the previous lab strain used as reference was 24 (15-35) ng Cry1Ab/cm^2, using the same batch of toxin (B2) **(Table 6)**. Probit lines

were compared for equality and parallelism using likelihood ratio tests in PoloPlus 1.0 (LeOra Software. 2002-2018), and hypotheses were not rejected (P = 0.195 and P = 0.699, for equality and parallelism tests, respectively).

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). In the light of the results, the population from Galicia has been established as the reference population from the 2018 season onwards, and it will be refreshed periodically with new individuals collected from the same region. This will facilitate the availability of a susceptible population that has never been exposed to Bt.

2.4. Cry1Ab protein

Two batches of Cry1Ab protein have been used since the start of the MON 810 monitoring plan (2004) to the present. 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 and B2-6 in July 2018. Stock solutions were prepared from the original and kept in the freezer at -80ºC until used. Aliquots were thawed only when the bioassay was ready to be performed. To prepare the test concentrations, a sodium bicarbonate buffer (50 mmol/l) with pH 10.25 was used. The lot of Cry1Ab toxin sent in July 2018 (B2-6) has been used for the bioassays of this season.

2.5. Bioassays

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

The bioassays were carried out in accordance with the methods described by Farinós et al. (2004), using “Bio-Ba-128” plastic trays (Color-Dec Italy, Capezzano Pianore, Italy). Each tray contains 128 wells, where 0.5 ml of rearing diet is placed and flattened, corresponding to a surface of 1.77 cm^2 and a height of about 10 mm. Once solidified, 50 µl of a solution containing different concentrations of Cry1Ab were added to the surface of the diet. The controls consisted of the sodium bicarbonate buffer solution used to dilute

the toxin. After drying the wells under a laminar flow hood, one neonate larva was placed in each well using a fine paintbrush and it was covered with a breathing adhesive cover "Bio-Cv-16" (Color-Dec Italy, Capezzano Pianore, Italy). The trays were incubated in rearing chambers at 25 ± 1ºC, 70 ± 5% relative humidity and total darkness. Measured endpoint of the test in both species was moulting inhibition (moulting inhibition concentration, MIC) relative to the negative control after 7 days of exposure, where moulting inhibition equals larvae that have either died or not moulted to the second 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. Each replicate consisted of 32 larvae per concentration (64 for controls), giving a total of 96 larvae for each concentration tested (192 for controls). For each replicate neonate larvae from different oviposition cages were used. The susceptibility of the laboratory strains of *S. nonagrioides* and *O. nubilalis* to Cry1Ab was assessed using the batch B2-6 of protein. The MIC_{50} value obtained for *S. nonagrioides* was compared with those of the same population in previous years. The MIC_{50} value was determined for the third time for the new 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 2018 and on the reference laboratory strain of *S. nonagrioides,* which served as control. The methodology of the bioassay was the same as that explained above (Section 2.4.1.), with the exception that

only DC (1091 ng Cry1Ab/cm^2) and control (sodium bicarbonate buffer solution) are tested. A minimum of 1000 neonates per field population (zone) were treated, and 100 neonates per zone were used as controls. Moulting inhibition was recorded after 7 days.

To ensure that as many field collected individuals as possible were represented in the bioassay, and to calculate the detection limit for resistance allele frequency in this study, the following parameters were quantified: number of field collected larvae, number of emerged adults, number of oviposition cages settled and used in bioassays and number of neonates used in the bioassays from each oviposition cage (treated and controls). The number of egg masses used for the bioassays was maximized by doing the bioassay at a daily basis, whenever neonates of an oviposition cage emerged. This procedure forced to (i) estimate in advance the number of oviposition cages to be set, (ii) determine the approximate number of neonates to be used from each oviposition cage (treated and controls), and (iii) prepare a lot of small bioassay arenas to conduct the bioassay little by little. For all that, the DC bioassay with the F1 generation extended for about six 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.

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 after 10 days.

It was ensured that all the 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 neonates of *O. nubilalis*: 10-15 neonates per plant were fed ad libitum on maize tissue, and mortality one week later should be 100% for a plant to be used in bioassays. 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 if necessary at the end of the bioassays.

Firstly, all second-instar larvae recovered alive after 7 days in the DC bioassay were gathered, and those coming from the same oviposition cage were placed in plastic boxes of 9 cm diameter and 3 cm height. 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 a new DC bioassay and plant bioassay, as explained in sections 2.5.2. and 2.5.3.

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

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

2.6. Statistical analysis

The results of moulting inhibition of laboratory populations at different concentrations of Cry1Ab (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 average response of 50% obtained is comprised between at least 2 concentrations above it and 2 concentrations below it, from all the concentrations tested.

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

3. Results and Discussion

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

3.1. Collection of larvae

The technicians involved in the collection of field larvae for *S. nonagrioides* and *O. nubilalis* during 2018 growing season carried out about 300 hours of fieldwork, travelled over 2900 km and made in total three rounds of trips to three different field zones to collect a sufficient number of larvae for the bioassays.

A total of 1490 last-instar larvae of *S. nonagrioides* were collected between September and October 2018 from three different Zones in NE Spain (516, 553 and 421 larvae from the Zones 1, 2 and 3, respectively; **Table 3**). A map showing the sampling points for *S. nonagrioides* is displayed in **Annex IIa.** Larvae were searched in seven, five and six fields in the Zones 1, 2 and 3, respectively, although they were successfully collected in three fields in each Zone (**Figure 1, Annex IIb**). The maximum distance between successfully sampled fields was about 10, 5 and 2 Km within the Zones 1, 2 and 3, respectively (**Annex IIb**).

Larvae of *O. nubilalis* were collected between September and October 2018 from three Zones in the Northeast of Spain, yielding a total of 1144 larvae (480, 367 and 297 larvae from the 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 (seven in the Zone 1 and five in the Zones 2 and 3), larvae were mainly gathered in three fields in the Zone 1 and in two fields in the Zones 2 and 3 (**Figure 1, Annex IIIb**). The maximum distance between successfully sampled fields was about 10, 12 and 1 Km within the Zones 1, 2 and 3, 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 829 neonates by a dose-response bioassay, resulting in a MIC_{50} value of 19 (13-26) ng Cry1Ab/cm^2 (**Table 5, Figure 2a**). This value is approximately in the middle of the range of MIC_{50} values obtained since 2011 with the same batch of toxin (between 5 and 30 ng Cry1Ab/cm^2; **Table 6**).

For the Cry1Ab susceptibility assessment bioassay of the laboratory strain of *O. nubilalis,* 854 neonates were used. The MIC_{50} value obtained was 4.0 ng Cry1Ab/cm^2 (**Table 5, Figure 2b**), similar to the MIC_{50} values observed in the 2016 and 2017 seasons (5.4 and 3.9 ng Cry1Ab/cm^2, respectively) 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, such as we have observed historically during this monitoring program, are not unusual. Different reasons have been proposed, such as diverse geographical sources of individuals, varying testing personnel, different protein preparations, etc. (Robertson et al., 1995; Marçon et al., 1999; Da Silva et al., 2016 ; Farinós et al., 2018). Even so, MIC values of the control laboratory strains have been in general very consistent in the interval of years examined using the same batch of toxin (B2), being the maximum magnitude of variation 6- and 7-fold for *S. nonagrioides* (**Table 6**) and *O. nubilalis*, (**Figure 3),** respectively.

3.3. Diagnostic concentration bioassays

From the 1490 last-instar larvae of *S. nonagrioides* collected in three zones in the Ebro valley in 2018, 853 (57%), combining larvae and pupae, died in the process of rearing in the laboratory, mainly during the diapause period, and 53 adults (4%) did not emerge in the date range for oviposition cages **(Table 7).** Thus, 584 adults (39%), emerged between 9th January and 1st February 2019, were placed in 60 oviposition cages for mating. The offspring of 554 (95%) 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 2018 is 0.042 (4.2%), calculated considering the model developed by Andow and Ives (2002).

Of the total F1 neonates originated from the field collected larvae, 3449 were used in the bioassays. The DC (1091 ng Cry1Ab/cm^2) caused a mean (± S.E.) moulting inhibition of 98.65% ± 0.40% (97.85%, 99.06% and 99.05% in larvae from Zones 1, 2 and 3, respectively; **Table 8**). This value was not significantly different from either the expected value of 99% (t = - 0.8038, df = 2, p = 0.253) or the value of moulting inhibition (97.75%) caused to neonates of the laboratory strain of *S. nonagrioides* after treatment with the same DC (t = - 2.1759, df = 2, p = 0.081) (**Table 9**). Due to the fact that this year the moult inhibition data have been logit-transformed, the values of the two previous

campaigns (2016 and 2017) have been reanalyzed using this same transformation and the results evaluated again. After the reanalysis, significant differences in the moult inhibition values between the field populations compared to the laboratory population or to the expected value of 99% could be observed in 2017 **(Table 9)**. However, this did not happen in 2016 or 2018. Therefore, so far no trend has been observed in terms of changes in the susceptibility of populations from the Ebro valley to the Cry1Ab toxin.

It is noteworthy that in both the 2017 and 2018 campaigns, the percentages of moulting inhibition of *S. nonagrioides* obtained with the laboratory susceptible strain was below the expected value of 99% (97.69% and 97.75%, respectively), and only in 2016 was this value (99.20%) over the 99% (**Table 9**). Fluctuations of about 6-fold for both LC_{50} and MIC_{50} were also found in the laboratory strain during the period that monitoring was performed by means of dose-response bioassays (2004–2015), although no trends were observed over time (**Table 6**). This finding highlights the importance of maintaining a susceptible laboratory strain against which the field populations should be compared, enabling the correct interpretation of the results. Thus, MIC_{50} and LC_{50} values of field populations recorded between 2004 and 2015 were compared with the values of a susceptible laboratory strain (Farinós et al., 2018), and moulting inhibition values recorded from 2016 to present in DC bioassays are also compared with values obtained with the laboratory population in the same DC bioassay.

3.4. Larval development on MON 810 tissue: plant bioassays

10,294 F1 first-instar larvae of *S. nonagrioides*, from the populations collected in three zones in the Ebro valley in 2018, and 3,430 from the laboratory strain were fed *ad libitum* on MON 810 tissue. As a control, 543 neonates of these zones and 160 neonates of the susceptible strain were reared on conventional maize. After 10 days, no larva fed on Bt maize was able to moult to the second larval instar, regardless of its origin, whereas 90% and 97.5% of the larvae fed on conventional maize, coming from the field or from the laboratory population, respectively, had moulted to second or third larval instar **(Table 10a)**.

3.5 Confirmatory experiments

A total of 40 larvae reached the second larval instar in the DC bioassay, which were then individualized in boxes and fed on Bt leaves. Five of these larvae (from two different oviposition cages, from the Zones 1 and 2) moulted to the third larval instar, so their

siblings (about 125 per oviposition cage) were raised on an artificial diet up to the next generation (F2), with which other DC and plant bioassays were done **(Table 11)**.

In total, 128 neonates were treated with the diagnostic concentration (1091 ng/cm^2), 4 of which moulted to the second larval instar after 7 days. They were then fed on MON 810 maize, and none of them moulted to the third larval instar after 10 days. On the other hand, 1200 neonates were fed on MON 810 maize and 60 neonates, used as controls, on conventional maize. After 10 days, none of the larvae fed on Bt tissue were able to moult, whereas 57 larvae (95%) fed on conventional maize had moulted to second or third larval instar **(Table 10b)**.

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 2018 has been focused for the third time in the Ebro valley, in the Northeast (NE) of Spain, where the adoption rate of Bt maize in 2018 was over 60%. A total of 1490 last-instar larvae of *S. nonagriodes* and 1144 larvae of *O. nubilalis* were collected in three sampling zones for each species. Larvae of *O. nubilalis* were sent to the laboratory BTL GmbH Sagerheide (Germany) for testing their susceptibility to the Cry1Ab protein.

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

3. From the 1490 larvae of *S. nonagrioides* collected, 637 adults (43%) emerged, of whom 584 mated. The offspring of 95% of these adults (554) 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 the 37% 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 2018 is 0.042 (4.2%).

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

number of larvae harvested.

5. The treatment with the DC caused moulting inhibition of 98.65% (S.E. 0.40%) to F1 neonates from the field collected larvae of the NE Spain. This outcome was not significantly different from either the expected value of 99% ($t = -0.8038$, $df = 2$, $p = 0.253$) or the value of moulting inhibition (97.75%) caused to neonates of the laboratory strain of *S. nonagrioides* after treatment with the same DC ($t = -2.1759$, $df = 2$, $p = 0.081$).

6. None of the 10,294 neonates of the F1 generation of the field collected populations that were reared on MON 810 leaves was able to moult to the second larval instar.

7. The susceptible laboratory strains of *S. nonagrioides* and strain of *O. nubilalis* showed susceptibility levels to the batch B2-6 of the Cry1Ab toxin (MIC_{50} values of 19 and 4 ng Cry1Ab/cm^2, respectively) comparable with those obtained for laboratory strains in previous years.

5. Concluding remarks

In the last three seasons, a considerable increase in effort in the collection of a rising number of last-instar larvae of *S. nonagrioides* over time 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%.

The moulting inhibition (98.65%) caused to F1 neonates of *S. nonagrioides* from larvae collected in the Ebro valley in 2018 after treatment at a diagnostic concentration (DC) was not significantly lower than either the hypothetical value of 99% or the value of moulting inhibition caused to neonates of a laboratory strain using the same DC (97.75%).

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.

6. References

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

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

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

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

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

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

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

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

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms) 2017. Scientific Opinion on the annual post-market environmental monitoring (PMEM) report on the cultivation of genetically modified maize MON 810 in 2015 from Monsanto Europe S.A. EFSA Journal 2017;15(5):4805, 27 pp. https://doi.org/10.2903/j.efsa.2017.4805

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

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

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

EuropaBio. 2012. Harmonised insect resistance management (IRM) plan for cultivation of Bt maize (single insecticidal traits) in the EU. https://ec.europa.eu/food/sites/food/files/plant/docs/gmo_rep-stud_mon-810_report-2015_app-06.pdf (accessed 13 July 2017).

EuropaBio. 2017. Harmonised insect resistance management (IRM) plan for cultivation of Bt maize (single insecticidal traits) in the EU. https://ec.europa.eu/food/sites/food/files/plant/docs/gmo_rep-stud_mon-810_report-2017_app-06.pdf

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

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

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

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

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

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

LeOra Software. 2002-2018. *Polo Plus 1.0 Probit and Logit analysis*. LeOra Software, Petaluma, California.

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

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

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

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

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

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

7. Tables and figures

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

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

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

Components	Amount	Provider
Distilled H_2O	1 l	
Agar	24 g	Conda Pronadisa
Maize flour	168 g	Santiveri
Wheat germ	42 g	Santiveri
Yeast	45 g	Santiveri
Ascorbic acid	9 g	Panreac
Benzoic acid	3 g	Merck Millipore
Nipagin (Methyl p-hydroxybenzoate)	1.5 g	Sigma-Aldrich
Sorbic acid	1.2 g	Sigma-Aldrich

Table 3. *Sesamia nonagrioides* larvae collection details for the 2018 season in the Ebro valley (NE Spain)

Zone	Field	Province [a]	Postal Code	Date	Surface (Ha) [b]	Distance to the nearest MON810 field (m) [d]	No of larvae collected
1	2018-Lanaja 1	HU	22250	25-Sep-18	41.9	5	172
	2018-Lanaja 2	HU	22250	25-Sep-18	15.9	2500	0
	2018-Lanaja 3	HU	22250	26-Sep-18	7.7	500	169
	2018-Lanaja 4	HU	22250	25-Sep-18	10	0	0
	2018-Lanaja 5	HU	22250	25-Sep-18	7.1	-	0
	2018-Cantalobos	HU	22251	25-Sep-18	8.6	20	175
	2018-San Juan de Flumen	HU	22213	26-Sep-18	6.4	300	0
	Total						**516**
2	2018-Peñalba	HU	22592	18-Sep-18	31.8	0	0
	2018-Candasnos 1	HU	22591	19-Sep-18	6	600	206
	2018-Candasnos 2	HU	22591	18-Sep-18	6.8	0	181
	2018-Candasnos 3	HU	22591	18-Sep-18	9.9	2500	166
	2018-Candasnos 4	HU	22591	18-Sep-18	20.3	1200	0
	Total						**553**
3	2018-Mendigorria 1	NA	31150	16-Oct-18	17.8	0	0
	2018-Mendigorria 2	NA	31150	16-Oct-18	5.1	0	76
	2018-Mendigorria 3	NA	31150	16-Oct-18	0.9 [c]	0	44
	2018-Mendigorria 4	NA	31150	16-Oct-18	3.5 [c]	0	104
	2018-Mendigorria 5	NA	31150	16-Oct-18	1.5 [c]	0	197
	2018-Mendigorria 6	NA	31150	16-Oct-18	1.6 [c]	0	0
	Total						**421**
	GRAND TOTAL						**1490**

[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 2018 season in the Ebro valley (NE Spain)

Zone	Field	Province [a]	Postal Code	Date	Surface (Ha) [b]	Distance to the nearest MON810 field (m) [c]	No of larvae collected
1	2018-Lanaja 1	HU	22250	25-Sep-18	41.9	5	20
	2018-Lanaja 2	HU	22250	25-Sep-18	15.9	2500	0
	2018-Lanaja 3	HU	22250	26-Sep-18	7.7	500	130
	2018-Lanaja 4	HU	22250	25-Sep-18	10	0	0
	2018-Lanaja 5	HU	22250	25-Sep-18	7.1	n.i.	0
	2018-Cantalobos	HU	22251	25-Sep-18	8.6	20	121
	2018-San Juan de Flumen	HU	22213	26-Sep-18	6.4	300	209
	Total						**480**
2	2018-Peñalba	HU	22592	18-Sep-18	31.84	0	143
	2018-Candasnos 1	HU	22591	19-Sep-18	6	600	214
	2018-Candasnos 2	HU	22591	18-Sep-18	6.8	0	0
	2018-Candasnos 3	HU	22591	18-Sep-18	9.9	2500	10
	2018-Candasnos 4	HU	22591	18-Sep-18	20.3	1200	0
	Total						**367**
3	Aibar 1	NA	31460	17-Oct-18	14	n.i.	11
	Aibar 2	NA	31460	17-Oct-18	10	n.i.	12
	Aibar 3	NA	31460	17-Oct-18	6	n.i.	0
	Sangüesa 1	NA	31400	17-Oct-18	0.5	n.i.	159
	Sangüesa 2	NA	31400	17-Oct-18	1.1	n.i.	115
	Total						**297**
	GRAND TOTAL						**1144**

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

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

Species	Toxin batch	n	Slope ± SE	X^2	d.f.	MIC_{50}[a] (FL 95%)	MIC_{90}[a] (FL 95%)
S. nonagrioides	B2-6	829	1.6 ± 0.1	42.2	19	19 (13-26)	116 (76-224)
O. nubilalis	B2-6	854	1.7 ± 0.1	22.1	19	4.0 (3.2-5.0)	17.5 (13.7-24.0)

[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 2018. 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)

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

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

Fields	Field larvae collected	Dead larvae and pupae	Adults out of the date range for oviposition cages[a]
Zone 1	516	318 (62%)	27 (5%)
Zone 2	553	369 (67%)	17 (3%)
Zone 3	421	166 (39%)	9 (2%)
Total	1490	853 (57%)	53 (4%)

[a] Adults that did not emerge between 9th January 1st February 2019.

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	516	171 (33%)	19	17	68-85	153 (30%) (89%)	1120	98.13	142	12.68	97.85
	Zone 2	553	167 (30%)	20	18	69-91	160 (29%) (96%)	1148	99.22	143	16.78	99.06
	Zone 3	421	246 (58%)	21	20	108-133	241 (57%) (98%)	1181	99.15	143	11.19	99.05
	All zones[g]	1490	584 (39%)	60	55	245-309	554 (37%) (95%)	3449	98.84	428	13.55	98.66
Laboratory	-	-	319	20	13	70-124	194	924	98.05	163	13.50	97.75

[a] Adults emerged between 9th January and 1st February 2019. Those emerged before or after these dates were discarded. 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, the percentages with respect to the number of collected larvae and with respect to the number of emerged adults are in brackets (in this order).
[e] MI, moulting inhibition: larvae that have not reached the second larval instar
[f] Calculated using Abbot's formula (Abbot, 1925).
[g] Results obtained pooling the data of the three zones.

Table 9. Moulting inhibition 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

[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 and F2 generations of *S. nonagrioides* after 10 days feeding on Bt (MON 810) or not-Bt (conventional) maize tissue.

10a. Growth of larvae of the F1 generation in plant bioassays.

	Zone 1		Zone 2		Zone 3		Laboratory	
	MON 810	Conventional	MON 810	Conventional	MON 810	Conventional	MON 810	Conventional
Nº of F0 oviposition cages used[a]	17	17	19	18	20	20	18	16
Nº of F1 neonates exposed[b]	3187	164	3427	185	3680	194	3430	160
Nº of moulted larvae (≥ L2)	0	145	0	162	0	182	0	156
% moulting (≥ L2)	0.00	88.41	0.00	87.57	0.00	93.81	0.00	97.50

10b. Growth of larvae of the F2 generation in confirmatory bioassays.

	Zone 1		Zone 2	
	MON 810	Conventional	MON 810	Conventional
Nº of F1 oviposition cages used	5	5	2	1
Nº of F2 neonates exposed[b]	850	50	350	10
Nº of moulted larvae (≥ L2)	0	48	0	9
% moulting (≥ L2)	0.00	96.00	0.00	90.00

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

Table 11. Larvae that were able to moult to the second larval instar (L2) in the DC bioassay and then molted 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] (%)
Northeast Spain	Zone 1	1120	21 (1.88)	3 (0.27)
	Zone 2	1148	9 (0.78)	2 (0.17)
	Zone 3	1181	10 (0.85)	0 (0.00)
	All zones	3449	40 (1.16)	5 (0.14)
Laboratory	-	924	18 (1.95)	0 (0.00)

[a] Number of larvae that moulted to L2 in the DC bioassay, and then fed on MON 810 maize.
[b] Number of larvae that moulted to L3 after feeding on MON 810. There were no L4 survivors.

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 2018. A collection at a field within a zone was considered successful if at least 50 larvae were gathered.

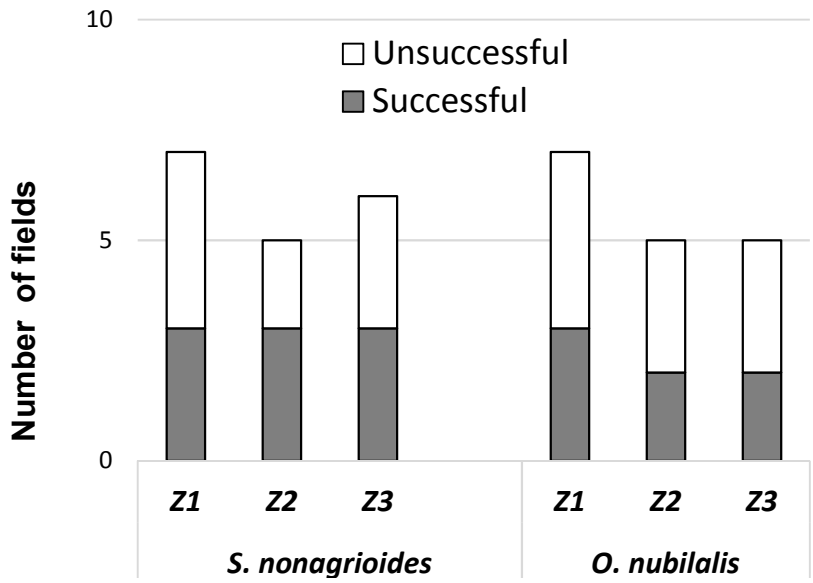


Figure 2. Fitted curves of susceptibility to the toxin Cry1Ab of the laboratory populations of *S. nonagrioides* and *O. nubilalis* (PoloPlus 1.0, LeOra Software 2002-2018). Response is moulting inhibition after seven days feeding on treated diet. **a:** *S. nonagrioides*. **b:** *O. nubilalis*.

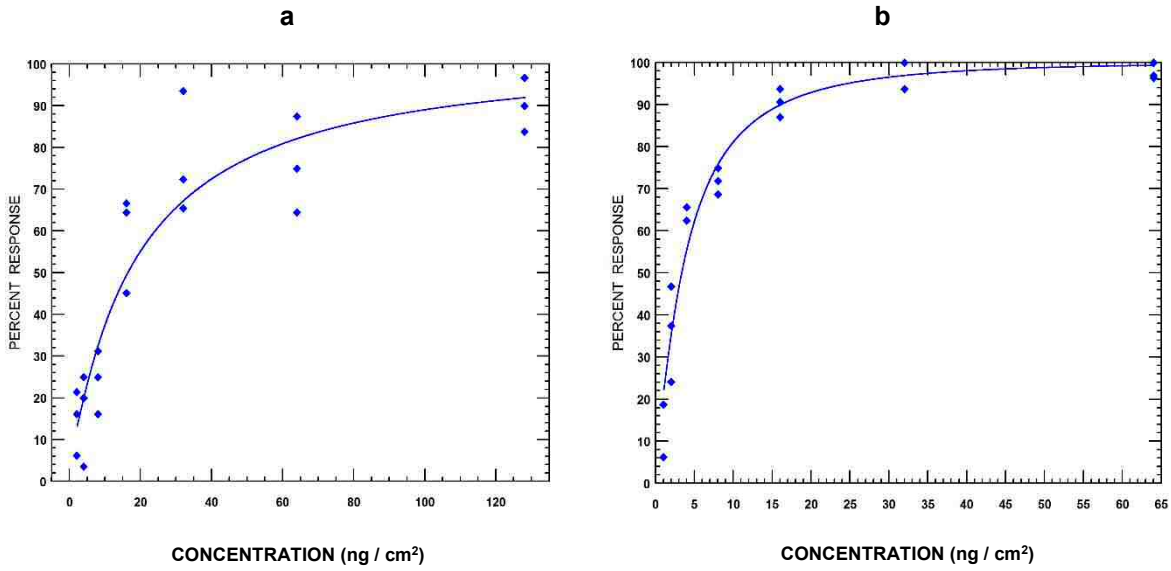
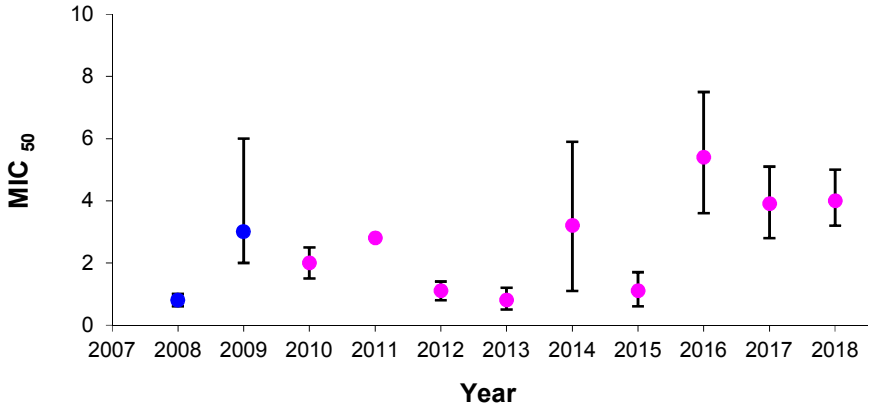
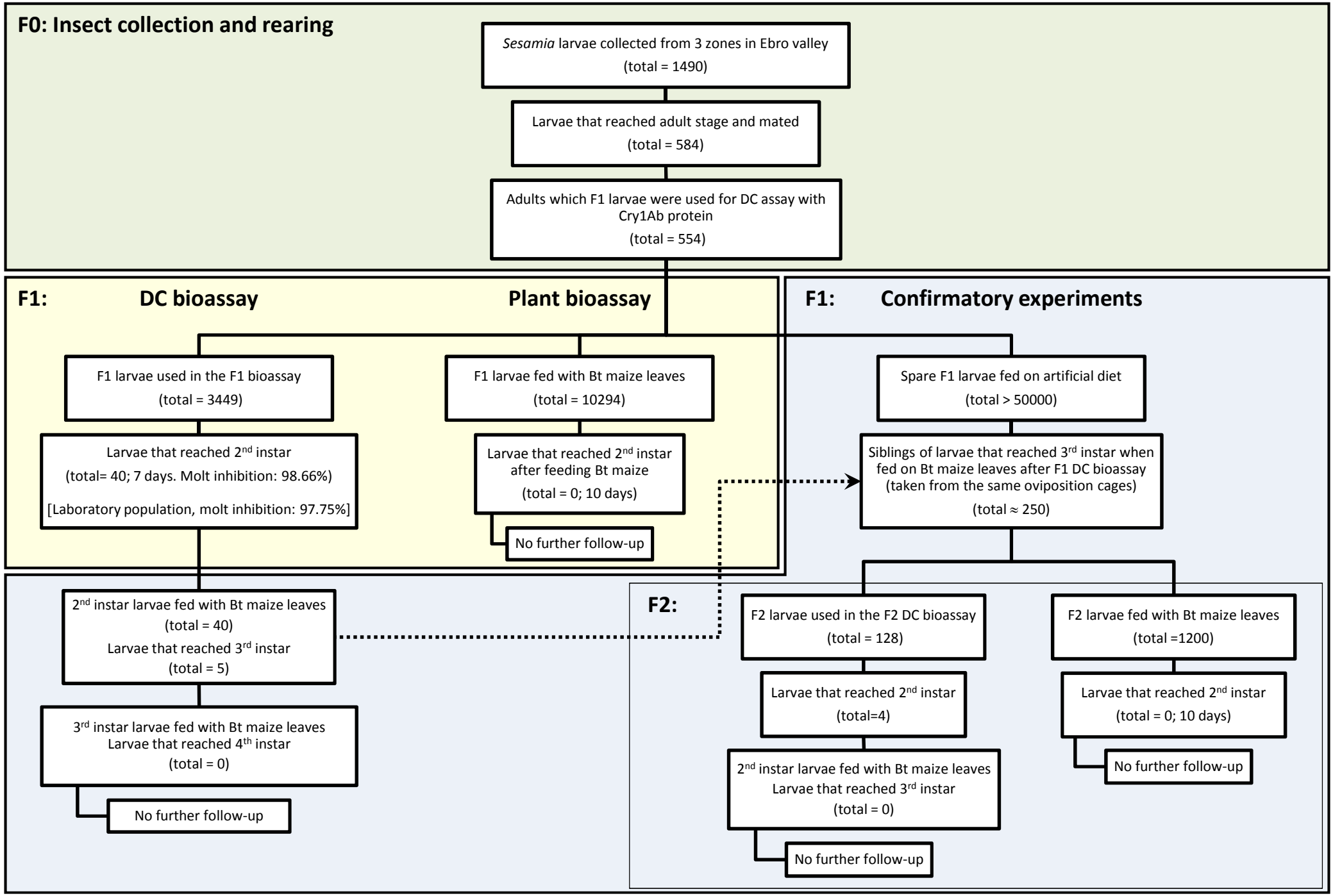


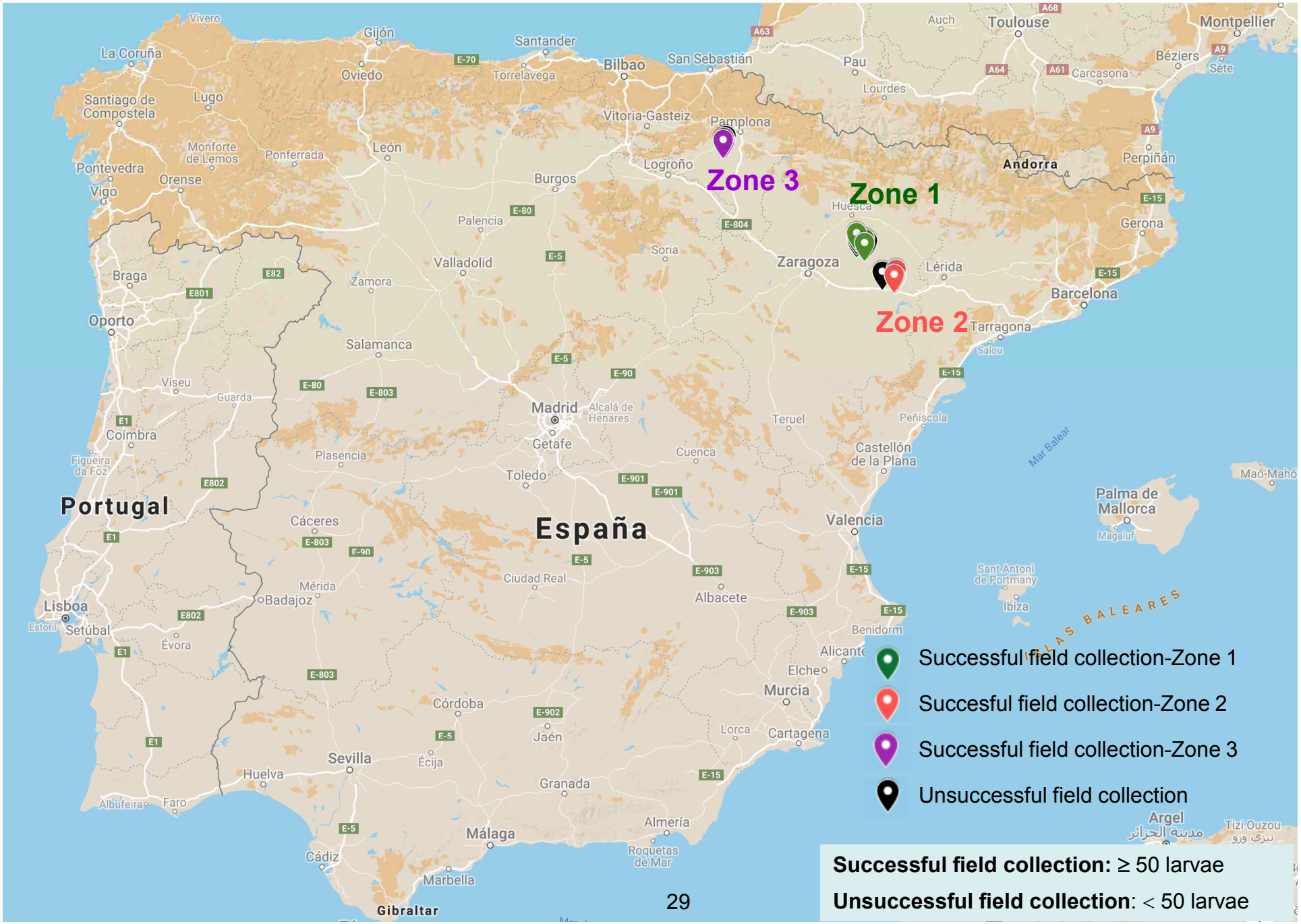
Figure 3. Susceptibility to Cry1Ab toxin measured by MIC_{50} values of a laboratory population of *O. nubilalis.* Colors indicate the B1 (blue) and B2 (pink) toxin batches.



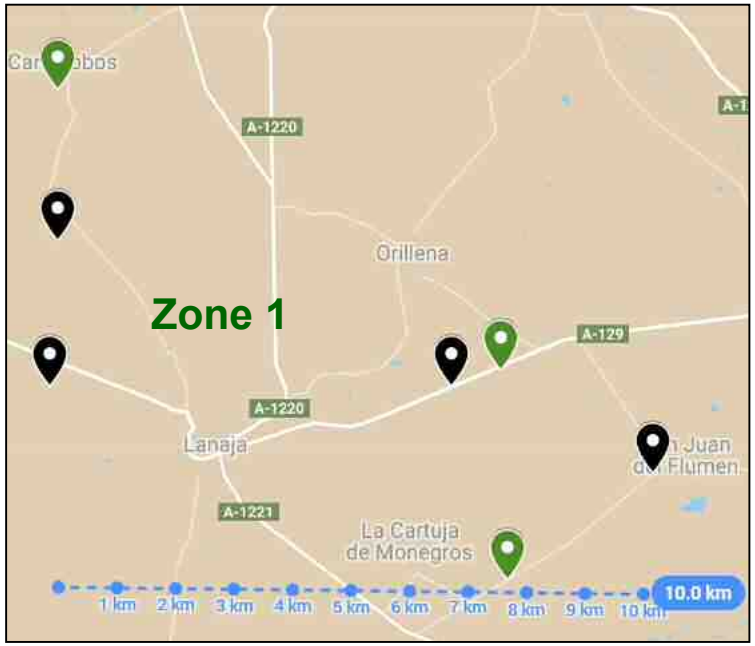
Annex I. Stepwise approach followed to do the bioassays



ANNEX IIa. Collection of *S. nonagrioides* larvae in the Ebro valley in 2018



ANNEX IIb. Collection of *S. nonagrioides* larvae in the Ebro valley in 2018



- Successful field collection-Zone 1
- Succesful field collection-Zone 2
- Successful field collection-Zone 3
- Unsuccessful field collection

ANNEX IIIa. Collection of *O. nubilalis* larvae in the Ebro valley in 2018

ANNEX IIIb. Collection of *O. nubilalis* larvae in the Ebro valley in 2018

- Successful field collection-Zone 1
- Succesful field collection-Zone 2
- Successful field collection-Zone 3
- Unsuccessful field collection