Monitoring of *Sesamia nonagrioides* resistance to MON 810 maize in the European Union: lessons from a long-term harmonized plan

Short running title:

Monitoring of *S. nonagrioides* resistance to MON 810 maize in the EU

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Abstract

BACKGROUND: MON 810 maize, which expresses the insecticidal protein Cry1Ab, is a highly effective method to control *Sesamia nonagrioides* (Lefèbvre), a key maize pest in Mediterranean countries. Monitoring programs to assess the potential development of resistance of target pests to Bt maize are mandatory in the European Union (EU). Here we report the results of the *S. nonagrioides* resistance monitoring plan implemented for MON 810 maize in the EU between 2004–2015 and reassess the different components of this long-term harmonized plan.

RESULTS: No major shifts in the susceptibility of *S. nonagrioides* to the Cry1Ab protein have occurred over time. The reassessment of this long-term program has identified some practical and technical constraints, allowing us to provide specific recommendations for improvement: (1) use reference strains instead of susceptibility baselines as comparators for field-collected populations; (2) shift from dose-response bioassays to diagnostic concentrations; and (3) focus monitoring on areas with high adoption rates, such as the Ebro basin in Spain.

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CONCLUSION: There are no signs of field resistance of *S. nonagrioides* to the Cry1Ab protein of MON 810 maize. Specific recommendations for improvement are provided, based on the knowledge and experience accumulated through the implementation of this unique EU-wide harmonized plan.

1 INTRODUCTION

MON 810 maize, which expresses the insecticidal protein Cry1Ab, was introduced into the European agricultural landscape in 2003. It targets two key pests: the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), which is present all over Europe, and the Mediterranean corn borer, *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera: Noctuidae), which is restricted to the Mediterranean area. For three years MON 810 hybrids shared the market with maize hybrids derived from Event 176 (Cry1Ab protein, cv. Compa CB, Syngenta), available in the EU from 1998 to 2005, but since 2006 MON 810 has been the only insect-resistant transgenic maize approved for cultivation in the EU. The adoption rates of MON 810 maize across Europe have been unequal, due in part to the different positions of the EU Member States on the release of genetically modified crops into the environment.[1–3] To date, eight EU countries have ever cultivated MON 810 hybrids (Fig. 1A). However, Spain is the only country where MON 810 hybrids have been commercialized continuously on a large scale, and since 2010 Spain has accounted for over 80% of the total MON 810 growing area in the EU (116,867 ha in 2016) (Fig. 1B). The majority of Bt maize is concentrated in three maize-growing areas, with the highest adoption rate in the Ebro basin in Northeast Spain (Fig. 2) where, since 2007, more than 60% of the maize hybrids cultivated have been MON 810 to prevent recurring damage by *S. nonagrioides.* This noctuid can complete a variable number of generations per year,[4,5] but larvae of the first generation are the most harmful to maize because they tunnel throughout the stem during the larval stage, damaging maize seedlings.

The widespread cultivation of Bt varieties and the prolonged exposure to Bt proteins represent a strong selection pressure for resistance in target pests.[6,7] Up to now, two noctuid moths, *Spodoptera frugiperda* (J. E. Smith) and *Busseola fusca* (Fuller), and one chrysomelid beetle, *Diabrotica virgifera virgifera* Le Conte, have evolved resistance in the field to Bt maize that expresses Cry proteins.[8–10] As a consequence, the Bt crops that caused the selection pressure have decreased efficacy for controlling the target pests [11,12] and in some areas they are no longer cultivated.[13] Interestingly, other Bt-crops with the same target species and the same protein or other with the same mode of action can also be potentially affected, being appropriate for resistance management the use of other Bt traits that are not affected by protein-specific resistance.

Insect resistance management (IRM) strategies are crucial to reduce the selection pressure and to delay the evolution of resistance of target pests to Bt crops.[14,15] The IRM strategy

applied for MON 810 maize in the EU is called the high-dose/refuge strategy, whose appropriate implementation has proved to be vital in maintaining the susceptibility of *S. nonagrioides* to the insecticidal protein and preserving the durability of this technology.[16] The level of expression of Cry1Ab in MON 810 plants most probably represents a high dose for *S. nonagrioides* since no larvae were found in MON 810 stems after many years of field sampling.[16,17] In addition, the compliance of Spanish farmers with refuge requirements has been higher than 60% since 2006, and over 80% since 2009.[16,18] Refuges are commonly deployed as blocks within Bt fields or adjacent to them. In contrast, the mixtures of Bt maize seeds with conventional maize seeds ("refuge in the bag") used in other areas, such as the US Corn Belt or Canada, are not recommended in the EU. The main reasons are the stimulation of *S. nonagrioides* larval dispersal by the Bt trait and the dispersal capacity of adults,[19] along with the fact that only single-trait Bt plants are cultivated in the EU, which might favour selection for resistance.[20]

A main element in IRM plans is the monitoring for target pests' susceptibility to the Bt proteins in areas with high adoption rates, to detect, in a timely manner, any resistance that evolves.[21–24] For MON 810 in the EU, EFSA defined a sampling zone of high Bt-maize adoption as a zone where MON 810 occupies more than 50% of the total maize cultivation for at least three consecutive years. [47] It is commonly accepted that effective monitoring programs should have well-established baseline susceptibility data, determined before or shortly after the Bt crop has been established in the field, to assess later shifts in susceptibility.[25–28] Baselines are also essential to establish the natural variability of pest populations in the distinct geographical areas to be assessed. In the EU, baselines of susceptibility to the Cry1Ab protein have been established for both *S. nonagrioides* and *O. nubilalis*.[17,25,29,30] Thereafter, the use of appropriate bioassays would enable estimation of changes in the susceptibility of the insects to the Cry proteins with respect to the baseline. The most commonly used approaches are dose-response bioassays, which measure changes in susceptibility at the population level, and the application of diagnostic concentrations, which allow discriminating between resistant and susceptible insects.[31–33] However, the use of baseline susceptibility can be undermined in long-term IRM programs by the unavailability of a single stable and effective batch and formulation of toxin to assure optimal performance over time.[30,33,34]

Insect resistance monitoring plans for Bt maize are mandatory in the EU (Directive 2001/18/EC[35]; Regulation (EC) 1829/2003[36]). Thus, monitoring programmes focussed on *S.*

nonagrioides and *O. nubilalis* have been conducted at the national level in Spain [16,17,34] and Germany.[30] The only long-term, EU-wide, harmonized IRM plan was proposed by the European Association for Bioindustries (EuropaBio) following the directives of the working group on IRM and submitted to the Competent Authorities of the Member States and the European Commission.[37,38] The purpose of this harmonized plan is to develop and use common methodology to monitor the potential development of resistance of *O. nubilalis* and *S. nonagrioides* to Cry1Ab following the cultivation of Bt maize varieties. The plan was implemented in 2003 and has been in place since then.

We report here the results of the *S. nonagrioides* resistance monitoring plan implemented for MON 810 maize cultivation in the EU during the period 2004–2015, based on the EuropaBio Harmonised IRM plan. The plan covers the four maize-growing areas in the EU where MON 810 hybrids have been grown and *S. nonagrioides* is present. The overview of this long-term harmonized programme has provided insights into its practical and technical constraints, allowing us to provide specific guidelines/recommendations for its enhancement and improvement. This information is crucial to the sustainability of long-term resistance monitoring plans, which will result in durability of this Bt maize technology.

2 MATERIALS AND METHODS

2.1 Insect sampling and rearing

Field samplings of *S. nonagrioides* populations were carried out in the three main areas in the EU where MON 810 has been cultivated from 2004 to 2015: Northeast Spain (NE-Ib), including areas of Catalonia, Aragon and Navarra that fall within the Ebro basin; Central-East Spain (C-Ib), particularly the province of Albacete; and Southwest Iberia (SW-Ib), comprising Extremadura and Western Andalusia in Spain and Southern Portugal (Fig. 3 and Supporting Information). Each area was monitored at least once every two years. Additionally, field populations of *S. nonagrioides* were collected from Southwest France (SW-Fr) in Midi-Pyrénées and Poitout-Charentes in the period 2005–2007 (Fig. 3 and Supporting Information). No further samplings were performed in this area because MON 810 maize cultivation was disallowed in France from 2007 onwards.

Larvae were collected in each area from a minimum of three fields per season. The fields were separated by at least 50 km except in the C-Ib area because the maize crops there were

concentrated in a relatively small zone. Samplings were carried out in refuges and fields of nontransgenic maize adjacent to Bt maize fields (MON 810 maize or Event 176 maize in 2004 and 2005, and MON 810 maize in all other years). The fields were previously selected by field technicians, based on evidence of damage caused by corn borers (after visual inspection or enquiries to growers). After checking that the observable damage was caused by *S. nonagrioides*, a minimum of 100 last-instar larvae were collected per field, resulting in at least 300 larvae per area per season. The larvae were gathered from plants just before maize harvesting (September to November, depending on the area and weather each year), by cutting the maize stalk. Only one larva per plant was taken to avoid gathering siblings. If the minimum number of larvae was not reached within a reasonable time, the search in that field was discontinued and a new field was searched in the vicinity. These larvae were kept and, if needed, they were later combined with those collected in the nearest field (spaced by no more than 5 Km).

In the laboratory the larvae were dipped in a solution containing 1% bleach, to avoid contamination by pathogens, and placed in 21×16×4 cm plastic boxes containing corn-based artificial diet modified from Poitout and Bùes[39] by the addition of 1.6 g of Wesson's salt mixture and 1 g methyl *p*-hydroxybenzoate (Sigma-Aldrich Co., St. Louis, MO, USA). Most of the larvae were in diapause or entered diapause after reaching the last larval instar when they were placed in a rearing chamber (Sanyo MLR-350 H, Sanyo, Osaka, Japan) at 15 ± 1 °C, 70 ± 10% relative humidity and a photoperiod of 12:12 hours of light:dark (L:D). Those that were not in diapause were reared on artificial diet until pupation under the standard conditions: 25 ± 1 °C, 70 ± 10% relative humidity and a photoperiod of 16:8 hours (L:D). When needed, diapause was interrupted by placing the larvae under conditions of 28 ± 1 °C, 70 ± 5% relative humidity and continuous light. Larvae pupated and the process continued in an insectarium under standard conditions. Pupae were sexed and 5 to 10 couples were placed in each of the oviposition cages, which consisted of ventilated methacrylate cylinders (30 cm high × 12 cm diameter) covering a pot with 5–8 maize plantlets. The males in each oviposition cage originated from the same location within an area, whereas the females originated from different locations within that area. After 7 days, eggs laid on the plants were collected and placed into ventilated plastic boxes containing a wet filter paper. The eggs were incubated under standard conditions. Neonate larvae (<24 h old) that were not allowed to feed were selected for the bioassays.

2.2 Reference strain of *S. nonagrioides*

A reference susceptible strain of *S. nonagrioides* that served as control in this study was established with individuals collected from different areas in Spain in 1998 (see González-Núñez *et al.*[29] for details). Every one to three years, the reference strain was refreshed by the addition of field-collected individuals to avoid the negative consequences of inbreeding. The field-collected individuals always originated from Cry1Ab-susceptible populations collected in non-Bt fields, with levels of susceptibility similar to that of the existing strain (verified by dose-response bioassays; see Section 2.4). To avoid introducing pathogens, field individuals incorporated into the reference strain were maintained in the laboratory for two to three generations and discarded if they presented signs of disease.

2.3 Cry1Ab protein

Two batches of Cry1Ab protein were used in the bioassays. The first batch (B1) was provided by Monsanto (St. Louis, MO, USA) in 2003 (concentration 2.03 mg/ml in sodium bicarbonate buffer, pH 10.5; purity 95%) and stored at −20 °C. Test concentrations were prepared in sodium bicarbonate buffer (50 mmol/l), pH 10.5. As a reduction in toxicity was observed in 2010 in bioassays with the reference strain, a new batch of protein was required.

The second batch (B2) was sent by Monsanto in 2011 (B2-1) and in 2014 (B2-2), at a concentration of 1.8 mg/ml in 50 mM sodium bicarbonate buffer, pH 10.25, and purity 91%. Aliquots at a lower concentration were prepared and kept in a freezer at −80 °C until use. The test concentrations were prepared in sodium bicarbonate buffer (50 mmol/l), pH 10.25.

2.4 Bioassays

2.4.1 Susceptibility to Cry1Ab in dose-response bioassays

The susceptibility to the Cry1Ab protein was tested by bioassays on the F1 progenies obtained from field-collected parents. The bioassays were carried out in accordance with the methods described by Farinós *et al.*,[17] using BAW128 plastic trays (Frontier Agricultural Sciences, Newark, DE, USA). Each tray contained 128 wells, in which 0.5 ml of rearing diet was placed and flattened. Once solidified, 50 µl of a solution containing different concentrations of Cry1Ab or sodium bicarbonate buffer for controls were applied to the surface of the diet. After drying the wells under a laminar flow hood, one neonate larva was

placed in each well using a fine paintbrush and the wells were covered with a breathing adhesive lid (BACV16; Frontier Agricultural Sciences). The trays were incubated in rearing chambers at 25 ± 1 °C, 70 ± 5% relative humidity and continuous darkness. Mortality and moulting inhibition were determined after 7 days of exposure, where mortality denoted larvae not showing any reaction when prodded and moulting inhibition denoted larvae that had either died or not moulted to the second instar.

To determine the susceptibility of each population, 7 to 10 different concentrations between 0.75 and 640 ng Cry1Ab/cm^2 were used. Three replicates were done for each concentration and the control. Each replicate consisted of 32 larvae per concentration (64 for controls), making a total of 96 larvae for each concentration tested (192 for controls). Neonate larvae from different oviposition cages were selected for each replicate. For a replicate to be included in the statistical analysis, the control mortality had to be less than 25%. A bioassay was considered valid if, from all the concentrations tested, there were 2 concentrations above and below the obtained average response of 50% in mortality or moulting inhibition (LC_{50} or MIC_{50}).

Mortality and growth inhibition data from dose-response bioassays were analyzed by probit analysis. The lethal concentrations (LC) and moulting inhibition concentrations (MIC) causing a response in 50% (LC_{50}, MIC_{50}) and 90% (LC_{90}, MIC_{90}) of each population were estimated together with their 95% confidence intervals (CI) using the POLO-PC program,[40] which automatically corrects for natural mortality (mortality in controls).

The susceptibility (MIC_{50}) of the reference strain of *S. nonagrioides* to Cry1Ab served as the control against which field populations were compared each year. MIC values of populations collected in 2005 and 2006 were compared to the values of the reference strain in 2004, and those of the population collected in 2009 were compared with the value estimated for the reference strain in 2008. The variability in susceptibility of the reference strain was determined by comparing LC_{50} and MIC_{50} values estimated every year with the values measured for the first time in 2004.

2.4.2 Diagnostic concentration

A diagnostic concentration (DC), defined here as the level causing 99% moulting inhibition of first-instar larvae (MIC_{99}), was estimated using probit analysis by pooling the results obtained in the dose-response bioassays performed with the field populations of *S. nonagrioides* collected in NE-Ib (2009 and 2011), C-Ib (2008, 2010 and 2012) and SW-Ib

(2010 and 2012). The validity of this concentration was tested in bioassays with the field populations collected in 2013, 2014 and 2015. For each population and year, three replicates of 32 neonates each (96 larvae) were tested at the diagnostic concentration following the same protocol and under the same conditions as in the case of dose-response bioassays, and moult inhibition was recorded after 7 days.

2.4.3 Larval survival on MON 810 tissue

A high number of larvae were screened from 2011 to 2015 by exposure to MON 810 maize tissue to determine if there were any resistant individuals in the field-collected populations. Approximately 1500 leftover neonates per population per year, generated from field collections and not used in bioassays, were exposed to MON 810 leaves. They were siblings of those used in the bioassays and they were not allowed to feed prior to the screen. Groups of ~100 larvae were transferred to plastic boxes provided with fresh MON 810 maize leaves without the central nerve and allowed to feed *ad libitum*. The leaves were replaced every 2 days if necessary. Survival was recorded 10 days later. Likewise, all surviving larvae from the dose-response bioassays (mostly first and second larval instar, but also some third instar larvae were found at the lowest concentrations tested) were exposed to MON 810 leaves. MON 810 maize was grown in the greenhouse and leaf material from plant growth stages V5–V8 was used in the experiment. The presence of the Cry1Ab protein in the plants was tested by ImmunoStrip® for Bt-Cry1Ab/1Ac (Agdia Inc, Elkhart, IN, USA).

2.5 Statistical analysis

The significance of changes in susceptibility in the field population was tested by the 95% confidence limits of lethal concentration ratios (LCR) or moult inhibition concentration ratios (MICR) at the LC_{50} or MIC_{50}, respectively.[41]

Moult inhibition means, obtained after treatment at the diagnostic concentration (MIC_{99}), were tested against the expected value of 99% using a one-sample *t*-test and a one-tailed probability distribution (IBM SPSS Statistics 23). Mean values were corrected with Abbott's formula [42] prior to analysis.

3 RESULTS

3.1 Insect collection

More than 12,000 larvae of *S. nonagrioides* were collected from 2004 to 2015 from the four areas monitored: 3605 (7 samplings) from NE-Ib, 2632 (6 samplings) from C-Ib, 2724 (7 samplings) from SW-Ib and 3336 (3 samplings) from SW-Fr (Supplementary Information). The efficiency in the collection of larvae varied among sampling areas and years. Collections were, in general, more difficult to accomplish in SW-Ib, where a higher number of fields were visited to obtain the minimum number of larvae required per field (n=100) (Fig. 4). The mean percentages of success in gathering larvae from a field per area per season were 80 ± 10%, 48 ± 10% and 31 ± 15 % for NE-Ib, C-Ib and SW-Ib, respectively. On average, the number of larvae collected per area per season was 515 ± 44, 439 ± 46 and 389 ± 122 in the NE-Ib, C-Ib and SW-Ib populations, respectively. Considering the low number of larvae captured in SW-Ib in 2004 and 2009 (83 and 59, respectively; Supplementary Information), we excluded these field populations from the analysis of susceptibility to the Cry1Ab protein.

3.2 Susceptibility of *S. nonagrioides* to the Cry1Ab protein

3.2.1 Susceptibility of the reference strain

The susceptibility of the reference strain of *S. nonagrioides* was evaluated by larval mortality and moult inhibition in different years and using different Cry1Ab batches (Table 1). LC_{50} values ranged between 12 and 69 ng Cry1Ab/cm^2 and LC_{90} values between 93 and 565 ng Cry1Ab/cm^2; in both cases, the magnitude of variation was 6-fold (Fig. 5). MIC_{50} values of the laboratory strain ranged between 5 and 28 ng Cry1Ab/cm^2, with a magnitude of variation of 6-fold. This variation was twice that obtained with MIC_{90} values (3-fold), which ranged between 42 and 120 ng Cry1Ab/cm^2. A bridging experiment confirmed that there were no significant differences in the toxicity of B2-1 and B2-2 when tested against the laboratory strain (Table 1, season 2013), as indicated by the resistance ratio at the MIC_{50} level (0.8, with a 95% confidence interval of 0.5–1.1).

Concentration ratios (LCR and MICR at the LC_{50} or MIC_{50} level, respectively) were calculated every year with respect to the first value measured (in 2004). The maximum difference in LCR value was observed in 2008 (3.1-fold), and for MICR in 2015 (1.5-fold). However, in neither case was a trend observed over time (Table 2).

3.2.2 Susceptibility of field populations

Values of LC and MIC of *S. nonagrioides* populations assessed between 2004 and 2015 are detailed in Table 1. LC_{50} values ranged between 19 and 482 ng Cry1Ab/cm^2 and MIC_{50} values ranged between 7 and 63 ng Cry1Ab/cm^2. In general, the magnitude of variation of MIC_{50} values was equal to or lower than that of LC_{50} values: 7- and 21-fold in NE-Ib, 3- and 5-fold in SW-Ib, and 2- and 2-fold in SW-Fr, respectively (Fig. 5). The only exception was C-Ib, with ranges of variation of 4- and 3-fold for MIC_{50} and LC_{50} values, respectively (Fig. 5). Likewise, the magnitude of variation of MIC_{90} values of populations within an area was in all cases equal to or lower than that of the corresponding LC_{90} values (Table 1). The higher heterogeneity of LC values in this species occurs because some of the larvae exposed to the Cry1Ab protein are able to survive to the end of the 7-day assay, though they do not moult and they are not expected to survive under natural field conditions. Accordingly, MIC values were used to assess shifts in the susceptibility of field populations to the Cry1Ab protein over time with respect to the susceptible reference strain, estimated by variations in the MICR at the MIC_{50} level (Fig. 6). The ratios ranged between 0.6 and 3.5-fold in NE-Ib, 0.4 and 2.6 in C-Ib, 0.6 and 2.6 in SW-Ib, and 0.9 and 2.2-fold in SW-Fr, with no clear shifts from 2004 to 2015.

3.2.3 Diagnostic concentration

The diagnostic concentration (DC) of Cry1Ab estimated for *S. nonagrioides* was 726 ng Cry1Ab/cm^2, with a 95% confidence interval of 548–1013. This value (MIC_{99}) represents the response of 6,646 neonates derived from larvae collected in maize fields from 2008 to 2012 in different locations of NE-Ib (2009 and 2011), C-Ib (2008, 2010 and 2012) and SW-Ib (2010 and 2012). After estimation of this DC, its validity was tested in the field populations collected in 2013, 2014 and 2015. The moult inhibition values (mean ± standard error) obtained were 97 ± 2% for the NE-Ib population collected in 2013; 96 ± 2% and 96 ± 1% for the SW-Ib and C-Ib populations, respectively, collected in 2014; and 100% for the NE-Ib population collected in 2015. Two of these values were not significantly different from the expected value of 99%: NE-Ib in 2013 ($t = -1.163$, $df = 2$, $p = 0.183$) and SW-Ib in 2014 ($t = -1.287$, $df = 2$, $p = 0.164$). However, moult inhibition was significantly lower in C-Ib in 2014 ($t = -3.886$, $df = 2$, $p = 0.03$). Data for NE-Ib in 2015 could not be analysed since mortality was 100%.

3.2.4 Survival of larvae on MON 810 leaves

Between 2011 and 2015 (five seasons), larvae of *S. nonagrioides* from the three areas monitored were screened by rearing them on MON 810 tissue. None of the survivors in the dose-response bioassays with Cry1Ab (4871 larvae) survived 10 days of feeding *ad libitum* on MON 810 maize leaves. Additionally, there were no survivors among the spare neonate larvae that were not used in dose-response bioassays (more than 10,000) exposed to MON 810 leaves (Table 3).

4 DISCUSSION

Insect resistance management (IRM) plans are critical to extending the durability of Bt crops. One of their main elements is monitoring for target pests' susceptibility to the Bt protein expressed in the crop. However, long-term strategies should be reviewed and refined, based on the knowledge acquired through research, together with laboratory and field experience accumulated through the implementation of the IRM plan itself.[23] In this study we made a reassessment of the long-term monitoring plan of *S. nonagrioides,* mandated by the European Commission, after the widespread planting of MON 810 maize in the EU. This information will provide insights that enable further improvement of the sensitivity and accuracy of current protocols.

The first step in monitoring programs is to estimate the target pest's baseline susceptibility just before or immediately after the release of a Bt crop, to evaluate the natural variability in susceptibility among field populations.[23–27] The baseline susceptibility is to be used as a comparator to assess future shifts in susceptibility. Accordingly, baselines of susceptibility for Spanish field populations of *S. nonagrioides* collected in 1998 and 1999 were determined.[17,29] However, the toxicity of the formulation of Cry1Ab protein used in the present study was different from those used in previous studies. This finding, together with some necessary changes to the protocol, discouraged the use of these baselines as comparators in our study. As an alternative, we used a reference laboratory strain as a comparator, tested under the same conditions and at the same time as the field-collected individuals. Thus, the maintenance of a susceptible reference strain for the duration of the monitoring program has proved to be essential in this study. The laboratory strain was regularly refreshed by the addition of new healthy individuals collected in non-Bt fields to preserve its vigour and to ensure that the population did not collapse. Infusion of wild individuals into an established laboratory strain is a standard method to keep long-term

populations and to prevent a reduction in their genetic diversity compared with field populations.[43,44] Fluctuations of about 6-fold for both LC_{50} and MIC_{50} were found in the laboratory strain in the period 2004–2015, but no trends were observed over time. Similar variations have been noted in other laboratory strains when studying their susceptibility to pesticides or insecticidal proteins.[44]

Standardization of Cry protein preparations is critical for accurately assessing possible changes in the susceptibility of field populations,[45] since different batches and formulations of Cry1Ab can have significant impacts on toxicity.[30,33,34] Ideally, one single batch of protein should be used throughout the duration of the monitoring program. In this study, the first batch of Cry1Ab protein (B1) was used for seven seasons (2004–2010). However, after this time a decrease in effectiveness was observed in a parallel study carried out with *O. nubilalis* (data not shown), making it necessary to use a second batch (B2). The loss of activity of B1 might have been caused by the storage conditions (−20 °C) and the fact that the batch was not subdivided when first received, thus being exposed to multiple freeze/thaw cycles prior to its use in later bioassays. In the case of the second batch, supplied at two different times (B2-1 and B2-2), the protein was kept at −80 °C and small aliquots were prepared when each supply was received, so the protein needed to be thawed only one more time when used for the bioassays. Bridging experiments could only be performed between B2-1 and B2-2, since B1 had lost its activity by the time B2-1 was provided. These results highlight the importance of proper maintenance and handling of the Cry protein supplies so that they function optimally for many seasons.

Periodic insect collection is another key element in monitoring programs. To ensure that the samples are representative of the local population, we aimed at collecting a minimum of 300 larvae at least once every two years from representative maize-growing areas in the EU, with the purpose of achieving a detection limit of 5% for resistance allele frequency.[46] However, since adoption of MON 810 has progressed differently in each of the four areas assessed (increasing up to about 80% in NE-Ib, maintenance below 40% in C-Ib and SW-Ib, and discontinued in France), we propose to focus future monitoring efforts in the Ebro Valley, in Northeast Spain, and moving from biennial to annual sampling . In this area, MON 810 hybrids represent more than 60% of the cultivated maize, thus being an EU area with increased probability of resistance development to Bt maize in *S. nonagrioides*. This focus would permit allocation of available resources and efforts to collecting higher numbers of insects from the most Bt-exposed field population of *S. nonagrioides* in the EU to reduce the

detection limit for resistance allele frequency below the current 5%. It has been suggested that, in order to capture variability in sensitivity, sampling locations should be pre-defined areas of approximately 10 km × 10 km within a geographical area where the Bt-maize adoption rate and the target pest pressure are high to very high.[47] However, there are different situations that make this requirement not always feasible. One of them concerns the agronomical practices in Northeast Spain. Field rotation is common for maize farmers, with only 55% of the area being cultivated with maize for two consecutive years in the period 2007–2010 in the Ebro valley.[16] Moreover, our results show that, at least for *S. nonagrioides*, the recommendation of pre-defined sampling locations is difficult to accomplish, as the yield of larvae collected in maize fields varied from year to year in all areas sampled. Thus, despite sampling 19 fields in SW Iberia in 2012, we could not collect enough number of larvae from any of them to perform bioassays. The efficiency in field samplings for the Ebro Valley was higher, but unsuccessful sampling efforts were also common in that region. The success in the collection of larvae can be influenced by a number of factors, including the expertise of the people involved in the process of search of infested fields and collection of larvae, as well as biotic (natural enemies, competition, etc.) and abiotic (weather, planting date, etc.) factors.[48–53] The widespread planting of Bt crops has been associated with the decline since 2002 of population levels of the European corn borer, *O. nubilalis,* in corn in the United States[54] and the cotton bollworm *H. armigera* (Hübner) in China.[55] In the case of *S. nonagrioides,* there is insufficient historical data on population dynamics to support the idea of area-wide suppression by Bt maize.

The estimate of LC_{50} or MIC_{50} values by dose-response bioassays on a regular basis is one of the most frequently used methods to detect shifts in the susceptibility to the Cry1Ab protein over time.[7,24,56–58] Although both measures (growth inhibition and mortality) are often correlated,[7] in our study the use of MICs gave a more consistent assessment of *S. nonagrioides* susceptibility to Cry1Ab. This is reflected in the lower magnitude of variation found for MIC values when compiling data from 2004 to 2015. Something similar was observed by Ali and Luttrell[59] who concluded that, at 7 days, MIC_{50} estimates seemed to be a better fit of the linear dose-response model than LC_{50} estimates for assessing *H. zea* and *H. virescens* susceptibility to Bt proteins.

Our results indicate no shifts over time in the susceptibility of *S. nonagrioides* to the Cry1Ab protein in the three main areas in the EU where MON 810 has been cultivated from 2004 to 2015. We found variation up to 7-fold for MIC_{50} values in the population from NE-Ib, and

lower variation for those from C-Ib (4-fold) and SW-Ib (3-fold). In the case of France, where MON 810 hybrids were cultivated for the period 2005–2007, variation in both MIC_{50} and LC_{50} was 2-fold. The observed changes in susceptibility to the Cry1Ab protein in different years are comparable to those previously reported for this species,[16,17,29] suggesting that these changes could be due to common natural variation. These differences are also in the range of those found in target pests of Bt crops in which evolution of resistance has not been detected.[33,56,60] The features of dose-response bioassays make them inefficient at detecting resistant individuals among survivors that have been exposed to sublethal concentrations, thus being insensitive to the small changes in resistance allele frequency that take place in the first stages of resistance.[33,61] To rule out the presence of resistant individuals in this study, near 15,000 larvae from the dose-response bioassays performed between 2011 and 2015 and spare larvae that were not used in the bioassays were screened by rearing them on MON 810 leaves, but no survivors were found. These results confirm that the MON 810 maize is still efficiently controlling *S. nonagrioides* and there are no signs of field resistance to the Cry1Ab protein. Moreover, these results provide conclusive evidence that MON 810 qualifies as high dose for this species, killing more than 99.99% of susceptible larvae.[7,62]

Another approach for detecting shifts in susceptibility to Bt proteins in monitoring programs is the use of diagnostic concentrations (DCs).[63,64] The use of DCs is suitable in conditions where the resistant trait represents "high dose" against the target pest, as it is the case of MON 810 against *S. nonagrioides*. We have determined a candidate DC (726 ng Cry1Ab/cm^2), corresponding to the MIC_{99},[32,60,64] by using data that represent the response of more than 6500 larvae in seven dose-response bioassays with populations collected from 2008 to 2012. When this DC was tested against neonates of field populations collected from 2013 to 2015, the results showed that the mortality obtained was significantly lower than the expected 99% in only one of the four area-year combinations tested. Our results suggest that a refinement of the DC is needed for its future use in the monitoring program, most probably by using the 95% upper limit of the estimated MIC_{99} value from a larger pool of mortality data analyzed by probit analysis.[45,65] An advantage of the DC technique is the fact that all individuals are tested at a concentration at which the percentage of mortality is correlated with resistance; thus, it is more efficient than dose-response bioassays for detecting resistance at low frequencies,[33] as is the case for *S. nonagrioides*.[66] The use of this approach also permits a higher number of field individuals to be tested with the same effort made in dose-response bioassays, thereby helping to decrease the current 5% detection limit for

resistance allele frequency. One limitation, however, is that those individuals heterozygous for recessive resistance alleles will not survive at the diagnostic concentration. The frequency of recessive Bt-resistant alleles can be estimated by F2 screens in natural populations.[67] However, this method has not been used for long-term monitoring programs because it is extremely labour-intensive.

5 CONCLUSIONS

The reassessment of the continuing monitoring program of *S. nonagrioides* has provided insights into the most appropriate monitoring techniques. Thus, it is very useful for long-term monitoring programmes to maintain a susceptible reference laboratory strain against which the susceptibility of the field-collected populations can be compared. In contrast, the use of susceptibility baselines as comparators is limited because it requires the use of the same Cry protein formulation over many years, which cannot always be accomplished. We have also found that MICs are less variable than LCs for *S. nonagrioides*. Moreover, it would be very convenient to shift from dose-response bioassays to the use of a diagnostic concentration and to focus insect sampling on the Ebro basin (Northeast Spain), which contains the highest levels of MON 810 maize cultivation in the EU. Both changes would permit testing higher numbers of insects from the most Bt-exposed field population, thus decreasing the detection limit for resistance allele frequency to below the current 5% and considerably improving the monitoring plan.

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FIGURE CAPTIONS

Figure 1. Historical status of MON 810 maize in the European Union (EU). (A) EU countries that have ever cultivated MON 810 maize (●) since its first deployment in 2003; (B) Area (ha) of MON 810 maize cultivated in Spain and the total in the EU since 2006. Source: ISAAA.[68]

Figure 2. Adoption rate of Bt maize between 2004 and 2016 in the three areas identified in Spain where the penetration of Bt maize has been significant: Northeast Spain (Aragon, Catalonia and Navarre), Central Spain (Castile-La Mancha and Madrid) and Southwest Spain (Extremadura and Andalusia). Lines show the percentage of Bt maize cultivation area with respect to the total maize cultivation area in each region. Data of 2015 and 2016 are provisional.
Source: Compiled by the authors from Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, Gobierno de España (http://www.mapama.gob.es/en/)

Figure 3. Sampling locations for *S. nonagrioides* between 2004 and 2015 (see Supporting Information for more details). Black dots represent the locations where larvae were collected.

Figure 4. Number of fields sampled for collecting larvae of *S. nonagrioides* since 2006 in three EU areas: Northeast Spain (NE-Ib), Central-East Spain (C-Ib) and Southwest Iberia (SW-Ib). A collection at a field/site was considered successful if at least 100 larvae were gathered.

Figure 5. Variation in LC_{50} and MIC_{50} values of *S. nonagrioides* reference (Ref.) strain and field populations [Northeast Spain (NE-Ib), Central-East Spain (C-Ib), Southwest Iberia (SW-Ib) and Southwest France (SW-Fr)] monitored from 2004 to 2015. Each value in the graph indicates the ratio of the highest and lowest values obtained for that collection area and measurement.

Figure 6. Ratios of moult inhibition concentration (MICR) at the MIC_{50} level of *S. nonagrioides* populations from Northeast Spain (NE-Ib), Central-East Spain (C-Ib) and Southwest Iberia (SW-Ib) and Southwest France (SW-Fr), with respect to a laboratory susceptible strain, from 2004 to 2015. Data for Northeast Spain in 2007–2013 are from Castañera *et al*.[16]

Table 1. Susceptibility to Cry1Ab protein of a reference strain and field populations of *Sesamia nonagrioides* between 2004 and 2015

Population[a]	Year	Cry1Ab batch	n	Slope ± SE	χ^2	d.f.	LC_{50}[b] (CI 95%)	LC_{90}[b] (CI 95%)	n	Slope ± SE	χ^2	d.f.	MIC_{50}[b,c] (CI 95%)	MIC_{90}[b, c] (CI 95%)
Reference strain	2004	B1	671	1.4 ± 0.1	42.1	16	**23 (14–32)**	179 (113–385)	575	1.7 ± 0.2	32.6	13	**18 (11–25)**	99 (66–208)
	2007	B1	765	1.1 ± 0.1	46.1	19	**39 (20–62)**	541 (297–1467)	669	1.7 ± 0.2	23.3	16	**16 (12–22)**	94 (70–149)
	2008	B1	863	1.4 ± 0.1	34.8	19	**69 (48–96)**	565 (358–1127)	863	1.6 ± 0.2	65.0	19	**19 (10–30)**	120 (76–255)
	2010	B1	1050	0.9 ± 0.7	69.8	25	**17 (10–27)**	476 (240–1396)	1050	1.3 ± 0.1	40.7	25	**8 (5–11)**	74 (52–117)
	2011	B2-1	1054	1.1 ± 0.1	40.6	25	**26 (19–34)**	383 (247–688)	1054	1.5 ± 0.1	75.7	25	**9 (6–13)**	68 (45–127)
	2012	B2-1	879	1.2 ± 0.1	31.2	22	**15 (11–21)**	175 (108–351)	879	1.4 ± 0.1	33.9	22	**7 (5–10)**	62 (41–107)
	2013	B2-1	953	1.3 ± 0.1	80.8	22	**16 (11–24)**	161 (88–423)	953	1.6 ± 0.1	65.9	22	**7 (5–10)**	48 (31–88)
	2013	B2-2	960	1.3 ± 0.1	56.8	22	**12 (8–17)**	112 (69–226)	960	1.6 ± 0.1	65.6	22	**5 (3–9)**	42 (26–87)
	2014	B2-2	956	1.5 ± 0.1	73.9	22	**31 (21–48)**	232 (122–717)	956	1.8 ± 0.2	74.1	22	**17 (11–25)**	91 (57–209)
	2015	B2-2	863	3.1 ± 0.2	50.1	19	**36 (30–44)**	93 (72–1135)	863	3.3 ± 0.2	84.5	19	**28 (21–36)**	67 (50–110)
NE-Ib	2004	B1	766	1.2 ± 0.1	11.0	19	**140 (110–178)**	1614 (1095–2687)	766	1.3 ± 0.1	39.9	19	**63 (34–99)**	570 (333–1318)
	2005	B1	639	1.1 ± 0.1	21.9	19	**23 (13–36)**	362 (242–649)	639	1.4 ± 0.2	20.8	19	**9 (3–15)**	76 (54–117)
	2007	B1	862	1.0 ± 0.1	20.8	19	**60 (36–89)**	1317 (704–3597)	862	1.5 ± 0.2	23.9	19	**14 (8–20)**	99 (70–158)
	2009	B1	1055	0.9 ± 0.1	23.4	25	**482 (330–802)**	12614 (5028–58507)	1055	1.4 ± 0.1	28.2	25	**22 (16–28)**	188 (138–277)
	2011	B2-1	1056	1.1 ± 0.1	43.9	25	**105 (76–148)**	1601 (896–3685)	1056	1.5 ± 0.1	70.1	25	**20 (14–27)**	135 (91–232)
	2013	B2-1	1274	1.1 ± 0.1	78.9	30	**78 (53–129)**	1176 (526–4313)	1274	1.4 ± 0.1	67.5	30	**19 (14–25)**	163 (108–287)
	2015	B2-2	920	1.2 ± 0.1	36.6	22	**73 (51–119)**	864 (395–3441)	920	1.8 ± 0.2	28.5	22	**17 (13–21)**	84 (63–124)
C-Ib	2004	B1	768	1.1 ± 0.1	36.2	19	**32 (18–48)**	523 (299–1278)	768	1.0 ± 0.1	30.5	19	**12 (5–22)**	248 (143–588)
	2006	B1	768	0.8 ± 0.1	34.8	19	**29 (14–49)**	1137 (513–4934)	768	0.8 ± 0.1	42.5	19	**7 (1–17)**	321 (156–1360)
	2008	B1	864	1.2 ± 0.1	18.1	19	**83 (57–112)**	1045 (662–2050)	848	1.6 ± 0.2	22.3	19	**28 (18–38)**	170 (124–259)
	2010	B1	991	1.2 ± 0.1	63.2	17	**42 (24–71)**	1834 (730–9243)	991	1.2 ± 0.1	63.2	37	**10 (6–14)**	119 (81–200)
	2012	B2-1	954	1.0 ± 0.1	56.0	22	**47 (30–82)**	891 (358–4870)	954	1.2 ± 0.1	106.4	22	**15 (8–25)**	160 (79–608)
	2014	B2-2	943	1.0 ± 0.1	44.2	22	**54 (34–105)**	1018 (376–6607)	943	1.3 ± 0.1	44.4	22	**15 (9–21)**	138 (81–329)
SW-Ib	2005	B1	996	1.0 ± 0.1	92.5	31	**19 (9–32)**	339 (183–986)	996	1.3 ± 0.1	87.1	31	**11 (5–18)**	110 (69–229)
	2007	B1	766	0.8 ± 0.1	15.1	19	**54 (35–78)**	1796 (956–4659)	670	1.1 ± 0.1	17.7	16	**17 (10–25)**	226 (153–385)
	2010	B1	960	1.6 ± 0.1	30.6	22	**24 (19–31)**	163 (119–245)	960	1.7 ± 0.1	48.8	22	**16 (11–21)**	86 (60–141)
	2012	B2-1	782	1.1 ± 0.1	88.8	38	**99 (57–212)**	1558 (542–15918)	782	1.7 ± 0.2	86.3	37	**29 (19–41)**	158 (101–339)
	2014	B2-2	957	1.2 ± 0.1	29.0	22	**76 (53–126)**	898 (402–3744)	957	1.5 ± 0.2	36.8	22	**31 (23–43)**	236 (140–569)
SW-Fr	2005	B1	766	0.9 ± 0.1	35.4	18	**37 (19–61)**	1021 (466–4412)	766	1.0 ± 0.1	48.3	19	**17 (6–30)**	284 (155–815)
	2006	B1	765	1.1 ± 0.1	71.5	19	**53 (23–95)**	776 (356–3681)	765	2.2 ± 0.2	44.6	19	**38 (27–50)**	144 (105–222)
	2007	B1	288	1.4 ± 0.3	7.0	5	**64 (20–127)**	516 (238–3426)	288	1.5 ± 0.2	3.9	5	**40 (23–61)**	284 (178–590)

[a] Northeast Spain (NE-Ib), Central-East Spain (C-Ib), Southwest Iberia (SW-Ib) and Southwest France (SW-Fr).
[b] 50% and 90% lethal concentrations (LC_{50} and LC_{90}) and moult inhibition concentrations (MIC_{50} and MIC_{90}) and their 95% confidence intervals (CI 95%) are expressed in ng Cry1Ab/cm^2.
[c] Data submitted to EFSA as part of the resistance monitoring program for *S. nonagrioides*, available in EFSA´s "Scientific Opinion on the annual post-market environmental monitoring (PMEM) report on the cultivation of genetically modified maize MON 810" reports[18,69] (except data from NE-Ib 2004, SW-Ib 2005 and SW-Fr).

Table 2. Lethal concentration ratio (LCR) and moult inhibition concentration ratio (MICR) at LC_{50} and MIC_{50} level, respectively, measured over time in a laboratory strain of *Sesamia nonagrioides*

Season	Batch of Cry1Ab protein	LCR (LC_{50})[a] (CI 95%)	MICR (MIC_{50})[a] (CI 95%)
2004	B1	1	1
2007	B1	1.7 (1.1–2.6)*	0.9 (0.6–1.3)
2008	B1	3.1 (2.2–4.4)*	1.1 (0.7–1.6)
2010	B1	0.8 (0.5–1.1)	0.4 (0.3–0.6)*
2011	B2-1	1.1 (0.8–1.6)	0.5 (0.4–0.7)*
2012	B2-1	0.7 (0.5–1.0)	0.4 (0.3–0.6)*
2013	B2-1	0.7 (0.5–1.0)	0.4 (0.3–0.6)*
2013	B2-2	0.5 (0.4–0.8)*	0.3 (0.2–0.5)*
2014	B2-2	1.4 (1.0–1.9)	1.0 (0.7–1.3)
2015	B2-2	1.6 (1.2–2.1)*	1.5 (1.2–2.0)*

[a] Lethal concentrations or moult inhibition concentrations are significantly different (*) ($P < 0.05$) from those in the first year of testing (2004) if the 95% confidence intervals (CI 95%) of LCR or MICR do not include 1.

Table 3. Number of larvae of *Sesamia nonagrioides* fed on MON 810 maize to detect resistant individuals in the progenies of field-collected populations from 2011 to 2015. None of these larvae survived exposure to MON 810 maize leaf tissue.

	Survivors of bioassays[b]						Leftover larvae[c]
Population[a]	2011	2012	2013	2014	2015	Total	Total
NE-Ib	721	-	1003	-	574	2298	~4500
C-Ib	-	491	-	803	-	1294	~3000
SW-Ib	-	432	-	847	-	1279	~3000

[a] Northeast Spain (NE-Ib), Central-East Spain (C-Ib) and Southwest Iberia (SW-Ib).
[b] All surviving larvae from the dose-response bioassays with Cry1Ab protein.
[c] Leftover neonates, generated from field collections and not used in bioassays.

Figure 1

A

	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Spain	●	●	●	●	●	●	●	●	●	●	●	●	●	●
France			●	●	●									
Portugal			●	●	●	●	●	●	●	●	●	●	●	●
Germany			●	●	●	●								
Czech Republic [a]			●	●	●	●	●	●	●	●	●	●	●	●
Slovakia [a]				●	●	●	●	●	●	●	●	●	●	●
Poland [a]					●	●	●	●	●					
Romania [a]					●	●	●	●	●	●	●	●	●	●

[a] Czech Republic, Slovakia and Poland became members of the European Union in May 2004 and Romania in January 2007.

B

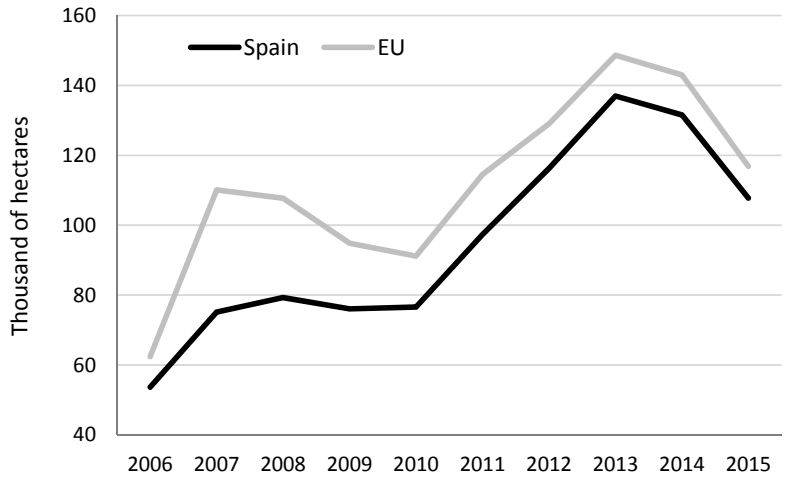

Figure 2

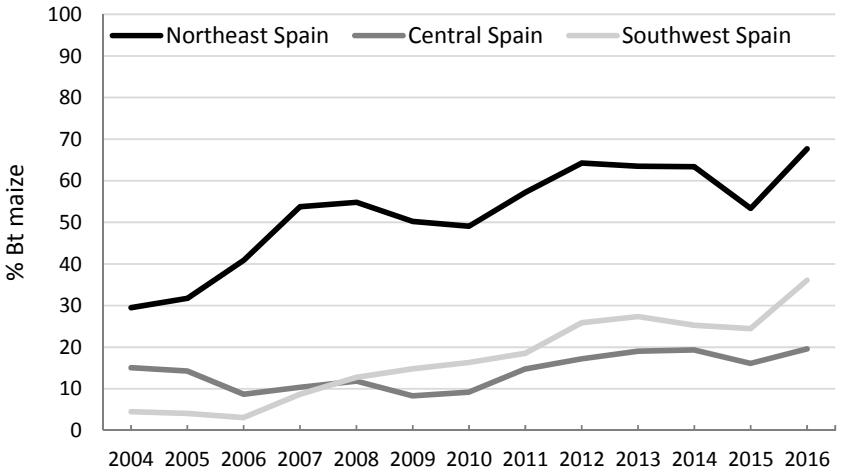

Figure 3

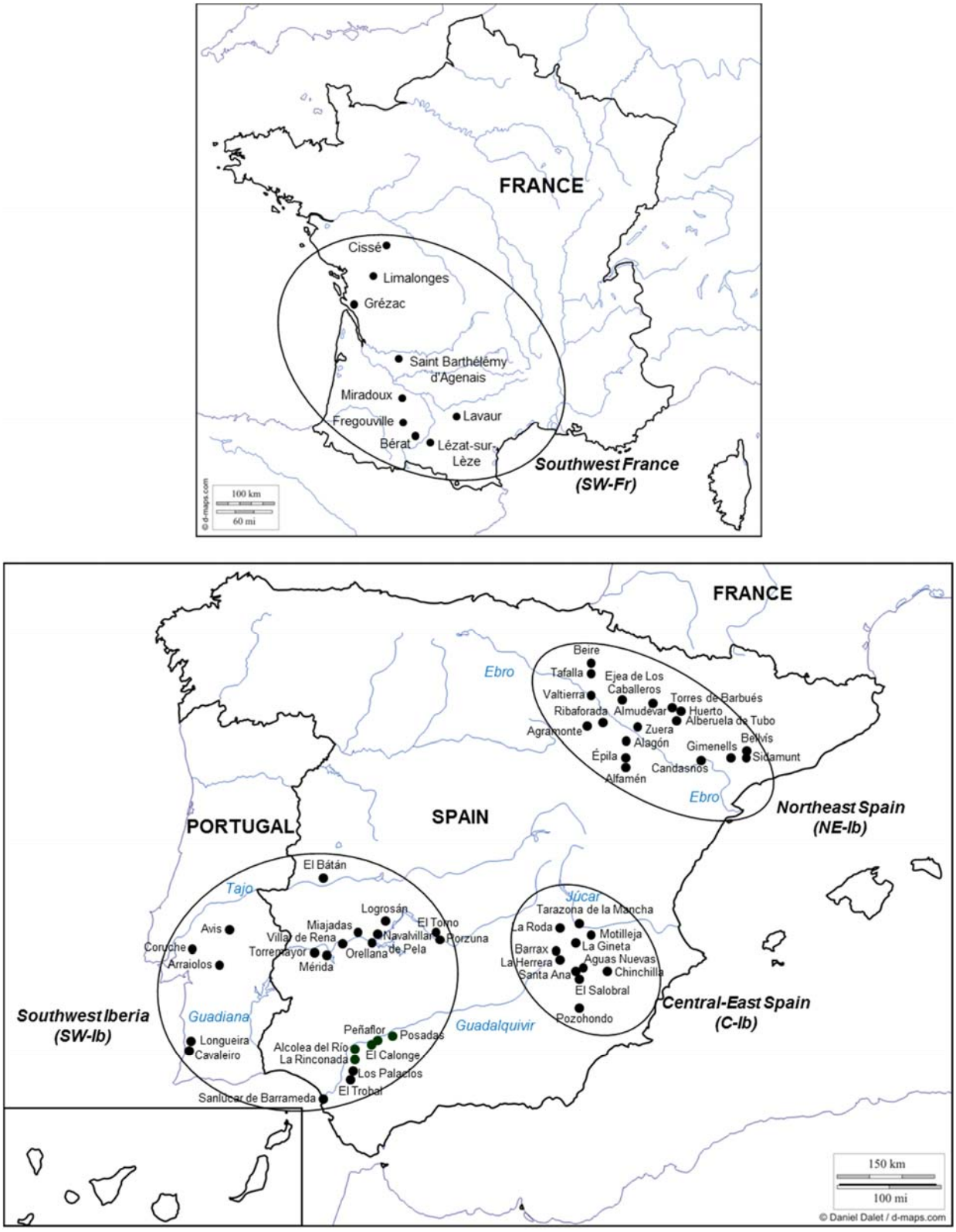

Figure 4

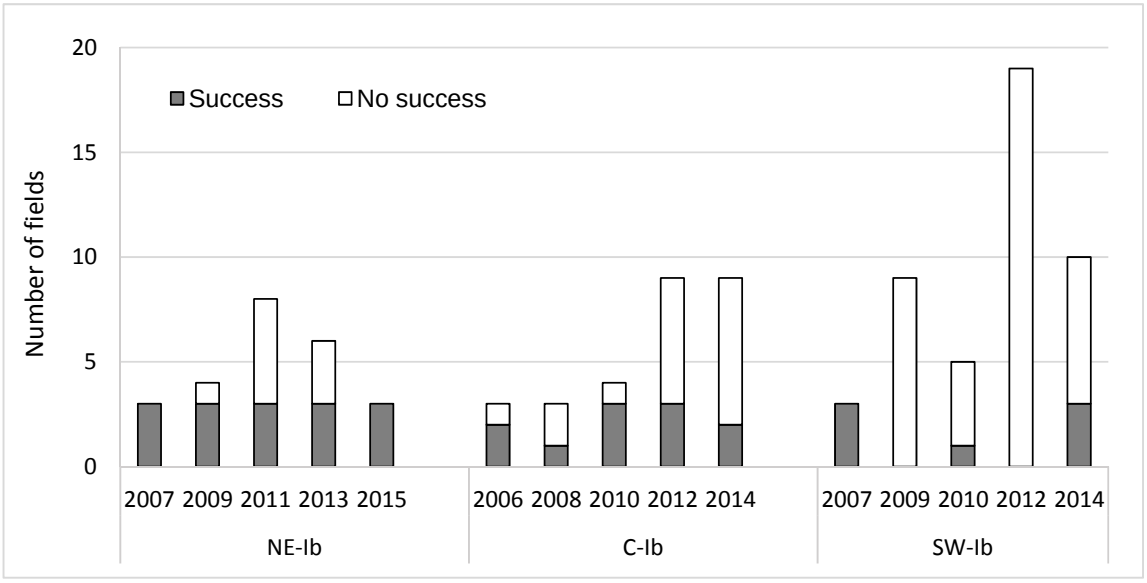

Figure 5

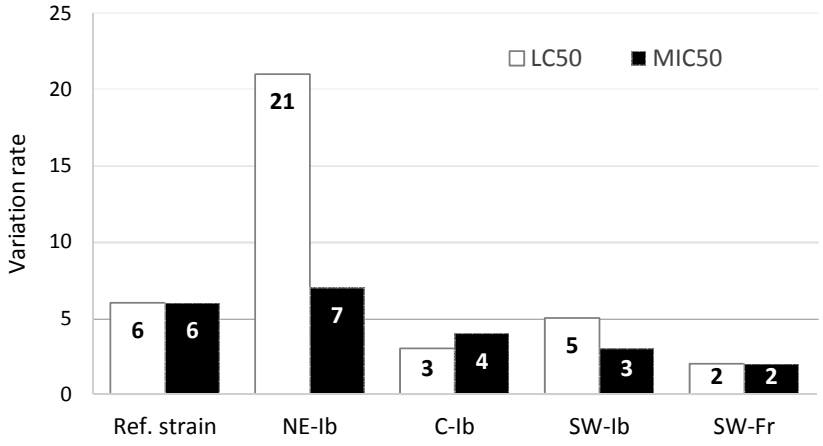

Figure 6