Appendix 4. Insect Resistance Monitoring in Iberian collections of *Ostrinia nubilalis* (ECB): 2013 Season

Report

Cry1Ab susceptibility in European origins of *Ostrinia nubilalis* (ECB)

- Results for 2013-2014 -

Date

23/07/2014

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Statement of Compliance with the Principles of Good Experimental Practice

The study described in this report was conducted in compliance with the most recent edition of:

- The Principles of Good Experimental Practice (GEP), (Plant Protection Products Ordinance, paragraph (5) of Article 1c, Germany).

Head of testing facility

Date / Signature: 23. July 2014 .....

Study director

Date / Signature: 23. July 2014 .....

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1 Introduction

Maize containing event MON 810 is genetically modified 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, Monsanto established an insect resistance monitoring program across Europe and in particular in areas where commercial activity of MON 810 is occurring or planned, *i.e.*, areas where the European target pests *O. nubilalis* and *S. nonagrioides* are prevalent. This monitoring program follows directions described in the plan of the industry working group on Insect Resistance Management (IRM) proposed to the Member State Competent Authorities and the European Commission (available since 2003 but published in 2007; ALCALDE et al., 2007 and subsequently updated as the EuropaBio harmonised IRM plan in September 2012). The current report focuses on the susceptibility monitoring of *O. nubilalis.*

The European corn borer (ECB), *Ostrinia nubilalis*, is native to southern Europe (BECK, 1987) and is believed to have been introduced into North America between 1909 and 1914 (VINAL, 1917), where multiple introductions have probably occurred (SHOWERS, 1993). Since then, *O. nubilalis* has rapidly spread across North America (CAFFREY & WORTHLEY, 1927; ROELOFS et al., 1985; HUDON & LEROUX, 1986). Apart from maize, more than 200 weeds and cultivated plants are known to serve as host plants for *O. nubilalis* (HODGSON, 1928; PONSARD et al., 2004). Before *Bt* maize was commercially available, *O. nubilalis* was one of the most damaging pests of maize in North America and Europe and was therefore a major target pest for control with genetically modified maize expressing *Bacillus thuringiensis* (*Bt*) proteins.

In accordance with the EuropaBio Harmonised IRM plan of September 2012 the baseline susceptibility of *O. nubilalis* to the Cry1Ab protein needs to be established after which subsequent routine monitoring for changes in susceptibility should be carried out. The objective is to detect, in a timely manner, shifts relative to baseline susceptibility that could result in inadequate protection against the target species. This program will enable early detection of potential development of resistance in *O. nubilalis* if it occurs, and this will allow the proposal and implementation of additional risk mitigation measures.

Previous baseline susceptibility to the Cry1Ab protein has been established for *O. nubilalis* populations collected in different maize grown areas in Spain (GONZALEZ-NUNEZ et al., 2000, FARINÓS et al., 2004), Germany (SAEGLITZ et al., 2006) and the United States (MARÇON et al., 1999a, b and 2000). The EU baseline results have been generated in areas where the MON 810 maize adoption by farmers was expected to be significant given the local abundance of the pests.

In accordance with the EuropaBio harmonized IRM plan, changes in the susceptibility of the target pests, which eventually could lead to resistance, will be reported on a biennial basis in areas where MON 810 is grown. Two exceptions were described: (1) in case the adoption of MON 810 remains below 20% in the given area, no data will be reported in future, and (2) in case MON 810 adoption equals or exceeds the theoretical maximum of 80% (due to the required 20% refuge implementation), susceptibility monitoring will be performed on a yearly basis. Samples were not taken in Southwest Iberia since they were the subject of last year's report. Therefore, the current report focuses on the resistance monitoring of *O. nubilalis* in Northeast Iberia and Central Iberia, the area where adoption of MON 810 was greater than 20%.

The objectives of the current report on the 2013 maize growing season are:

1) To determine the susceptibility of *O. nubilalis* in maize growing areas in Northeast Iberia and Central Iberia to the Cry1Ab protein expressed in MON 810 maize varieties.

2) In addition, preliminary studies were conducted to explore the feasibility of monitoring resistance of *O. nubilalis* to Cry1Ab using the diagnostic dose method. This method was established to be the most efficient method and as effective as the dose-response method to detect changes in susceptibility to Cry proteins (SIMS et al., 1996).

2 Materials and Methods

2.1 Insect collection

The three areas identified in the entire EU where adoption of MON 810 in 2013 was expected to be greater than 20% are the Ebro valley (defined in earlier reports as Iberia Northeast), Central Iberia (particularly the province of Albacete) and the Southwest Iberia area. For these areas data on the susceptibility of *O. nubilalis* to Cry1Ab has been collected since 2007. In 2013, it was the aim to collect samples from three sites that were separated by at least 50 km in both Central Iberia and Northeast Iberia. *O. nubilalis* samples were collected as larvae in naturally infested fields or refuges to MON 810 maize varieties fields following the Standard Operating Procedures (SOPs) as attached to the EuropaBio harmonized IRM plan. Collections were made by dissecting maize stalks in the field before harvest or in spring after diapause. If more than one larva per stalk was found, only one was taken to avoid collecting siblings (Figure 1). For each area, the aim was to collect 300 healthy larvae.

2.2 Insect culture

Field-collected *O. nubilalis* larvae were placed in plastic boxes containing corrugated cardboard and maintained in a growth chamber at 25°C, 90% RH and a photoperiod of 20:4h (L:D) on an agar-based wheat germ diet (Figure 2 and 3, Table 1). If the larvae did not pupate after a period of two weeks, they were assumed to have entered diapause and were transferred to another climatic chamber maintained at 8 ± 2°C, 70 ± 5% RH, and a photoperiod of 0:24h (L:D) until the time for collective emergence of adults in May.

O. nubilalis larvae from different sampling sites separated by at least 50 km were analysed. Collected insects from different sites within the area tested were reared or kept under diapause separately to avoid cross contamination with *Beauveria* sp. or *Nosema* sp.

Larvae surviving the diapause period were transferred to fresh containers and placed in incubators where the temperature was raised gradually from 15-25°C, humidity of 90% RH and a photoperiod of 20:4h (L:D) over a period of 10 days and kept at 25°C, humidity of 90% RH and a photoperiod of 20:4h (L:D) thereafter. Emerging adults were transferred to oviposition cages (Figure 4) and fed 15% honey water to increase fecundity (LEAHY & ANDOW, 1994). The insides of the cages were covered with filter paper (oviposition medium) that was changed twice a week. Egg masses were cut off and transferred to petri dishes with moistened filter paper. If necessary, egg masses were stored for up to seven days at 8 ± 2°C. Incubating egg masses were placed in an incubator for 20 h at 25 ± 2°C, 4 h at 20 ± 2°C, 90% RH and a photoperiod of 20:4h (L:D) (GUTHRIE et al., 1985).

Figure 1. Dissected maize stalk with larvae.

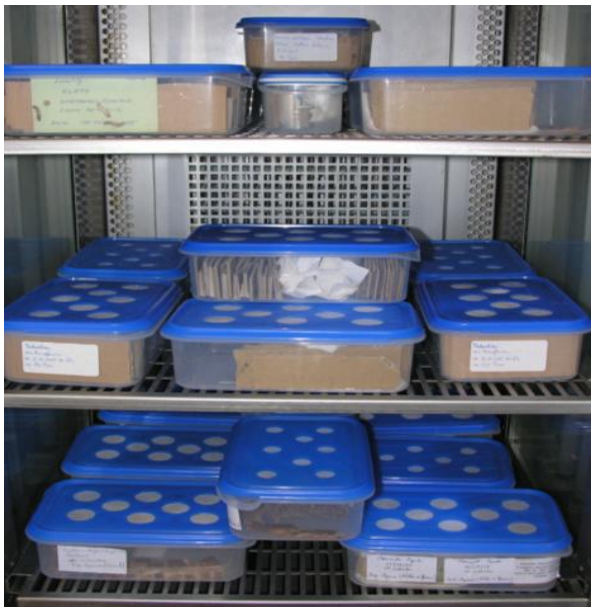


Figure 3. Growth chamber with plastic boxes containing diapausing ECB larvae

Figure 2. Corrugated cardboard with pupae.

Figure 4. Oviposition cages for adult ECB.

2.3 Bioassays

2.3.1 Susceptibility to Cry1Ab

Two batches of Cry1Ab protein have been used since the start of the MON 810 monitoring plan. The batch 2 (that was used for the campaign 2012-2013) was provided by Monsanto and was stored at -80°C until used (NBR: 11247229, 31/01/2012; concentration 1.64 mg/ml in 50 mM bicarbonate buffer, pH 10.25). To prepare the test concentrations, a bicarbonate buffer (25 mmol/l) with pH 10.5 was used. As this batch has reached the date of expiry a new batch (2a) was provided by Monsanto. The batch 2a (NBR: 11247229, 31/01/2015; concentration 1.64 mg/ml in 50 mM bicarbonate buffer, pH 10.25) was also stored at -80°C until being used. To prepare the test concentrations, a bicarbonate buffer (50 mmol/l) with pH 10.25 was used. To analyze if the two batches differed in efficacy a bridging experiment was done. The bioassays were performed in 128 well trays (Bio-Ba-128, Color-Dec, Italy). In each cell 1 ml of artificial diet was dispensed (see Table 1 for recipe). After the diet solidified, 100 µl of protein solution was applied to the surface and allowed to dry overnight at room temperature. To avoid contamination the trays were covered with a sheet of filter paper. Egg masses of each sampling location (offspring of field-collected larvae) were incubated and neonate larvae, within 12 h after hatching, were transferred to the cells. A single neonate was placed in each cell and confined with a cover (Bio-Cv-16, Color-Dec Italy) (Fig. 5). Eight concentrations and a control (bicarbonate buffer) were tested for each population. Those tested with the second batch were exposed to 0.2–28.22 ng Cry1Ab/cm² and those tested with the third batch to 0.2–28.22 ng Cry1Ab/cm². Field collected insects used in bioassays came from pooled samples of healthy insects collected in different fields within an area. For each collection area, >30 larvae were tested for each Cry1Ab concentration and 64 larvae for fed the control diet.

All assays were conducted at 25°C, 70% RH and a photoperiod of 0:24h (L:D). After seven days, larval mortality and developmental stage were recorded. Larvae that had not grown beyond first instar would not survive under field conditions (e.g. SIEGFRIED et al., 2000). As a result, the criterion for mortality used in this study accounts for both death and complete moulting (or growth) inhibition.

Table 1. *O. nubilalis* diet recipe

Component	Amount	Provided
Distilled H_2O	680 ml	
Benzoic acid	1 g	Carl Roth GmbH & Co. KG
Sorbic Acid	1 g	BioServ
Nipagin (methyl-paraben)	1 g	BioServ
Agar	16 g	Carl Roth GmbH & Co. KG
Maize powder	112 g	Gut & Gerne, BZ Bio-Zentrale
Wheatgerm	28 g	Frießinger Mühle GmbH
Brewer's yeast	30 g	Biolabor GmbH & Co.KG
Fumidil B	1 g	CEVA Salud Animal, S.A.
Ascorbic acid	3 g	BioServ
Vanderzant vitamin mix	2 g	BioServ

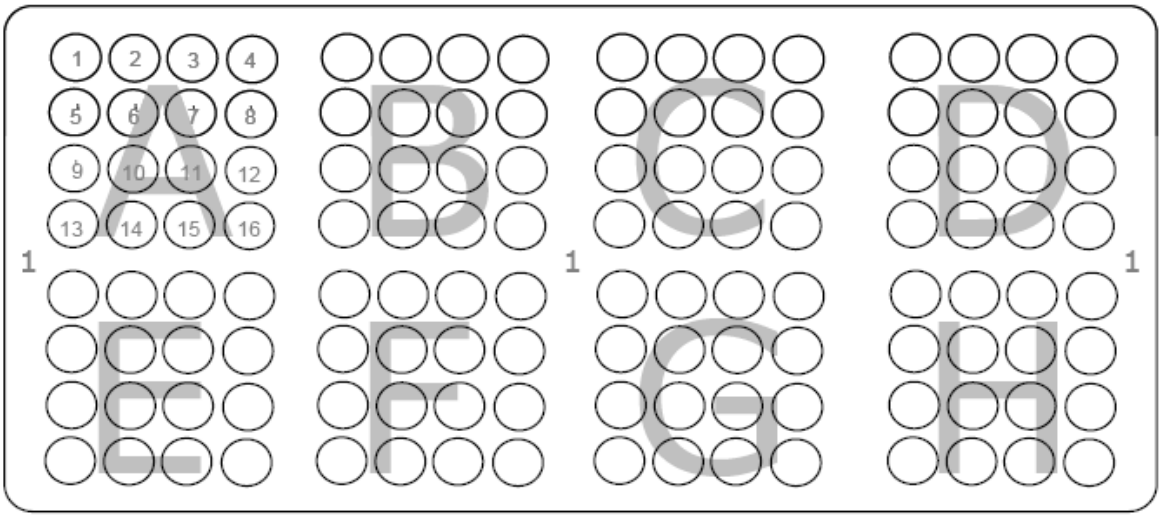


Figure 5. IDs of Bio-Ba-128 trays (tray number, field letter, well number; i.e.: 1.A.13)

2.3.2 Diagnostic dose

For the calculation of diagnostic dose the data for all experiments with ECB from 2005-2012 were used. These ECB collected in fields from Czech Republic, France, Germany, Italy, Panonia, Poland, Portugal, Romania and Spain representing the responses of 11,502 larvae. Using the average of the moulting inhibition concentrations (MIC) for 99% (MIC_{99}) the diagnostic dose for ECB larvae from Europe was calculated to be 28.22 ng/cm^2, and its half 14.16 ng Cry1Ab/cm². Data from bioassays with more than 20% response at the control after exposure to Cry1Ab have been neglected.

2.4 Statistical analysis

All statistical analyses were done using the computer program SYSTAT, Version 10.0, except for dose-response analysis where PoloPlus 1.0 was used (LeOra Software Company). The results obtained for growth inhibition at different concentrations of Cry1Ab were adjusted by probit weighted regression lines, and moulting inhibition concentrations (MICs) for 50% (MIC_{50}) and 90% (MIC_{90}) of each origin tested were estimated together with their 95% confidence limits using the POLOPC programme (LeOra Software, 1987). Mortality of the control must be below 20% for *O*. *nubilalis*, in order to be able to include the bioassay in the statistical analysis.

The measure of how well the data (response of *O. nubilalis* to different concentrations of protein) fit the assumptions of the Probit model is goodness-of-fit. To test goodness-of-fit, responses predicted by the Probit model were compared with responses actually observed in the bioassay (χ^2 test).

Hypothesis tests are essential for the interpretation of bioassay results. Three possible outcomes of comparing Probit regression lines are that lines are parallel but not equal (i.e., different intercepts), lines are parallel and equal, or lines are neither parallel nor equal. When lines are parallel but not equal, their slopes are not significantly different. This means that changes in activity per unit change in rate are the same. If regression lines are equal, they do not differ in either intercept or slope, meaning the populations being compared are equally affected.

3 Results and Discussion

3.1 Collection of ECB

The area where ECB larvae were collected in 2013 is shown in Table 2, and the location is displayed on a map in Annex I.

Table 2. *O. nubilalis* collection details for the 2013-2014 season.

Area	ID	Country	Collection site[a]	Collected	Eggs	Larvae	Tested
lab[b]	G 04	Germany		2005	x		2014
EsC	ES.20	Spain	ES-02162 La Herrera (AB)	09.2013		207	2014
	ES.21	Spain	ES-02639 Barrax (AB)	09.2013		225	2014
EsNE	ES.05	Spain	ES-22591 Candasnos (HU)	09.2013		145	2014
	ES.06	Spain	ES-31550 Ribaforada (NA)	09.2013		142	2014
	ES.07	Spain	ES-22270 Almudevar (HU)	10.2013		165	2014

[a] Spanish provinces: NA = Navarra, HU = Huesca, AB = Albacete; [b] reference strain

3.2 Susceptibility to Cry1Ab in the 2013-2014 campaign

To analyze if the Cry1Ab protein batches 2 and 2a differed in efficacy a bridging experiment was done with larvae of strain G.04, applying the same method as for the bioassays. The proteins of both batches did not significantly differ in their efficacy (Figure 6).

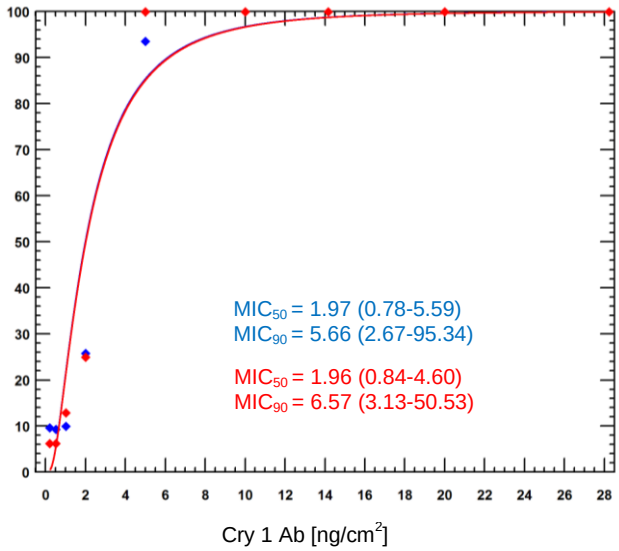
To determine the susceptibility of the field collections as well as the lab strain to Cry1Ab, larval mortality and larval moult inhibition data at the different concentrations of Cry1Ab tested were analyzed by Probit analysis. Fitted curves of susceptibility to the Cry1Ab protein of laboratory and field collections of *O. nubilalis* were generated taking into account the moulting inhibition concentration of neonate larvae after seven days feeding on treated diet (Figure 7). Moulting inhibition concentrations at 50% (MIC_{50}) and 90% (MIC_{90}) for *O. nubilalis* collected in a particular area are provided in Table 3. The significance of differences in susceptibility between the laboratory strain (originally collected in Niedernberg, Germany, and kept in culture since 2005) and the field collected insects was tested by determining the 95% confidence limits (CI) of MIC ratios (ROBERTSON et al., 2007). The moulting inhibition concentrations are considered significantly different from the laboratory strain ($P < 0.05$) when the MICR 95% confidence limits do not include 1. The MIC_{50} and MIC_{90} values for field-collections of ECB from both areas (EsC and EsNE) did no differ significantly to the reference strain in their susceptibility to Cry1Ab/cm² (Figure 7) as indicated by the inclusion of 1 in their MICR 95% confidence limits.

Table 3. Results from Probit analysis for the ECB origins collected in 2013.

Area	n	Slope ± SE	χ^2	D.f.	MIC_{50} (95% CI) [a]	MIC_{90} (95% CI) [a]
lab	281	2.438 ± 0.253	46.96	7	1.96 (0.84-4.60)	6.57 (3.13-50.53)
EsC	575	3.023 ± 0.226	19.07	16	2.40 (2.04-2.83)*	6.38 (5.18-8.34)*
EsNE	861	3.771 ± 0.258	78.84	25	2.48 (2.03-3.02)*	5.41 (4.27-7.61)*

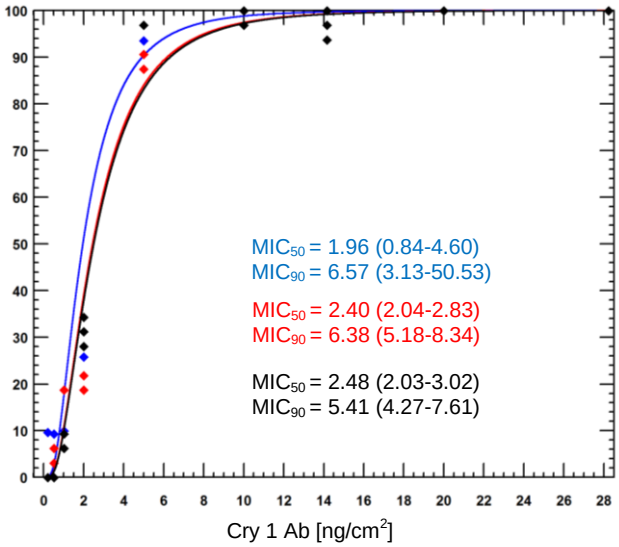
[a] 50% and 90% moulting inhibition concentrations (MIC_{50} and MIC_{90}) and their 95% confidence intervals (95%CI) are expressed in ng Cry1Ab/cm².

* Moulting inhibition concentrations are not significantly different ($P > 0.05$) to the laboratory strain.



	MIC ratios	
	($MICR_{50}$)	Conf. limits
Batch 2 vs. 2a	0.997	(0.733-1.355)
	($MICR_{90}$)	
Batch 2 vs. 2a	1.162	(0.741-1.823)

Figure 6. Fitted curves of susceptibility as percentage moult inhibition after seven days of feeding by ECB (G.04) when exposed to treated diet from the batches 2 (blue) and 2a (red) of protein Cry1Ab (PoloPlus, LeOra Software 2002-2009). According to the statistical analyses both curves have equal slopes and equal intercepts (P 0.05; χ^2: 0.93, d.f.: 2; tail probability: 0.627)



	MIC ratios	
	($MICR_{50}$)	Conf. limits
G04 vs. EsC	0.815	(0.626-1.062)
G04 vs. EsNE	0.791	(0.618-1.014)
	($MICR_{90}$)	
G04 vs. EsC	1.030	(0.703-1.510)
G04 vs. EsNE	1.214	(0.849-1.735)

Figure 7. Fitted curves of susceptibility as percentage moult inhibition after seven days feeding on treated diet of ECB (collected in 2013) to the batch 2a of protein Cry1Ab (PoloPlus, LeOra Software 2002-2009).

Reference laboratory strain (G.04, blue); Iberia Central (EsC, red); Iberia Northeast (EsNE, black)

3.3 Diagnostic dose

Not a single larva tested in 2014 survived the diagnostic dose for ECB larvae from Europe (determined to be 28.22 ng/cm^2). Only two larvae from Candasnos and one larva from Almudevar survived a dosage representing 50% of diagnostic dose.

3.4 Exposure to MON 810 tissue (confirmatory experiment)

It was planned that all *O. nubilalis* larvae from field collections that survived the bioassay at the highest dose should be assembled, transferred to plastic boxes in groups of approx. 50 larvae, provided with newly detached MON 810 maize leaves without the central nerve, and fed *ad libitum* to record any survivors. As for the season reported here, no surviving larvae were found after 10 days and confirmatory experiments were not necessary.

3.5 Historical susceptibility of corn borers to Cry1Ab

During 2008–2012, 28 samples of ECB from different areas were analyzed. Their susceptibility to Cry1Ab is shown in Table 4.

Table 4. Susceptibility of *O. nubilalis* neonates exposed to Cry1Ab as measured by the MIC over time for areas tested.

Area	Year	MIC_{50} (95% CI) [a]	MIC_{90} (95% CI) [a]
Iberia Central	2009[1]	3.09 (2.03-4.33)	11.98 (8.12-22.31)
	2011[2]	1.56 (1.27-1.91)	4.04 (3.12-5.91)
	2013[3]	2.40 (2.04-2.83)	6.38 (5.18-8.34)
Iberia Northeast	2008[1]	7.03 (4.89-10.03)	23.91 (15.76-46.84)
	2009[1]	6.40 (5.32-7.75)	13.68 (10.77-20.02)
	2011[2]	1.79 (1.54-2.07)	4.19 (3.45-5.48)
	2013[3]	2.48 (2.03-3.02)	5.41 (4.27-7.61)
Iberia Southwest	2008[1]	3.39 (2.94-3.89)	6.90 (5.79-8.89)
	2010[1]	5.76 (4.38-7.84)	11.85 (8.53-23.52)
	2012[2]	4.08 (2.99-5.50)	8.69 (6.30-15.56)

MIC moulting inhibition concentrations, CI confidence interval, [a] ng Cry1Ab/cm^2; [1] batch 1 of Cry1Ab, [2] batch 2 of Cry1Ab, [3] batch 2a of Cry1Ab

3.6 Susceptibility of the lab strain (G.04) to Cry1Ab

The laboratory strain G.04 was kept in sub-strains since 2011 and checked regularly for performance (size of adults, size of egg masses, and development of larvae). In 2011 applying a PCR based method (SAEGLITZ, 2004) infection with *Nosema* was identified for some individuals in one sub-strain, which have been eliminated. One sub-strain was used for the following years, until now. This sub-strain is producing good-quality egg masses and normal-sized adults. A recent PCR analysis showed that the reference strain G.04 kept since 2011 is not infected with microsporidia and especially not infected with *Nosema* (see Appendix II).

4 Conclusions

In 2013, 2 areas with 5 samples of ECB were analysed. Thus far, susceptibility to Cry1Ab has been assessed for one laboratory colony and ECB collected in maize fields in Spain. ECB larvae were exposed to artificial diet treated with increasing Cry1Ab concentrations, and mortality and growth inhibition were evaluated after 7 days. Variation in Cry1Ab susceptibility (MIC_{50} and MIC_{90}) of ECB collected in the Spanish fields during the campaign 2013-2014 was 0.97-fold and 1.2-fold respectively. Variation in Cry1Ab susceptibility (MIC_{50} and MIC_{90}) of field samples in comparison with the lab strain was up to 0.79-fold and 1.03-fold respectively. Significant differences in *Bt* susceptibility between ECB from both areas in Spain and the reference strain were not found. These results indicate that the observed variation in susceptibility reflects natural variation in *Bt* susceptibility among ECB origins. Any evidence for a decrease of Cry1Ab susceptibility of ECB during the monitoring duration could not be detected.

5 Acknowledgement

This report presents the results of research only and would not be possible without the kind help of all those who supplied insects: the Spanish group around Félix Ortega Alonso and Gema Pérez Farinos (CSIC, Madrid, ES).

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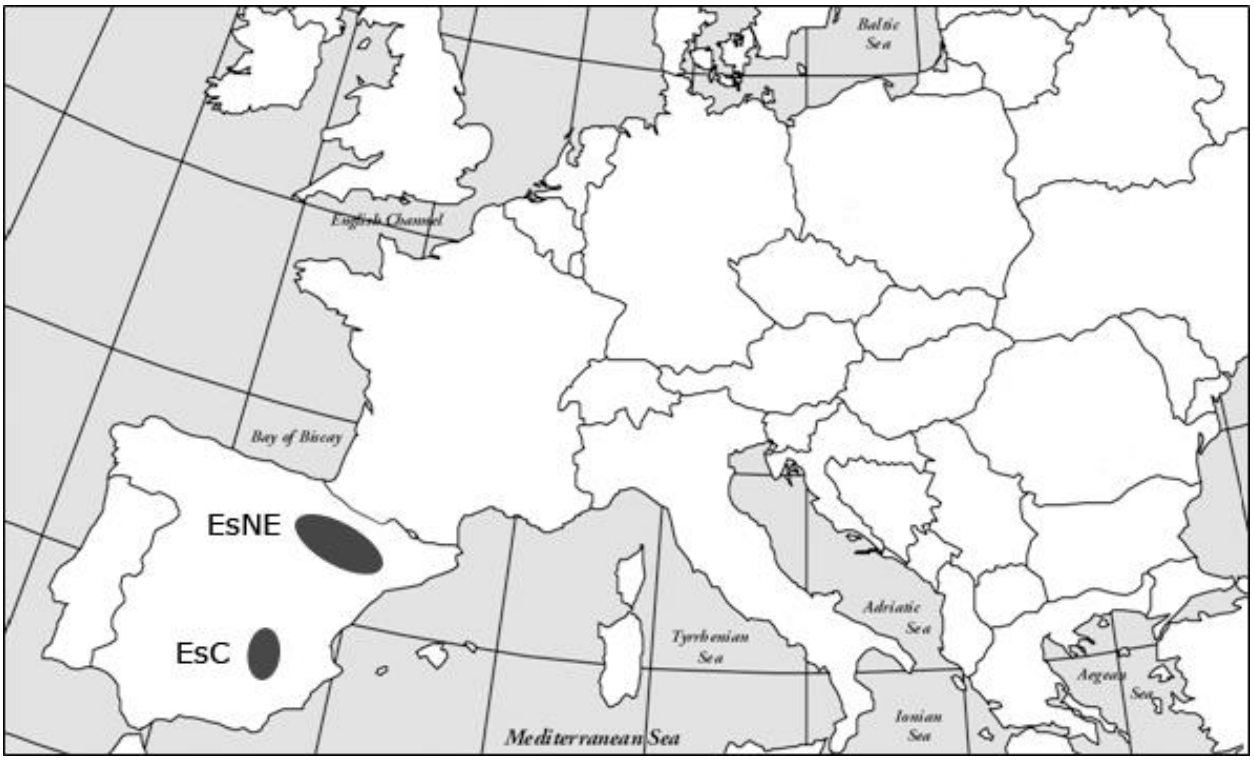
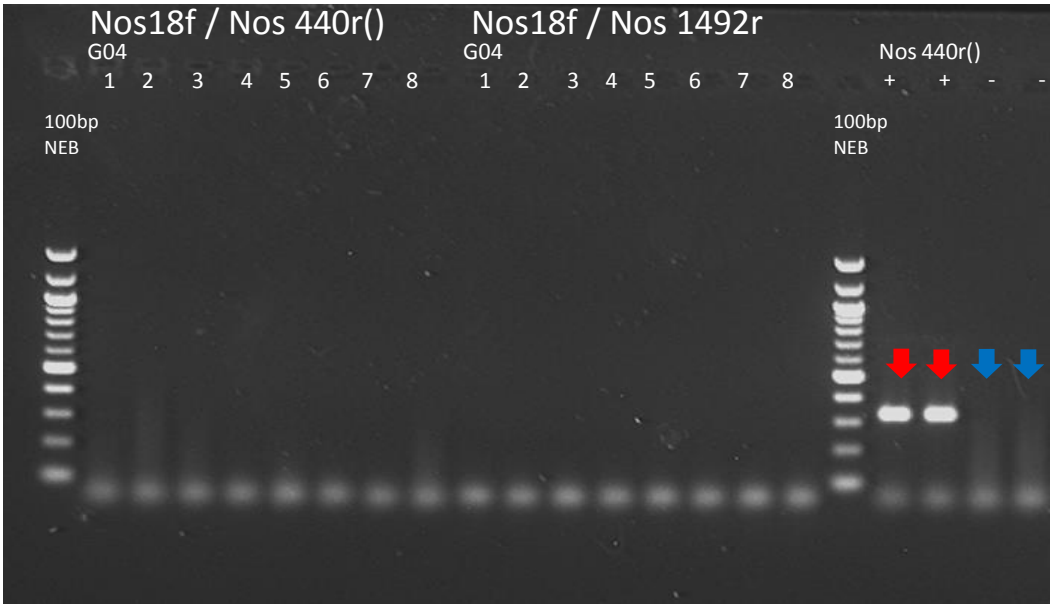
Annex I
Areas of collection activities for ECB in 2013



Area where ECB was sampled in 2013 (EsC Iberia Central, EsNE Iberia Northeast)

Annex II
Proof for stability and quality of the pest insect lab strain



PCR analyses with different markers for checking if the reference strain of ECB (G.04) is infected with *Nosema* (according to SAEGLITZ, 2004). Red arrows indicating positive control; blue arrows indicating negative control.