Appendix 8. Insect Resistance Monitoring in Iberian collections of *Ostrinia nubilalis* (ECB): 2015 Season

Report

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

- Results for 2015-2016 -

Date

31/07/2016

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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).

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Date / Signature: 31. July 2016 .....

Study director

Date / Signature: 31. July 2016 .....

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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), *O. 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 of MON 810 maize varieties expressing Cry1Ab 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 growing areas in Spain (GONZALEZ-NUNEZ et al., 2000, FARINÓS et al., 2004), Germany (SAEGLITZ et al., 2006) and the United States of America (USA) (MARÇON et al., 1999a, b and 2000). The European Union (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 adoptions 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 and Central Iberia, the area where adoption of MON 810 was greater than 20%.

The objectives of the current report on the 2015 maize growing season are:

1) To determine the susceptibility of *O. nubilalis* in maize growing areas in Northeast 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 and providing increased sensitivity compared to 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 2015 was expected to be greater than 20% are the Ebro valley (defined in earlier reports as Northeast Iberia), Central Iberia (particularly the province of Albacete) and the Southwest Iberia area. For these areas data on the susceptibility of *O. nubilalis* to Cry1Ab have been collected since 2007. In 2015, it was the aim to collect samples from three sites that were separated by at least 50 km in Northeast and Central 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). The aim was to collect 300 healthy larvae from the area sampled.

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 in Southwest Iberia 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 (details see tab. 2) 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 first heat-treated at 43°C for 40 min to reduce *Nosema* infections (SHOWERES et al., 2001) and then 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

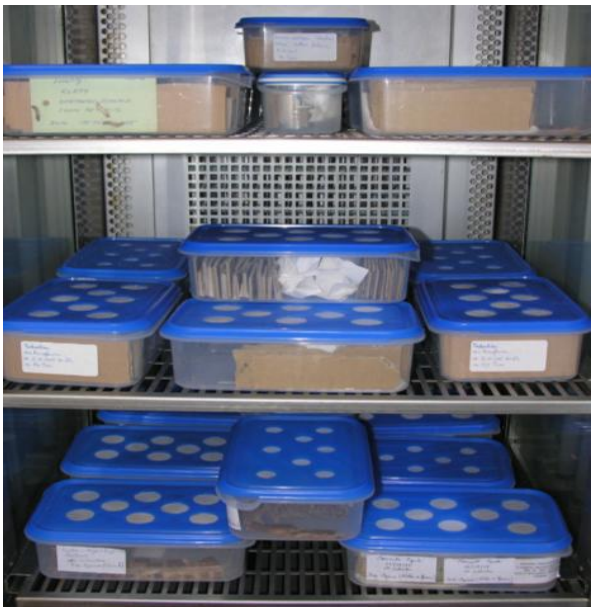


Figure 3. Growth chamber with plastic boxes containing diapausing ECB larvae

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 had 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. 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 site (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) (Figure 5). Eight concentrations (0.2–28.22 ng Cry1Ab/cm²) and a control (bicarbonate buffer) were tested for each population. Field collected insects used in bioassays came from pooled samples of healthy insects collected in different fields within an area. 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 reference strains of *O. nubilalis* to Cry1Ab was assessed using the same stock solution. Then MIC-values obtained for the reference strains were compared with that of the field populations.

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
Ascorbic acid	3 g	BioServ
Vanderzant vitamin mix	2 g	BioServ

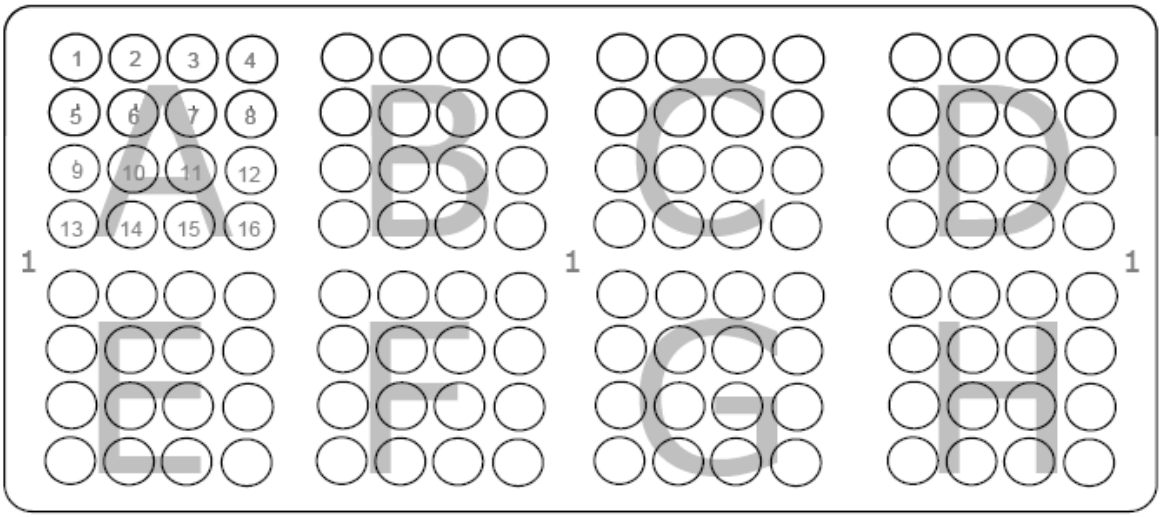


Figure 5. IDs of Bio-Ba-128 trays (tray number, field letter, well number; *e.g.*: 1.A.13)

2.3.2 Diagnostic dose

For the calculation of the diagnostic dose the data for all experiments with ECB from 2005-2012 were used. These include ECB collection 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/cm^2. 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 11.0, 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.

Additionally a Probit analyses using the data for real mortality was conducted to calculate LC-values.

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 2015 is shown in Table 2, and the locations are displayed on a map in Annex I.

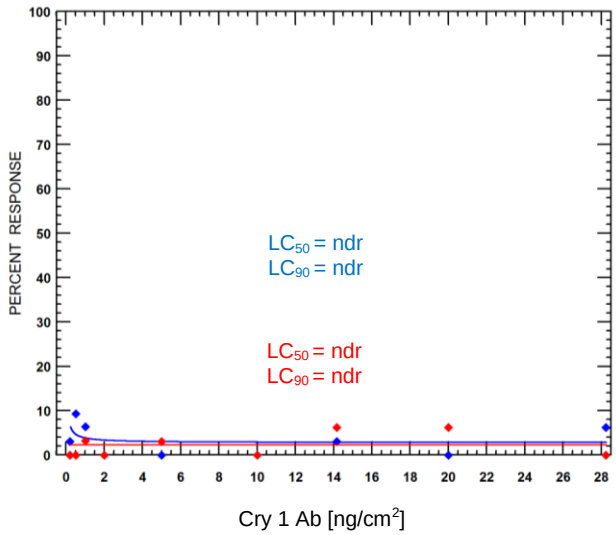
Table 2. *O. nubilalis* collection details for the 2015-2016 season.

Area	ID	Country	Collection site[a]	Collected	Eggs	Larvae
refG [b]	G.04	Germany		2005	x	
refES [c]	ES.ref	Spain		12.2015		145 (*75*)
IbC	ES.07_2015	Spain	ES-02162 La Herrera (AB)	09.2015		106 (*44*)
	ES.15_2015	Spain	ES- 02220 Motilleja (AB)	09.2015		178 (*72*)
	ES.08_2015	Spain	ES- 02328 Santa Ana (AB)	09.2015		159 (*64*)
IbNE	ES.13_2015	Spain	ES- 22212 Alberuela de Tubo (HU)	09.2015		132 (*52*)
	ES.05_2015	Spain	ES- 22591 Candasnos (HU)	09.2015		144 (*63*)
	ES.14_2015	Spain	ES- 31382 Mélida (NA)	09.2015		100 (*37*)

[a] Spanish provinces: AB = Albacete, HU = Huesca, NA = Navarra; [b] reference strain Germany, [c] reference strain Spain; italic number in brackets indicate number of larvae surviving the diapause period, reached the adult stage and mated

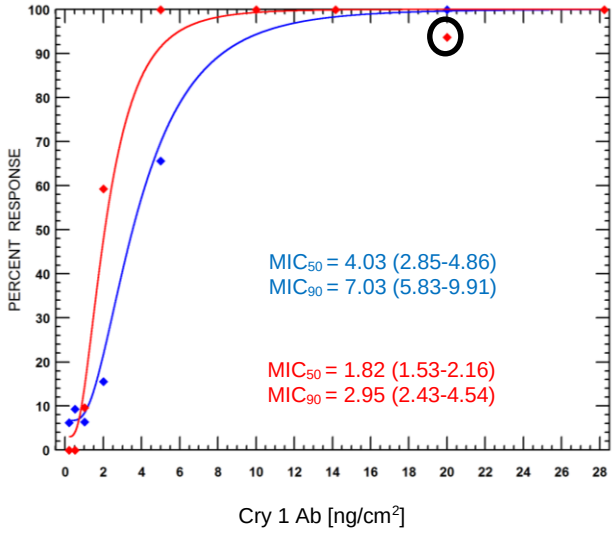
3.2 Susceptibility to Cry1Ab in the 2015-2016 campaign

To determine the susceptibility of the field collections as well as the reference strains to Cry1Ab, larval mortality and larval moult inhibition data at the different concentrations of Cry1Ab tested were studied by Probit analysis. The use of lethal concentrations was recommended by the EFSA GMO Panel (2012). Fitted curves of susceptibility to the Cry1Ab protein of laboratory and field collections of *O. nubilalis* were generated taking into account the lethal concentration (Figures 6, 8 & 10) and the moulting inhibition concentration of neonate larvae after seven days feeding on treated diet (Figures 7, 9, 11). As a dose-response relationship was not found for the mortality of any ECB origin this character will not be used for further discussions. Moulting inhibition concentrations at 50% (MIC_{50}) and 90% (MIC_{90}) for *O. nubilalis* collected in a particular area are provided in Table 3. A dose-response relationship was only calculated for the mortality of the reference strain ES.ref if an outliner-value was taken out of the calculation. This outliner-value is representing two larvae that moulted to the second larval stage after being exposed to a dose of 20 ng/cm^2 (indicated as a black circle in Figure 7). The significance of differences in susceptibility between the reference strains (G.04, originally collected in Niedernberg, Germany, and kept in culture since 2005; ES.ref collected in fields located in Galicia, Northwest Spain (near Barrantes (n=4 larvae), Ponteverda (n=135 larvae) and Ponte Caldelas (n=6 larvae), Spain in 2015) and the field collected insects was tested by determining the 95% confidence limits (CI) of MIC ratios (MICR) (ROBERTSON et al., 2007). The moulting inhibition concentrations are considered significantly different from the laboratory strains ($P < 0.05$) when the MICR 95% confidence limits do not include 1.



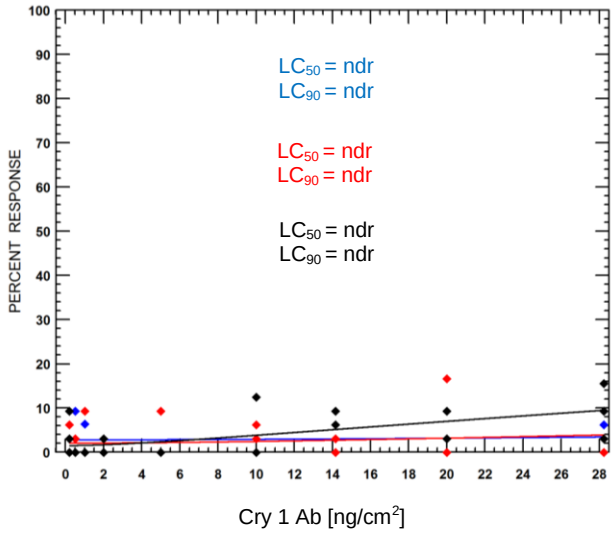
	LC ratios	
	(LCR_{50})	Conf. limits
G.04 vs. ES.ref	-	-
	(LCR_{90})	
G.04 vs. ES.ref	-	-

Figure 6. Fitted curves of susceptibility as percentage mortality after seven days feeding on treated diet of ECB reference strains to the batch 2a of protein Cry1Ab (PoloPlus, LeOra Software 2002-2009).
(Reference strain G.04: blue; reference strain ES.ref: red)



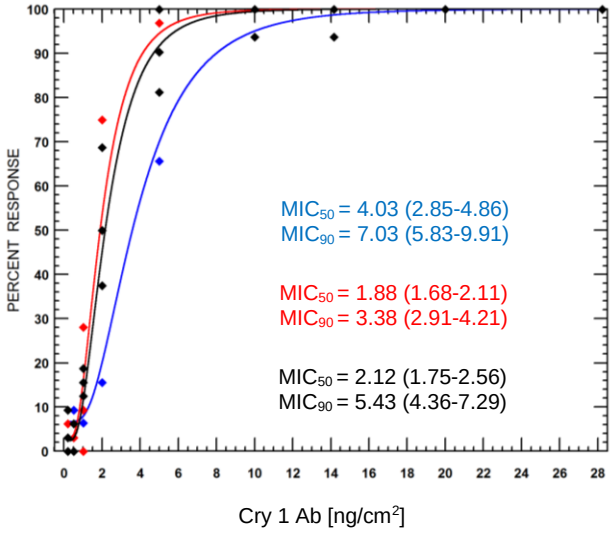
	MIC ratios	
	($MICR_{50}$)	Conf. limits
G.04 vs. ES.ref	2.209	(1.674-2.915)
	($MICR_{90}$)	
G.04 vs. ES.ref	2.383	(1.680-3.380)

Figure 7. Fitted curves of susceptibility as percentage moult inhibition after seven days feeding on treated diet of ECB reference strains to the batch 2a of protein Cry1Ab (PoloPlus, LeOra Software 2002-2009).
(Reference strain G.04: blue; reference strain ES.ref: red; the black circle is indicating an outliner that was not used for the calculation)



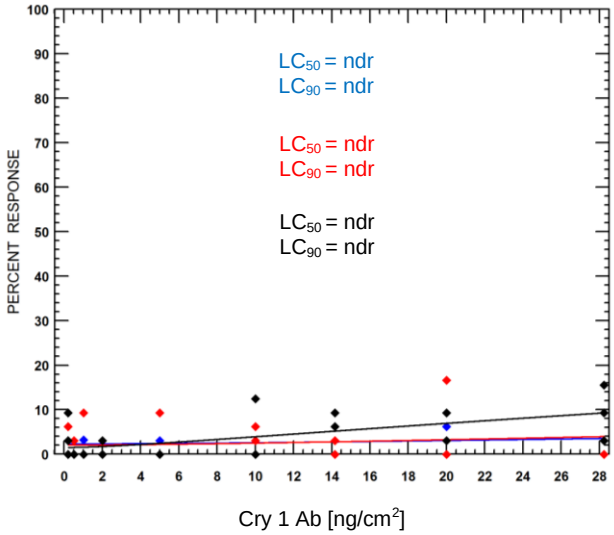
	LC ratios	
	(LCR_{50})	Conf. limits
G.04 vs IbC	-	-
	(LCR_{90})	
G.04 vs. IbC	-	-
	(LCR_{50})	Conf. limits
G.04 vs IbNE	-	-
	(LCR_{90})	
G.04 vs. IbNE	-	-

Figure 8. Fitted curves of susceptibility as percentage mortality after seven days feeding on treated diet of ECB (collected in 2015) to the batch 2a of protein Cry1Ab in comparison to the reference strain G.04 (PoloPlus, LeOra Software 2002-2009).
(Reference strain G.04: blue; IbC: red; IbNE: black)



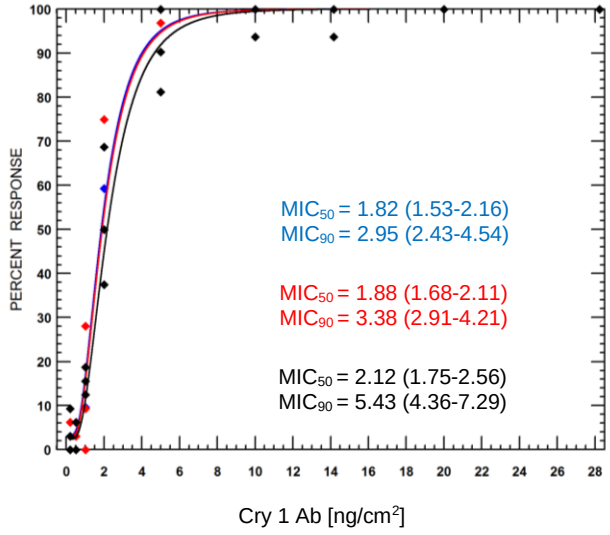
	MIC ratios	
	($MICR_{50}$)	Conf. limits
G.04 vs IbC	2.142	(1.669-2.750)
	($MICR_{90}$)	
G.04 vs. IbC	2.081	(1.577-2.746)
	($MICR_{50}$)	Conf. limits
G.04 vs IbNE	1.897	(1.463-2.461)
	($MICR_{90}$)	
G.04 vs. IbNE	1.295	(0.977-1.717)

Figure 9. Fitted curves of susceptibility as percentage moult inhibition after seven days feeding on treated diet of ECB (collected in 2015) to the batch 2a of protein Cry1Ab in comparison to the reference strain G.04 (PoloPlus, LeOra Software 2002-2009).
(Reference strain G.04: blue; IbC: red; IbNE: black)



	LC ratios	
	(LCR_{50})	Conf. limits
ES.ref vs IbC	-	-
	(LCR_{90})	
ES.ref vs. IbC	-	-
	(LCR_{50})	Conf. limits
ES.ref vs IbNE	-	-
	(LCR_{90})	
ES.ref vs. IbNE	-	-

Figure 10. Fitted curves of susceptibility as percentage mortality after seven days feeding on treated diet of ECB (collected in 2015) to the batch 2a of protein Cry1Ab in comparison to the reference strain ES.ref (PoloPlus, LeOra Software 2002-2009).
(Reference strain ES.ref: blue; IbC: red; IbNE: black)



	MIC ratios	
	($MICR_{50}$)	Conf. limits
ES.ref vs IbC	0.97	(0.81–1.17)
	($MICR_{90}$)	
ES.ref vs. IbC	0.87	(0.64–1.19)
	($MICR_{50}$)	Conf. limits
ES.ref vs IbNE	0.86	(0.71–1.05)
	($MICR_{90}$)	
ES.ref vs. IbNE	0.54	(0.40–0.74)

Figure 11. Fitted curves of susceptibility as percentage moult inhibition after seven days feeding on treated diet of ECB (collected in 2015) to the batch 2a of protein Cry1Ab in comparison to the reference strain ES.ref (PoloPlus, LeOra Software 2002-2009).
(Reference strain ES.ref: blue; IbC: red; IbNE: black)

The MIC_{50} and MIC_{90} values for field-collections of ECB from IbC and the MIC_{50} value for field-collections of ECB from IbNE differed significantly to the reference strain G.04 in their susceptibility to Cry1Ab/cm² as indicated by the lack of inclusion of 1 in their MICR 95% confidence limits (Figure 9). In comparison to the reference strain ES.ref the MIC_{90} value for field-collections of ECB from IbNE differed significantly (Figure 11).

Table 3. Results from Probit analysis for the ECB origins collected in 2015.

Area	n	Slope ± SE	χ^2	D.f.	MIC_{50} (95% CI) [a]	MIC_{90} (95% CI) [a]
refG	351	5.303 ± 1.271	2.37	7	4.03 (2.85-4.86)	7.03 (5.83-9.91)
refES	351	6.140 ± 1.406	4.23	7	1.82 (1.53-2.16)	2.95 (2.43-4.54)
IbC	1054	5.040 ± 0.522	29.71	25	1.88 (1.68-2.11)	3.38 (2.91-4.21)
IbNE	1055	3.144 ± 0.221	52.97	25	2.12 (1.75-2.55)	5.43 (4.36-7.29)*

[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 significantly different ($P < 0.05$) to the reference strain G.04.

3.3 Diagnostic dose

From both reference strains and from each origin sampled 32 larvae were exposed to the diagnostic dose for ECB larvae from Europe (determined to be 28.22 ng/cm^2) and also to a dosage representing 50% of diagnostic dose. Not a single larva tested in 2016 survived the diagnostic dose but two larvae from IbNE (Alberuela de Tubo) survived a dosage representing 50% of diagnostic dose. These larvae were used for a confirmatory experiment.

3.4 Exposure to MON 810 tissue (confirmatory experiment)

All *O. nubilalis* larvae from field collections that survived the bioassay at the highest dose or diagnostic dose were subject to a confirmatory experiment where they were assembled, transferred to plastic boxes, provided with newly detached MON 810 maize leaves without the central nerve, and fed *ad libitum* to record any survivors. For the season reported here, only two surviving larvae were found after 7 days of exposure to a dosage representing 50% of diagnostic dose (14.11 ng/cm^2) and two exposed to a dosage of 20 ng/cm^2. A confirmatory experiment was conducted with these larvae. Each of them died after feeding on *Bt* maize within 2 days.

3.5 Historical susceptibility of corn borers to Cry1Ab

During 2008–2016, 37 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)
	2015[3]	1.88 (1.68-2.11)	3.38 (2.91-4.21)
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)
	2015[3]	2.12 (1.75-2.55)	5.43 (4.36-7.29)
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)
	2014[3]	1.32 (0.94-1.74)	3.80 (2.78-6.21)

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 reference strain G.04 to Cry1Ab

The reference strain G.04 was kept in the laboratory in sub-strains since 2011 and checked regularly for performance (size of adults, size of egg masses, and development of larvae). In 2011, by performing 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 subsequent years until now. This sub-strain is producing good-quality egg masses and normal-sized adults. A PCR analysis (done in March 2015) showed that the reference strain G.04 kept since 2011 is not infected with microsporidia or with *Nosema* (Fig. A2).

3.7 Susceptibility of the reference strain ES.ref to Cry1Ab

The reference strain ES.ref was established as a second reference strain with ECB collected in December 2015 in fields without *Bt* maize. This strain has also been checked for infection with *Nosema.* Applying the PCR based method (SAEGLITZ, 2004) there were no individuals identified as being infected with microsporidia or with *Nosema* (Fig. A2). Not taking into consideration the two larvae reaching the next developmental stage after being exposed to 20 ng/cm^2 this reference strain showed higher susceptibility than the other reference strain (G.04).

4 Conclusions

In 2016, ECB larvae from 2 areas each with 3 samples of ECB collected in 2015 were analysed. Thus far, susceptibility to Cry1Ab has been assessed for two reference strains and ECB collected in maize fields in Central as well as Northeast Iberia. ECB larvae were exposed to artificial diet treated with increasing Cry1Ab concentrations, and mortality and growth inhibition were evaluated after 7 days. Calculation of LC-values was not possible as a dose response relationship could not be identified and the highest dose tested did not induce mortality above 20%. Variation in Cry1Ab susceptibility (MIC_{50}) of ECB collected in the Central and Northeast Iberian fields during the campaign 2015-2016 was 1.64-fold and 1.68-fold, respectively. Variation in Cry1Ab susceptibility (MIC_{90}) of ECB collected in the Central and Northeast Iberian fields during the campaign 2015-2016 was 1.49-fold and 2.85-fold, respectively. Variation in Cry1Ab susceptibility (MIC_{50} and MIC_{90}) of field samples in comparison with the reference strain G.04 was up to 0.65-fold and 1.11-fold, respectively. Variation in Cry1Ab susceptibility (MIC_{50} and MIC_{90}) of field samples in comparison with the reference strain ES.ref was up to 1.44-fold and 2.65-fold, respectively. Significant differences in *Bt* susceptibility between ECB from Northeast and Central Iberia and the reference strain G.04 but also between Northeast Iberia and ES.ref were found. The 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.

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Annex I
Areas of collection activities for ECB in 2015

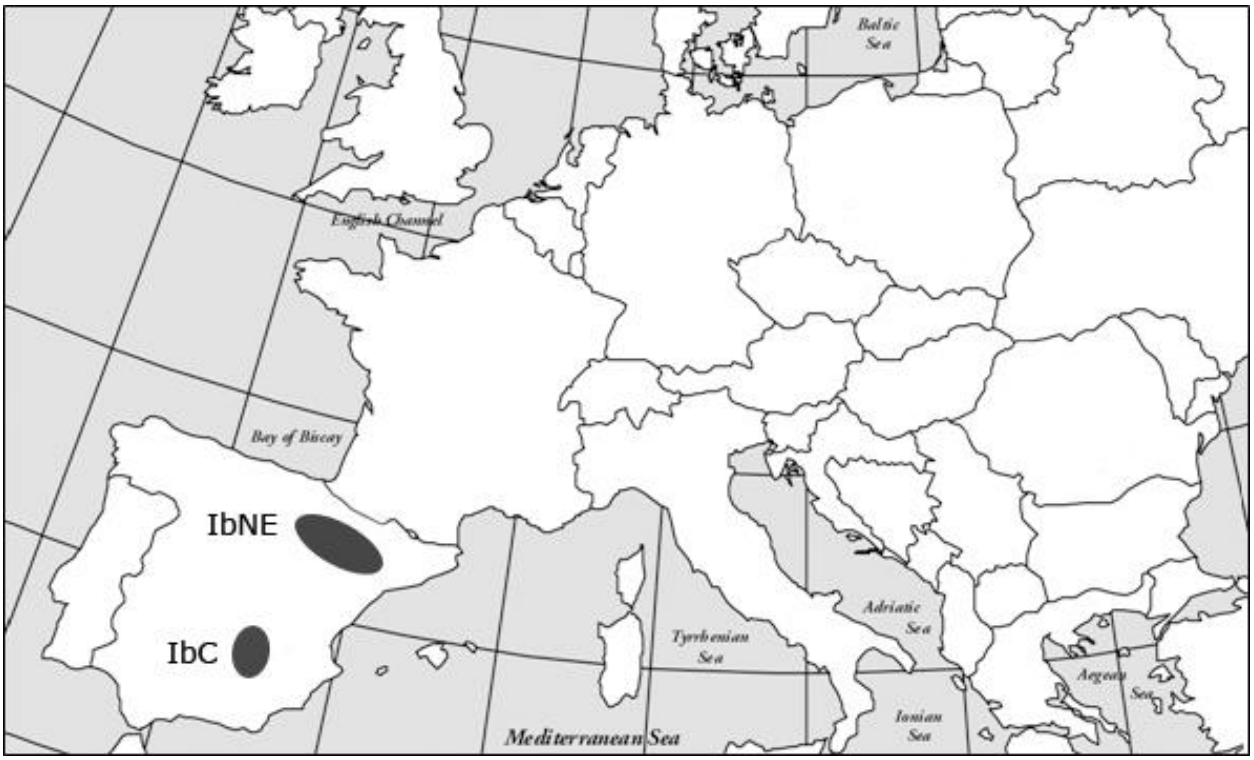

Figure A1. Area where ECB was sampled in 2015 (Iberia Central and Northeast)

Annex II
Proof for stability and quality of the pest insect reference strains

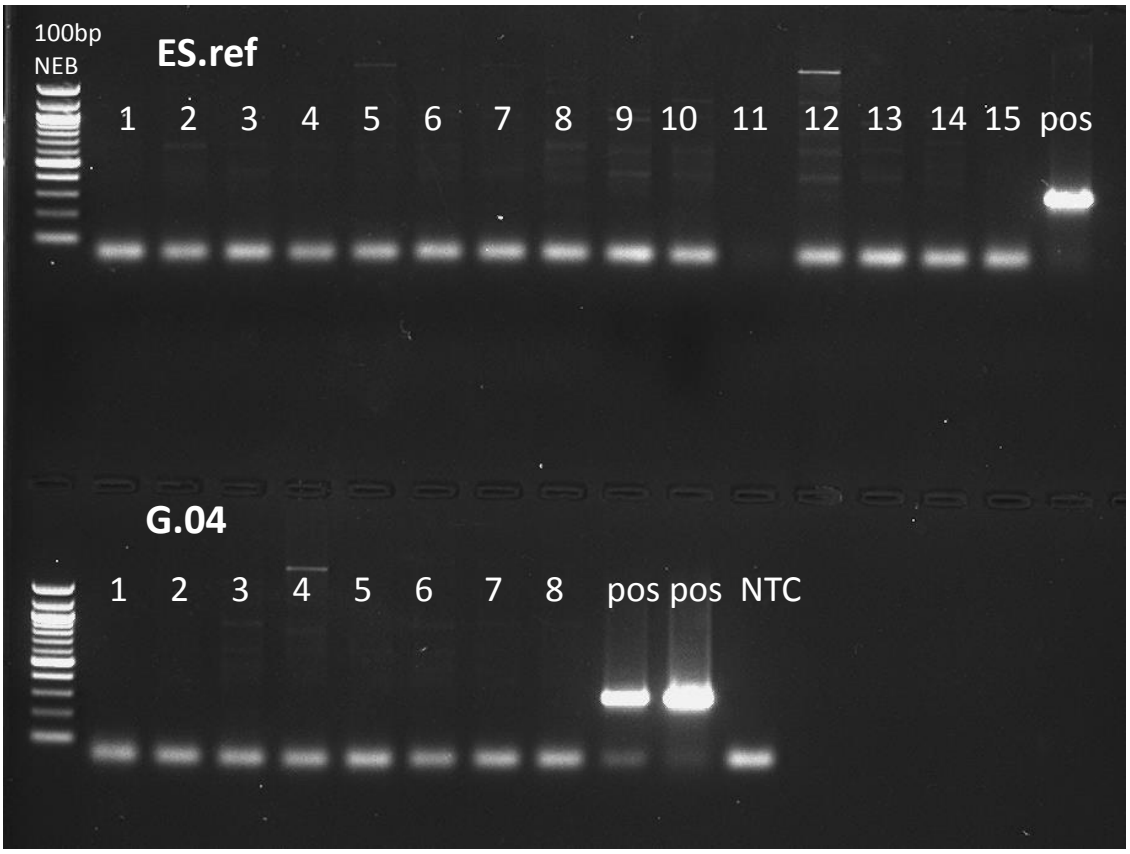

Figure A2. PCR analyses for checking if the reference strains of ECB (ES.ref (Spain) and G.04 (Germany)) is infected with *Nosema* (according to SAEGLITZ, 2004).

(pos: positive control, NTC no template control; ES.ref 1-7: two singe larvae each, ES.ref 8 one single pupa, ES.ref 9-15 one single larva each; G.04 1 one single larva, G.04 2-8 one pupa each; ES.ref 11 PCR reaction failed)