Report

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

- Results for 2016-2017 -

Date

21/08/2017

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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: 21. August 2017 ....

Study director

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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 2012 and 2017). 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 2017 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, have been reported in the previous years on a biennial basis in areas where MON 810 is grown. As the diagnostic dose method has been established for *O. nubilalis* populations collected in different maize growing areas in Europa (THIEME et al., 2017) this method was applied for the season reported here. Samples were taken in Northeast Iberia, the area where adoption of MON 810 was greater than 20%.

The objectives of the current report on the 2016 maize growing season are:

1) To determine the susceptibility of *O. nubilalis* in maize growing areas in Northeast Iberia to the Cry1Ab protein 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) As requested by EFSA a "negative control" using leaf segments of maize was applied.

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 2016, it was the aim to collect samples from three sites that were separated by at least 50 km in 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). The aim was to collect 350 healthy larvae from the area sampled.

2.2 Insect culture

Two reference strains are kept in culture. 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).

Larvae and adults of these animals are maintained in a climate cabinet at 25°C, a humidity of 90% RH and a photoperiod of 20:4 h (L:D). Egg masses to incubate were first heat-treated at 43°C for 40 min to reduce *Nosema* infections (SHOWERS et al., 2001) and then placed in an incubator at 25 ± 2°C until all larvae hatched.

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

These field-collected larvae were placed in plastic boxes containing corrugated cardboard and artificial agar-based diet (Figure 2 and 3, Table 1) and maintained in a growth chamber at 20°C, 70% RH and a photoperiod of 20:4h (L:D) for 5 days. Temperature then was decreased to 15°C for 12 days at a photoperiod of 12:12h (L:D). Afterwards the larvae were transferred to another climatic chamber and 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.

Larvae surviving the diapause period were transferred to fresh containers and placed in a climate chamber where temperature was raised gradually from 15 to 25°C at a humidity of 90% RH and a photoperiod of 20:4h (L:D) over a period of 10 days and kept at 25°C, a 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 daily. 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 (SHOWERS et al., 2001) and then placed in an incubator at 25 ± 2°C until all larvae hatched.

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 (50 mmol/l) with pH 10.25 was used. This batch had reached the date of expiry therefore 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. As this batch had also reached the date of expiry a new batch (2b) was re-supplied by Monsanto. The batch 2b (NBR: 11247229, 31/01/2020; 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 concentration, a bicarbonate buffer (50 mmol/l) with pH 10.25 was used. The bioassays were performed in 128 well trays (BAW128, Frontier Agricultural Sciences). 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 (BACV16, Frontier Agricultural Sciences) (Figure 5). A single discriminating concentration (28.22 ng Cry1Ab/cm^2) and a control (bicarbonate buffer) were tested for each population. 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 ngCry1Ab/cm^2 (*see* report 2013). Data from bioassays with more than 20% response at the control after exposure to buffer have been neglected. 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 discriminating concentration of Cry1Ab tested were studied. In total 1562 larvae were exposed to the discriminating concentration. In the control (50 mM bicarbonate buffer, ph 10.25) 223 larvae were tested.

Field collected insects used in bioassays came from pooled samples of healthy insects collected in different fields within an area. 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 moulting inhibition used in this study accounts for both death and complete moulting (or growth) inhibition.

Table 1. *O. nubilalis* diet recipe

Component	Amount	Provided
Distilled H2O	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

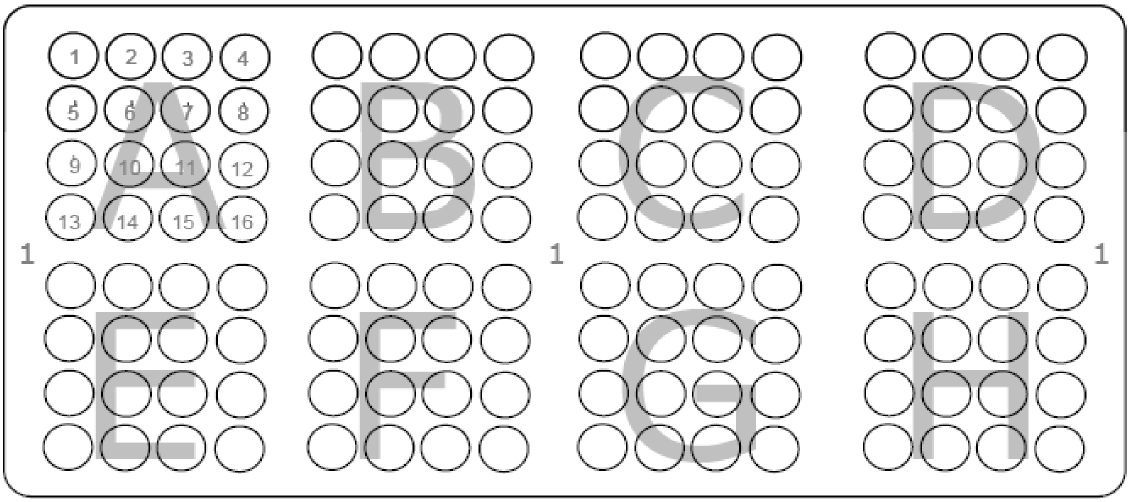


Figure 5. IDs of BAW128 trays (tray number, field letter, well number; *e.g.*: 1.A.13)

2.4 Exposure to maize tissue not expressing Bt protein (negative control)

As requested by EFSA a negative control was performed in 128 well trays (BAW128, Frontier Agricultural Sciences). 1 ml of an agar solution (2.5 %) was dispensed in each cell. After solidification a detached disc of maize leave (variety Golden Bantam) without central nerve and 13 mm in diameter and a neonate larva not older than 12 hours was added to each cell which then was closed with a lid (BACV16, Frontier Agricultural Sciences). The trays were kept in a climate cabinet at 25°C. After 3 days mortality and developmental stage of the larvae were recorded.

2.5 Susceptibility of the reference strains G.04 and ES.ref to Cry1Ab

To prepare the test concentration, a bicarbonate buffer (50 mmol/l) with pH 10.25 was used. The bioassays were performed in 128 well trays (BAW128, Frontier Agricultural Sciences). In each cell 1 ml of artificial diet was dispensed (see above 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 reference strain 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 (BACV16, Frontier Agricultural Sciences).

Each strain was tested with eight concentrations (0.2–28.22 ng Cry1Ab/cm²) and a control (bicarbonate buffer). Per concentration 32 larvae were tested (64 for controls). 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.

2.6 Exposure to MON 810 tissue (confirmatory experiment)

For larvae developing beyond first larval stage at the diagnostic dose (28.22 ng/cm²) a confirmatory experiment is planned. Therefore these larvae should be fed *ad libitum* with MON 810 leaves. The exposure to MON 810 is to be performed in 128 well trays (BAW128, Frontier Agricultural Sciences). 1 ml of an agar solution (2.5 %) is dispensed in each cell. After solidification a detached disc of maize leave (MON 810) without central nerve and 13 mm in diameter and a larva that grew beyond first larval stage will be added to each cell which then will be closed with a lid (BACV16, Frontier Agricultural Sciences). The trays were kept in a climate cabinet at 25°C.

2.7 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.

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. The significance of differences in the susceptibility of the reference strains was tested by determining the 95% confidence limits (CL) of the MIC ratios (MICR) (ROBERTSON et al., 2007). Concentrations are significantly different ($P < 0.05$) if the MICR 95% confidence limits do not include 1.

3 Results and Discussion

3.1 Collection of ECB

The area where ECB larvae were collected in 2016 is shown in Table 2, and the locations are displayed on a map in Annex I. In total 30 oviposition cages were used. Of the 1111 larvae of ECB collected in the Ebro valley Spain, 554 adults survived the diapause period, reached the adult stage and mated, meaning that 49.9 % of the field collected larvae were represented in the bioassay. Therefore, the detection limit for resistance allele frequency in 2016 was 4.25 % calculated based on the model developed by ANDOW & IVES (2002).

Table 2. *O. nubilalis* collection details in Spain for the 2016-2017 season.

Area	ID	Collection site[a]	Collected	Eggs	Larvae
refG [b]	G.04	German reference	2005	x	
refES [c]	ES.ref	Spanish reference	12.2015		145 (*75*)
IbNE	ES.13_2016	ES-22250 Lanaja 1 (HU)	12.-15.09.2016		166 (*75*)
	ES.13_2016	ES-22250 Lanaja 3 (HU)	12.-15.09.2016		78 (*25*)
	ES.13_2016	ES-22250 Lanaja 5 (HU)	12.-15.09.2016		112 (*56*)
	ES.13_2016	ES-22200 Sariñena 1 (HU)	12.-15.09.2016		122 (*49*)
	ES.11_2016	ES-50100 La Almunia de Doña Godina 1 (Z)[d]	03.-05.10.2016		393 (*230*)
	ES.11_2016	ES-50100 La Almunia de Doña Godina 3 (Z)[d]	03.-05.10.2016		
	ES.11_2016	ES-50100 La Almunia de Doña Godina 1 (Z)	20.10.2016		
	ES.14_2016	ES-31150 Mendigorria 1 (NA) [e]	06.-07.10.2016		192 (*83*)
	ES.14_2016	ES-31150 Mendigorria 2 (NA) [e]	06.-07.10.2016		
	ES.14_2016	ES-31140 Artajona 1 (NA)	06.-07.10.2016		48 (*36*)

[a] Spanish provinces: HU = Huesca, NA = Navarra, Z = Zaragoza; [b] reference strain Germany, [c] reference strain Spain; [d] larvae of these fields were merged and sent together, [e] larvae of these fields were merged and sent together; 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 2016-2017 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 discriminating concentration of Cry1Ab tested (determined to be 28.22 ng/cm^2) were studied. Of the 1562 larvae exposed to the discriminating concentration 3 larvae died, 1547 survived but did not reach the 2nd larval stage, and 12 reached the second larval stage and were used for a confirmatory experiment. The resulting moulting inhibition was 99.23 %. Of 223 larvae exposed to the control 1 larva died and 222 developed to older larval stages larvae. The moulting inhibition calculated was 0.45 %.

3.3 Exposure to maize tissue not expressing Bt protein (negative control)

192 fresh hatched neonate larvae of *O. nubilalis* were fed ad libitum with maize cv. Golden Bantam for three days. Two of the larvae died (1.04 %), 157 reached the second larval stage and 33 (17.19 %) didn´t reach the second larval stage but were healthy and fed on the maize leaves. It might be possible that the exposure time was too short for each larva to reach the next developmental stage.

3.4 Exposure to MON 810 tissue (confirmatory experiment)

All *O. nubilalis* larvae from field collections that survived the bioassay at the diagnostic dose were subject to a confirmatory experiment. For the season reported here, 12 larvae developed to the second larval stage after 7 days of exposure to a dosage representing the diagnostic dose (28.22 ng/cm^2). The confirmatory experiment conducted showed that each of these larvae died after feeding on *Bt* maize within 5 days.

3.5 Historical susceptibility of corn borers to Cry1Ab

During 2008–2016, 44 samples of ECB from different areas were analyzed. Their susceptibility to Cry1Ab is shown in Table 3.

Table 3. Susceptibility of *O. nubilalis* neonates exposed to Cry1Ab as measured by the MIC or response to DC (RDC) for areas tested.

Area	Year	RDC (%)	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)
	2016[4]	99.23		
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, [4] batch 2b of Cry1Ab

3.6 Susceptibility of the reference strains G.04 and ES.ref to Cry1Ab

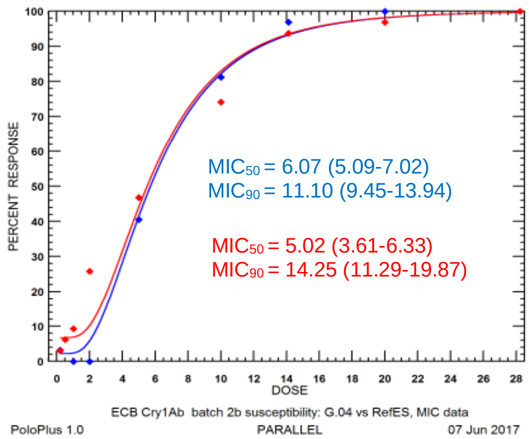
The reference strain G.04 was kept in the laboratory in sub-strains since 2011 and checked regularly for performance (size of adults, size of egg masses, and development of larvae). In 2011, by performing a PCR based method (SAEGLITZ, 2004), infection with *Nosema* was identified for some individuals 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. The last PCR analysis (done in June 2016) showed that the reference strain G.04 is not infected with microsporidia or with *Nosema* (Fig. A2).

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).

Fitted curves of susceptibility to the Cry1Ab protein of laboratory reference strains of *O. nubilalis* were generated taking into account the moulting inhibition concentration of neonate larvae after seven days feeding on treated diet (Figures 6). 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* kept as reference strains area are provided in Table 4.

A dose-response relationship was calculated for the moulting inhibition of the reference strains ES.ref and G.04. 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) was tested by determining the 95% confidence limits (CI) of MIC ratios (MICR) (ROBERTSON et al., 2007). The MIC_{50} and MIC_{90} values for the reference strains of ECB from Germany (G04) and Spain (ES.ref) did not differ significantly in their susceptibility to Cry1Ab/cm² (Figure 6) as indicated by the inclusion of 1 in their MICR 95% confidence limits.



Cry 1 Ab [ng/cm²]

	MIC ratios	
	($MICR_{50}$)	Conf. limits
G.04 vs. ES.ref	1.211	(0.886-1.654)
	($MICR_{90}$)	
G.04 vs. ES.ref	0.779	(0.506-1.083)

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

Table 4. Results from Probit analysis for the ECB reference strain tested in the season 2016-2017.

Area	n	Slope ± SE	χ^2	D.f.	MIC_{50} (95% CI) [a]	MIC_{90} (95% CI) [a]
refG	351	4.893 ± 0.685	5.66	7	6.07 (5.09-7.02)	11.10 (9.45-13.94)
refES	351	2.825 ± 0.421	4.45	7	5.02 (3.61-6.33)*	14.25 (11.29-19.87)*

[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 reference strain G.04.

4 Conclusions

In 2016, ECB larvae from 1 area with 3 sample sites of ECB were analyzed. Thus far, susceptibility to Cry1Ab has been assessed for two reference strains and ECB collected in maize fields in Northeast Iberia. ECB larvae were exposed to artificial diet treated with the discriminating concentration, and mortality and growth inhibition were evaluated after 7 days. Of the 1562 larvae exposed to the discriminating concentration 3 larvae died, 1547 survived but did not reach the 2nd larval stage, and 12 reached the second larval stage and were used for a confirmatory experiment. The resulting effect of Cry1Ab on moulting inhibition (this criterion used accounts for both death and complete moulting (or growth) inhibition) was 99.23 %. In the confirmatory experiment, all of these larvae died after feeding on *Bt* maize within 5 days. Therefore, no evidence for a decrease of Cry1Ab susceptibility of ECB during the monitoring duration could not be detected.

5 Acknowledgement

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Annex I
Areas of collection activities for ECB in 2016

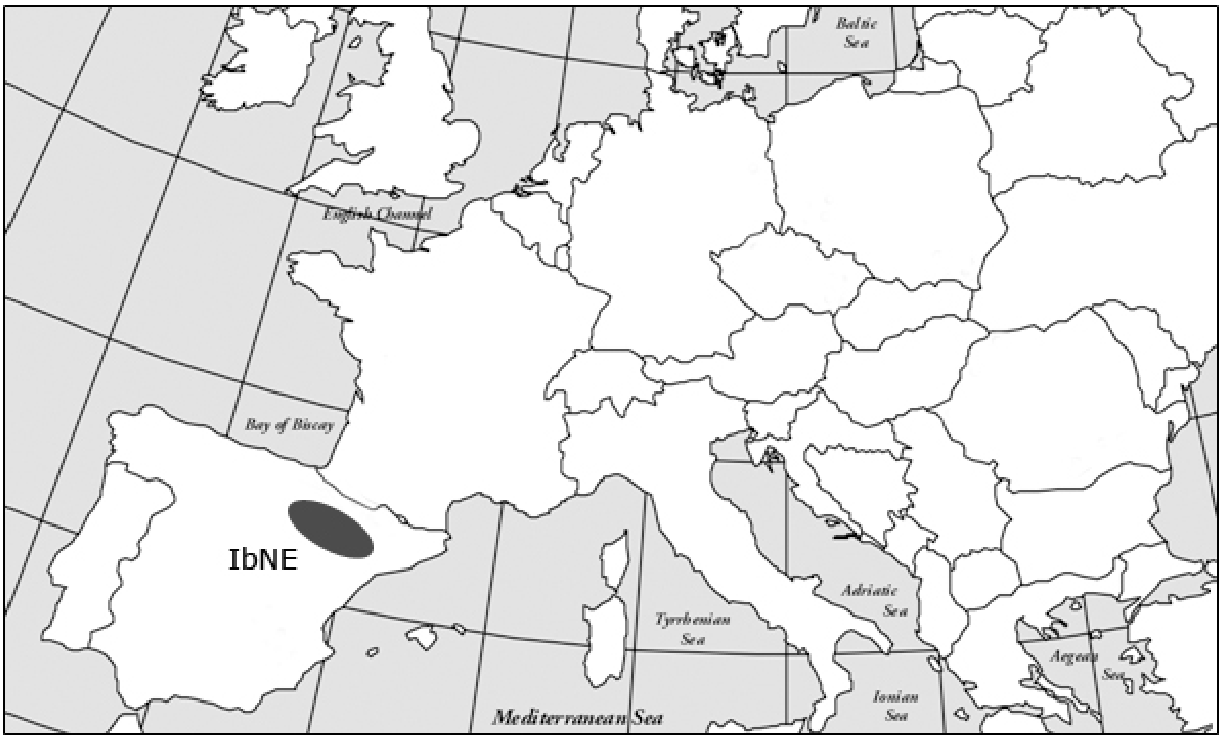

Figure A1. Area where ECB were sampled in 2016 (Iberia Northeast)

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

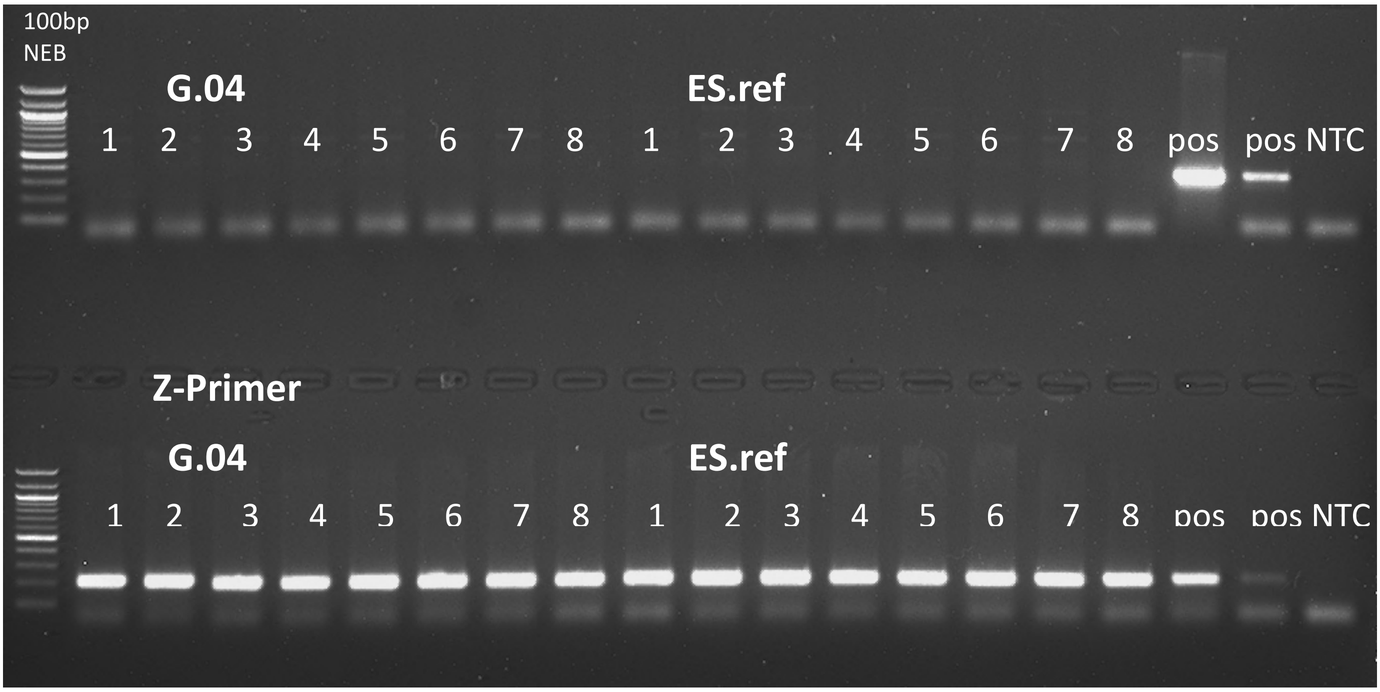

Figure A2. PCR analyses for checking if the reference strains of G.04 (Germany) and ECB (ES.ref (Spain)) are infected with *Nosema* (according to SAEGLITZ, 2004).

(pos: positive control, NTC: no template control; G.04: 1-4 each with five larvae (Lv1) pooled, 5-8 each with 10 larvae (Lv1) pooled; ES.ref: 1-4: each with five larvae (Lv1) pooled, 5-8 each with 10 larvae (Lv1) pooled; Z-Primer was applied to check that the DNA was not destroyed)