Report

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

- Results for 2018-2019 -

Date

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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: 10. September 2019 .....

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, Bayer 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, 2017 and 2019). 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 2019 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 60%.

The objectives of the current report on the 2018 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.
3) As requested by EFSA a “positive control” using leaf segments of maize expressing *Bt* protein 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 2018, it was the aim to collect samples from three sites that were separated by at least 50 km in Northeast Iberia. The monitoring efforts have been concentrated in Northeast Iberia based on EFSA opinion as there is high adoption rate of MON 810 in this region (> 60%) and so, here is resistance more likely to develop. *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 each sampling site.

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 isolated as bioassays were to be conducted separately for each sampling site and 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 Bayer 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 expiry date specified by the manufacturer, therefore a new batch (2a) was provided by Bayer. 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 specified by the manufacturer a new batch (2b) was re-supplied by Bayer. 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. What has been used as batch 2 (2, 2a, 2b) are different “batches” of a protein but all from the same original production i.e., they are all sub-samples from the same protein source. As they were stored and delivered at different times (with different expiry dates specified by the manufacturer), experiments were done demonstrating equivalence in biological activity of the protein batches 2a and 2b. This information was provided to EFSA during Q/A two years before.

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/cm²) 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/cm² (*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 1,768 larvae from north-Iberian collection sites were exposed to the discriminating concentration. In the control (50 mM bicarbonate buffer, pH 10.25) 368 larvae were tested.

Field collected insects used in bioassays came from 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.

Cry1Ab bioassays as well as control and negative control bioassays ran in parallel. All those larvae not used for these bioassays were exposed to maize plants expressing *Bt* protein as positive control as described in section 2.3.4.

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

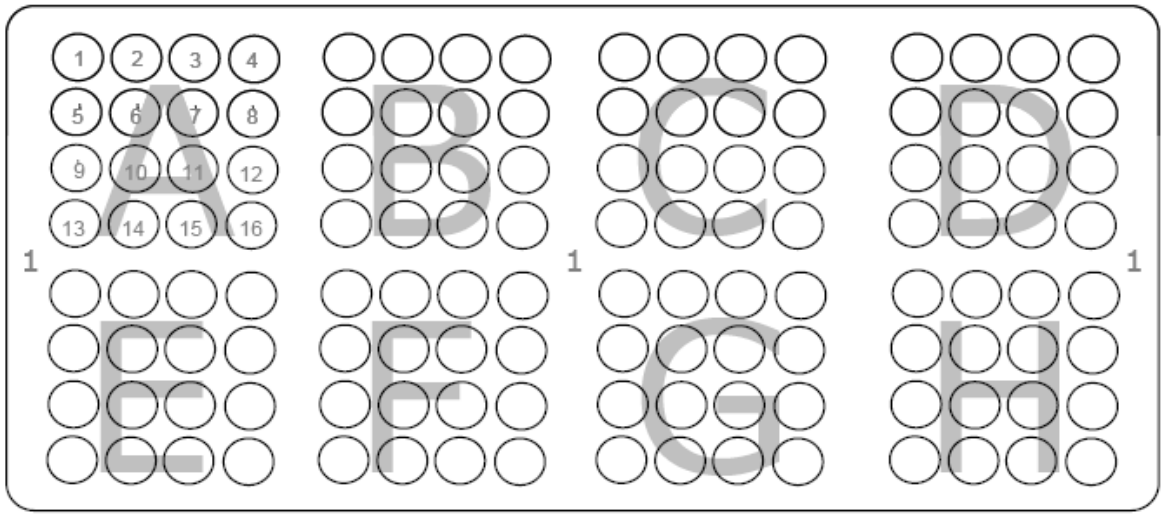


Figure 5. IDs of BAW128 trays (tray number, field letter, well number; *e.g.*: 1.A.13)

2.3.2 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) for up to seven days. 1 ml of an agar solution (2.0 %) containing sorbic acid, benzoic acid and methyl paraben (each 0.1 %) to prevent leaf degradation and microbial contamination 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.

2.3.3 Exposure to non-GM maize tissue (negative control)

As requested by EFSA (2018) a negative control was performed in 128 well trays (BAW128, Frontier Agricultural Sciences). 1 ml of an agar solution (2.0 %) containing sorbic acid, benzoic acid and methyl paraben (each 0.1 %) to prevent leaf degradation and microbial contamination 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 (from IbNE, the population that was also used for analysing the susceptibility to Cry1Ab) 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 seven days mortality and developmental stage of the larvae were recorded. Cry1Ab bioassays and negative control bioassays ran in parallel, but MON810 was only applied for larvae reaching the 2nd larval stage at the diagnostic dosage of the Cry1Ab bioassay, to check if they would survive the much higher content of protein present in MON810.

2.3.4 Exposure to maize tissue expressing *Bt* protein (positive control)

As requested by EFSA (2018) a positive control was performed with MON 810 leaves. The exposure to MON 810 is to be performed in plastic boxes with a diameter of 12 cm for up to seven days. 30 ml of an agar solution (2.0 %) containing sorbic acid, benzoic acid and methyl paraben (each 0.1 %) to prevent leaf degradation and microbial contamination is dispensed in each box. After solidification 3 detached leaves of maize (MON 810) without central nerve and a maximum of 300 freshly hatched larvae not older than 12 hours were added to each box. The boxes were kept in a climate cabinet at 25°C.

2.3.5 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 nine 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.

2.4 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 use the bioassay for 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 2018 is shown in Table 2, and the locations are displayed on a map in Annex I. In total 61 oviposition cages were used. Of the 1,144 larvae of ECB collected in the Ebro valley Spain, 534 specimens survived the diapause period, reached the adult stage and mated, meaning that 46.7 % of the field collected larvae were represented in the bioassay. Therefore, the detection limit for resistance allele frequency in 2018 was 4.32 % calculated based on the model developed by ANDOW & IVES (2002).

Table 2. *O. nubilalis* collection details in Spain for the 2018-2019 season.

Area	ID	Collection site[a]	Distance to the nearest MON810 field (m) [d]	Collected	Eggs	Larvae [e]
refG [b]	G.04	German reference		2005	x	
refES [c]	ES.ref	Spanish reference		12.2015		145 (*75*)
IbN	ES.13_201	ES-22250 Lanaja 1 (HU)	5	25.09.2018		20 (*15*)
	ES.13_201	ES-22250 Lanaja 3 (HU)	500	26.09.2018		130 (*40*)
	ES.13_201	ES-22251 Cantalobos (HU)	20	25.09.2018		121 (*55*)
	ES.13_201	ES-22213 San Juan de Flumen	300	26.09.2018		209 (*96*)
	ES.05_201	ES-22592 Peñalba (HU)	0	18.09.2018		143 (*53*)
	ES.05_201	ES-22591 Candasnos 1 (HU)	100	19.09.2018		214 (*126*)
	ES.05_201	ES-22591 Candasnos 3 (HU)	n.d.	18.09.2018		10 (*6*)
	ES.14_201	ES-31460 Aibar 1 (NA)	n.d.	17.10.2018		11
	ES.14_201	ES-31460 Aibar 2 (NA)[d]	n.d.	17.10.2018		12 (*14*)
	ES.14_201	ES-31400 Sangüesa 1 (NA)	n.d.	17.10.2018		159 (*75*)
	ES.14_201	ES-31400 Sangüesa 1 (NA)	n.d.	17.10.2018		115 (*54*)

[a] Spanish provinces: HU = Huesca, NA = Navarra; [b] reference strain Germany; [c] reference strain Spain; [d] there could be other nearer fields that are not known by the technician and/or the farmer, "0" means that it is adjacent to a MON 810 field, "n.d." means that we do not have this information; [e] larvae sent to Germany after removing those that were damaged or seemed to have some pathogens; 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 2018-2019 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 (Table 3).

Table 3. Response of *O. nubilalis* larvae in the 2018-2019 season.

Coll. Site	Test*	Larvae used (n)	Dead	L1	L2	L3	L4	MORT (%)	MI (%)
Lanaja 1	Cry1Ab	80	4	76				5.00	100.00
	Control	32	0	0	2	16	14	0.00	0.00
	- control	32	1	0	13	18		3.13	3.13
	+ control	508	508					100.00	100.00
Lanaja 3	Cry1Ab	200	6	194				3.00	100.00
	Control	48	0	0	1	25	22	0.00	0.00
	- control	48	0	0	23	25		0.00	0.00
	+ control	950	950					100.00	100.00
Cantalobos	Cry1Ab	176	6	169	1			3.41	99.43
	Control	32	0	0	0	12	20	0.00	0.00
	- control	32	0	0	11	21		0.00	0.00
	+ control	1,277	1277					100.00	100.00
San Juan de	Cry1Ab	288	8	280				2.78	100.00
Flumen	Control	48	0	0	0	26	22	0.00	0.00
	- control	48	0	0	18	30		0.00	0.00
	+ control	1,410	1410					100.00	100.00
Peñalba	Cry1Ab	192	19	173				9.90	100.00
	Control	32	0	0	1	21	10	0.00	0.00
	- control	32	0	0	15	17		0.00	0.00
	+ control	704	704					100.00	100.00
Candasnos 1	Cry1Ab	288	11	275	2			3.82	99.31
	Control	48	0	0	1	23	24	0.00	0.00
	- control	48	0	0	19	29		0.00	0.00
	+ control	1,485	1485					100.00	100.00
Candasnos 3	Cry1Ab	64	7	57				10.94	100.00
	Control	32	0	0	1	15	16	0.00	0.00
	- control	32	0	0	18	14		0.00	0.00
	+ control	692	692					100.00	100.00
Aibar 1+2	Cry1Ab	96	1	95				1.04	100.00
	Control	32	0	0	0	21	11	0.00	0.00
	- control	32	0	1	18	13		0.00	3.13
	+ control	495	495					100.00	100.00
Sangüesa 1	Cry1Ab	224	7	217				3.13	100.00
	Control	32	0	0	1	20	11	0.00	0.00
	- control	32	0	0	12	20		0.00	0.00
	+ control	1,518	1518					100.00	100.00
Sangüesa 2	Cry1Ab	160	7	153				4.38	100.00
	Control	32	0	0	0	15	17	0.00	0.00
	- control	32	1	0	13	18		3.13	3.13
	+ control	1,228	1228					100.00	100.00
Total	Cry1Ab	1,768	76	1689	3			4.30	**99.83**
	Control	368	0	0	7	194	167	0	
	- control	368	2	1	160	205		0.54	
	+ control	10,267	10,267					100	

* Bioassays: "Cry1Ab" = 28.22 ng/cm2 Cry1Ab protein, "Control" = 50 mM bicarbonate buffer (pH 10.25), "- control" = non-GM maize leaves, "+ control" = MON 810 leaves. Total number of larvae tested: 12,771. Test duration: 7 days

Of the 1768 larvae exposed to the discriminating concentration 76 larvae died, 1689 survived but did not reach the 2nd larval stage, and 3 reached the second larval stage and were used for a confirmatory experiment (Table 3). The resulting moulting inhibition was 99.83 %. Of 368

larvae exposed to the control not a single larva died, each larva developed beyond first larval stage (L2 n=7 (1.90%), L3 n=194 (52.72%), L4 n=167 (45.83%)). Any moulting inhibition was not observed.

3.3 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 (Table 3). For the season reported here, 3 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 7 days.

3.4 Exposure to non-GM maize tissue (negative control)

368 freshly hatched neonate larvae of *O. nubilalis* from the same population as tested in Cry1Ab bioassays and in MON810 leaf bioassays were fed with maize cv. Golden Bantam for seven days. Two of the larvae died (0.54 %), 160 reached the second larval stage (43.48 %) and 205 (55.71 %) developed to the third larval stage (Table 3). Since the feeding activity of the larvae was very high and additional leaf material was not added, many larvae remained in the 2nd larval stage.

3.5 Exposure to maize expressing *Bt* protein (positive control)

10,267 freshly hatched neonate larvae of *O. nubilalis* not used for the bioassays mentioned above were transferred to MON810 for seven days. Each of the larvae died (100 %) in the first larval stage (Table 3).

3.6 Historical susceptibility of corn borers to Cry1Ab

During 2008–2018, 63 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 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		
	2017[4]	99.19		
	2018[4]	99.83		
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)

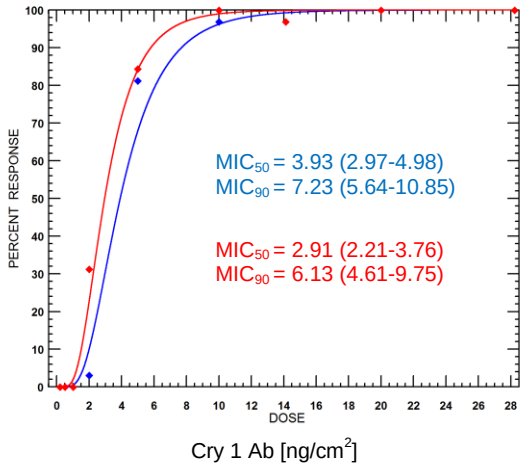
MIC moulting inhibition concentrations, CI confidence interval, [a] ng Cry1Ab/cm^2; [1] batch 1 of Cry1Ab, [2] batch 2 of Cry1Ab, [3] batch 2a of Cry1Ab, [4] batch 2b of Cry1Ab

3.7 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 July 2019) showed that the reference strain G.04 is not infected with microsporidia or with *Nosema* (Figure A2).

The strain ES.ref was established as a second reference strain with ECB collected in December 2015 in the Northwest region of Spain where *Bt* maize has not been cultivated. 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*. (Figure 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 (Figure 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 are provided in Table 5. The results for both strains appear to continue to be well within the historical range for the populations tested.



	MIC ratios	
	($MICR_{50}$)	Conf. limits
G.04 vs. ES.ref	1.355	(1.059-1.732)
	($MICR_{90}$)	
G.04 vs. ES.ref	1.179	(0.855-1.626)

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)

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} values for the

reference strains of ECB from Germany (G04) and Spain (ES.ref) differed significantly in their susceptibility to Cry1Ab/cm² (Figure 6) as indicated by the exclusion of 1 in their MICR 95% confidence limits.

Table 5. Results from Probit analysis for the ECB reference strain tested in the season 2018-2019.

Area	n	Slope ± SE	χ^2	D.f.	MIC_{50} (95% CI) [a]	MIC_{90} (95% CI) [a]
refG	352	4.849 ± 0.645	10.09	7	3.93 (2.97-4.98)	7.23 (5.64-10.85)
refES	352	3.949 ± 0.479	9.70	7	2.91 (2.21-3.76)*	6.13 (4.61-9.75)

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

4 Conclusions

In 2018, ECB larvae from one area with three 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 1,768 larvae exposed to the discriminating concentration 76 larvae died, 1,689 survived but did not reach the 2nd larval stage, and 3 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 99.85 %. In the confirmatory experiment, all of these larvae died after feeding on *Bt* maize within seven days. Therefore, no decrease in Cry1Ab susceptibility of ECB has been observed during the monitoring duration..

5 Acknowledgement

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Annex I
Areas of collection activities for ECB in 2018

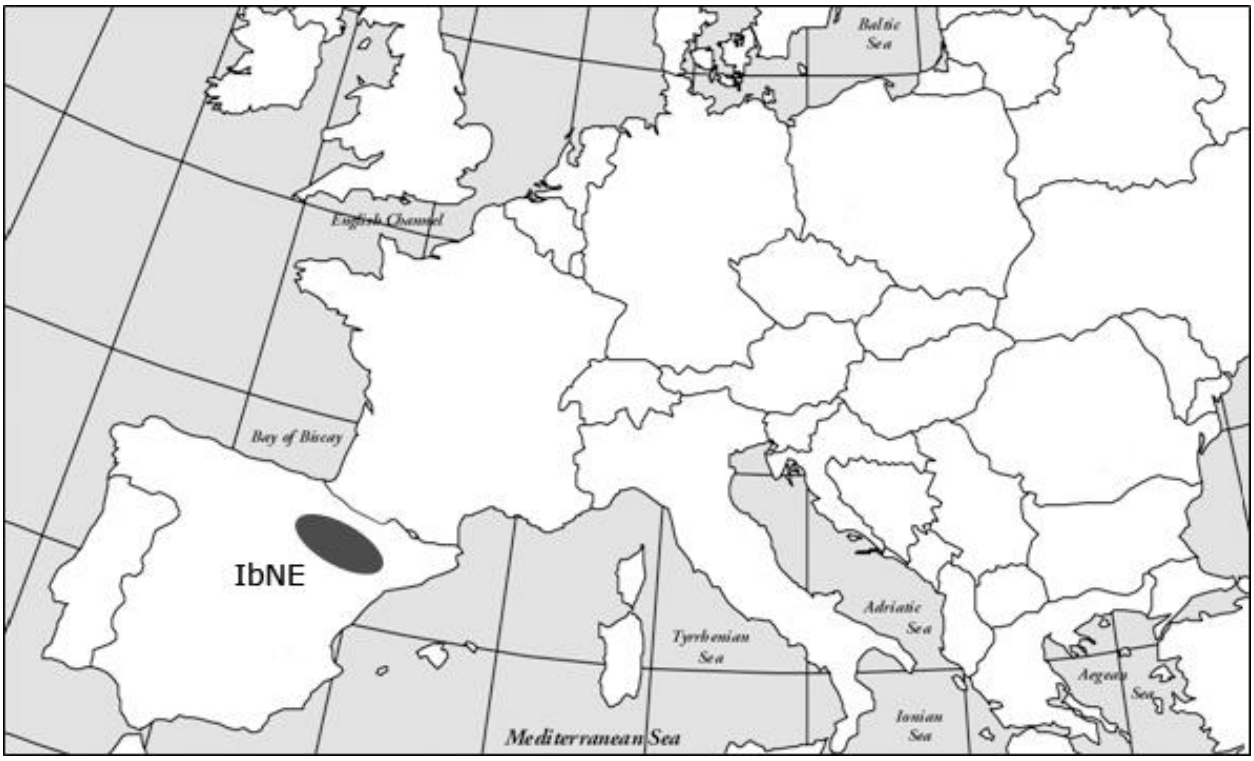

Figure A1. Area where ECB were sampled in 2018 (Iberia Northeast)

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

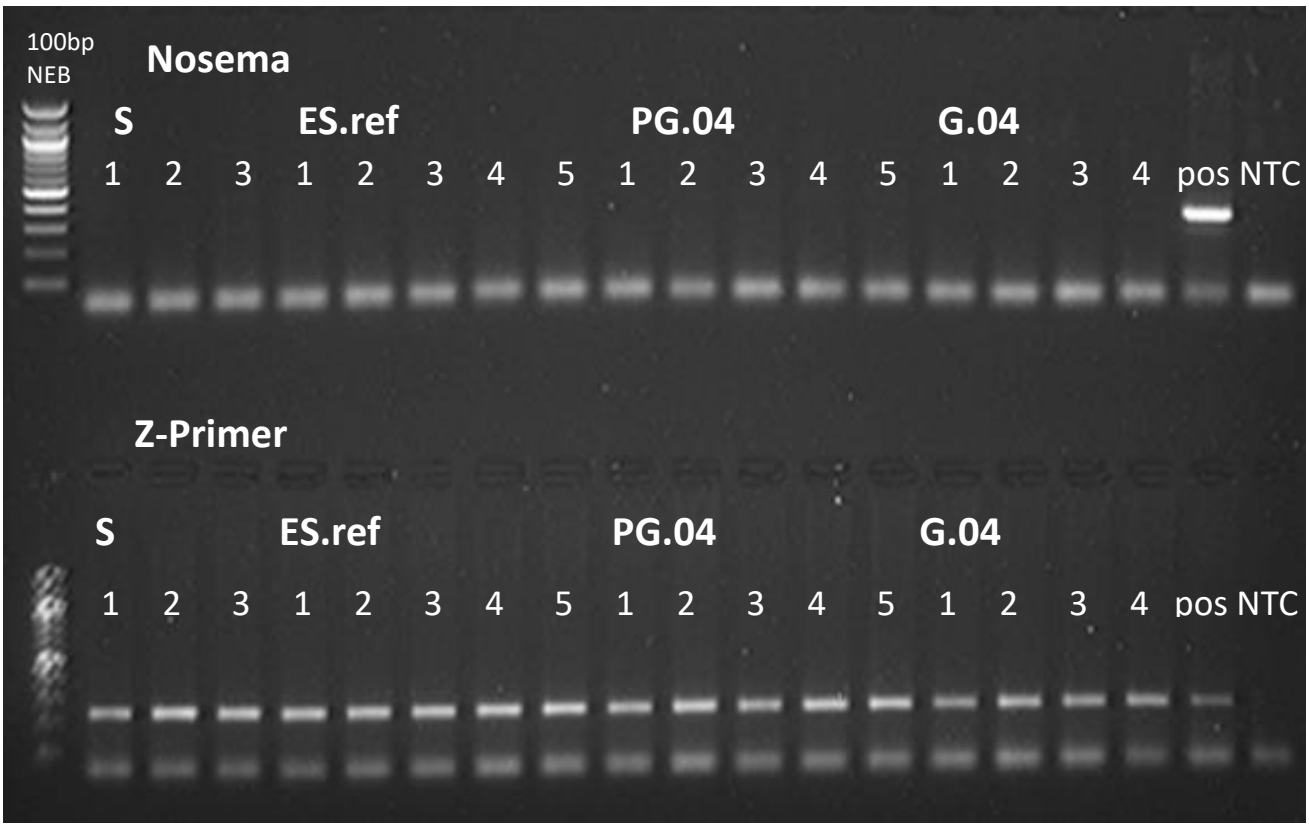

Figure A2. PCR analyses for checking if the reference strains of G.04 (Germany) and ECB (ES.ref (Spain)) or samples collected in the field (S = Sangüesa and P = Peñalba) are infected with *Nosema* (according to SAEGLITZ, 2004).

(pos: positive control, NTC: no template control; G.04: 1-4 each with 10 larvae (Lv1) pooled; ES.ref: 1-5 each with 10 larvae (Lv1) pooled; S 1 with 10 larvae (Lv1), 2-3 each with egg masses pooled; P: 1-5 each with 10 larvae (Lv1) pooled; Z-Primer was applied to check that the DNA was not destroyed)