

Report

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

- Results for 2022-2023 -

Date

29.09.2023

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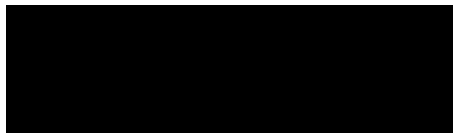
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Statement of Compliance with the Principles of Good Experimental Practice

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

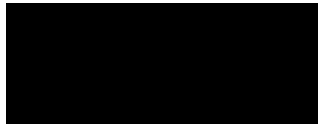
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Contents

1. Introduction.....	5
2. Materials and Methods	6
2.1 Insect collection	6
2.2 Insect culture	6
2.3 Bioassays	8
2.3.1 Susceptibility to Cry1Ab	8
2.3.2 Exposure to MON 810 tissue (confirmatory experiment)	9
2.3.3 Exposure to non-GM maize tissue (negative control)	10
2.3.4 Exposure to maize tissue expressing <i>Bt</i> protein (positive control)	10
2.3.5 Susceptibility of the reference strains G.04 and ES.ref to Cry1A	10
2.4 Statistical analysis	10
3. Results and Discussion	11
3.1 Collection of ECB.....	11
3.2 Susceptibility to Cry1Ab in the 2022-2023 campaign	13
3.3 Exposure to MON 810 tissue (confirmatory experiment)	14
3.4 Exposure to non-GM maize tissue (negative control)	14
3.5 Exposure to maize expressing <i>Bt</i> protein (positive control)	14
3.6 Historical susceptibility of corn borers to Cry1Ab	14
3.7 Susceptibility of the reference strains G.04 and ES.ref to Cry1Ab.....	15
4. Conclusions.....	16
5. Acknowledgement	16
6. References.....	17
Annex I	19
Annex II	20
Annex III	21
Annex IV.....	22
Annex V.....	23

Tables and Figures

Table 1.	<i>O. nubilalis</i> diet recipe	9
Table 2.	<i>O. nubilalis</i> collection details for the 2022-2023 season.	12
Table 3.	Development of <i>O. nubilalis</i> collected in Spain for the 2022-2023 season.....	13
Table 4.	Response of <i>O. nubilalis</i> larvae in the 2022-2023 season	14
Table 5.	Susceptibility of <i>O. nubilalis</i> neonates exposed to Cry1Ab as measured by the MIC or response to DD (RDD) for areas tested.....	15
Table 6.	Results from Probit analyses for the ECB reference strains tested in the season 2022-2023.....	16
Table A1.	Test whether there is a significant difference between the value for MIC 99 % (1.47062891) and the confidence intervals for MIC's of ECB sampled in 2022.....	21
Figure 1.	Dissected maize stalk with larvae.....	7
Figure 2.	Corrugated cardboard with pupae	7
Figure 3.	Growth chamber with plastic boxes containing diapausing ECB larvae.	7
Figure 4.	Oviposition cages for adult ECB.....	7
Figure 5.	IDs of Bio-Ba-128 trays	9
Figure 6.	Fitted curves of susceptibility as percentage moult inhibition after seven days of feeding by ECB (Es.ref) when exposed to treated diet from the batches 2c (blue) and 2d (red) of protein Cry1Ab (PoloPlus, LeOra Software 2002-2022).....	13
Figure 7.	Fitted curves of susceptibility as percentage moult inhibition after seven days feeding on treated diet of ECB reference strains to the batch 2d of protein Cry1Ab (PoloPlus, LeOra Software 2002-2020)	16
Figure A1	Flow-chart showing the activities with ECB in 2022-2023.....	19
Figure A2	Area where ECB were sampled in 2022.....	20
Figure A3	PCR analyses for checking if the reference strains of G.04 (Germany) and ECB (ES.ref (Spain)) are infected with <i>Nosema</i> (according to SAEGLITZ, 2004)....	22
Figure A4	The ImmunoStrip test used to check whether the <i>Bt</i> plants used contain <i>Bt</i>	23

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 EuropaBio¹ harmonised IRM plan in 2012, 2017 and 2019 and later on as CropLife Europe harmonised IRM plan in 2023) as well as EFSA recommendations (EFSA 2017, 2018). 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%.

¹ Please note that CropLife Europe has taken over all the responsibilities of EuropaBio in handling the harmonised IRM plan as of 1st January 2021.

The objectives of the current report on the 2022 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.
- 4) As requested by EFSA a statistical analysis was performed to demonstrate whether there is a significant difference between the value for MIC 99 % (1.47062891) and the confidence intervals for MIC's of ECB sampled.

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. But since 2016, 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. Thus, in 2019, 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 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), Pontevedra (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 Table 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 exchanged 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 $8 \pm 2^\circ\text{C}$. 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 $25 \pm 2^\circ\text{C}$ until all larvae hatched.



Figure 1. Dissected maize stalk with larvae

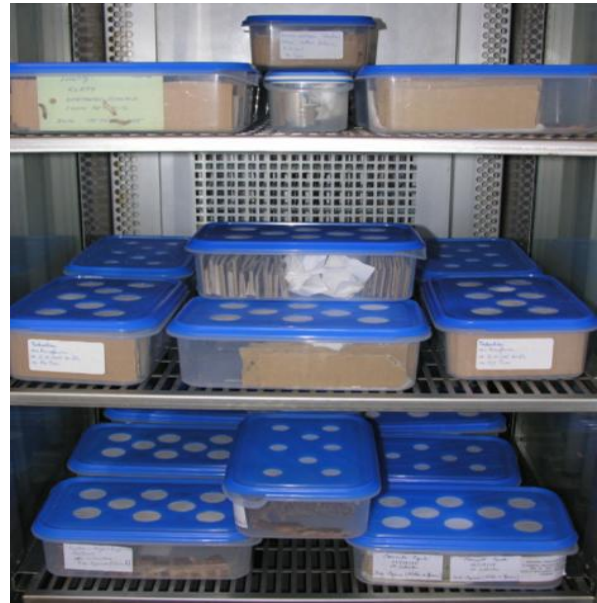


Figure 3. Growth chamber with plastic boxes containing diapausing ECB larvae



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 Monsanto² 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 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 specified by the manufacturer 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. As this batch had also reached the date of expiry specified by the manufacturer a new batch (2c) was re-supplied by Bayer. The batch 2c (NBR: 11247229-381, 31/01/2023; 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 (2d) was re-supplied by Bayer. The batch 2d (9 items, Barcode (BID) 82826, 82827, 828229-82835, Box Barcode NS72Z7FQ-0-0, Lot No. 7811; 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, 2c, 2d) 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. Because they were stored and delivered at different times (with different expiration dates given by the manufacturer), experiments were performed in previous reports (e.g., 2c vs. 2d), demonstrating the equivalence of the biological activity of the protein batches.

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 were 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 (DD) the data for all experiments with ECB from 2005-2012 were used. These include ECB collection in fields from Czech Republic, France, Germany, Hungary, Italy, Poland, Portugal, Romania, Slovakia and Spain representing the responses of 11,502 larvae. Using the average of the moulting inhibition concentrations (MIC) for 99% (MIC₉₉) 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 1,536 larvae from north-Iberian collection sites were exposed to the DD. In the control (50 mM bicarbonate buffer, pH 10.25) 220 larvae were tested. As requested by EFSA, the laboratory reference strains were included as negative controls for the diagnostic dose bioassay.

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

² **Disclaimer:** Monsanto has become the member of the Bayer group as of 21 August 2018. The owner of this report is Bayer Agriculture BV.

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

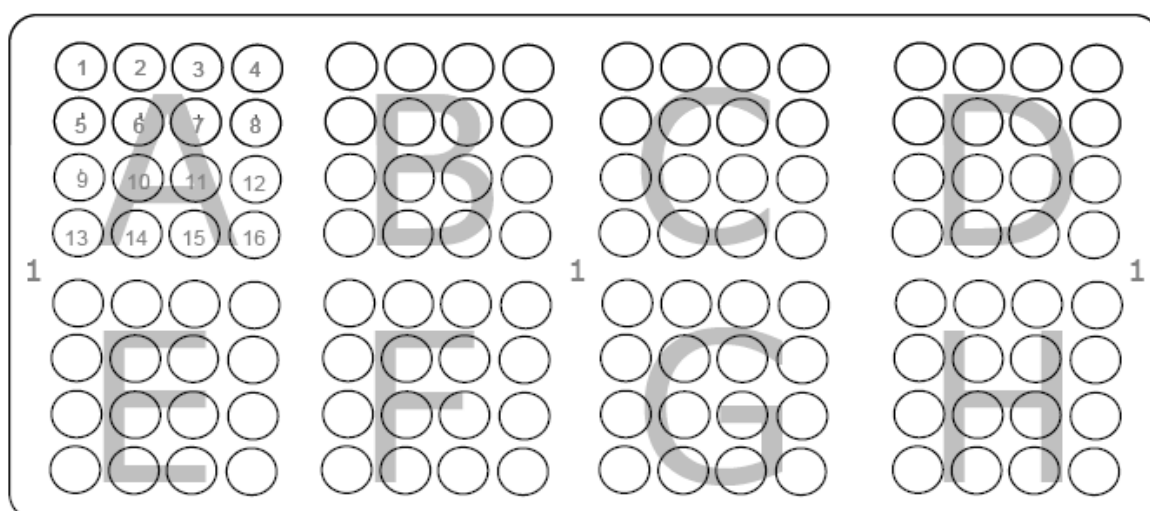


Figure 5. IDs of BAW128 trays (tray number, field letter, well number; e.g.: 1.A.13)

2.3.2 Exposure to MON 810 tissue (confirmatory experiment)

For larvae developing beyond the first larval stage at the diagnostic dose (28.22 ng/cm²), a confirmatory trial was performed. Therefore, these larvae were fed ad libitum with MON 810 leaves. Exposure to MON 810 took place in 128-well trays (BAW128, Frontier Agricultural Sciences) for up to seven days. In each cell, 1 ml of an agar solution (2.0 %) containing sorbic acid, benzoic acid and methylparaben (0.1 % each) was added to prevent leaf degradation and microbial contamination. After solidification, a detached maize leaf disc (MON 810) without a central nerve and with a diameter of 13 mm and a larva that had grown beyond the first larval stage was added to each cell, which was then sealed with a lid (BACV16, Frontier Agricultural Sciences). The trays were kept in a climate-controlled 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.

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.

To ensure the validity of this experiment, the ImmunoStrip test was used to check whether the *Bt* plants used actually contain *Bt*. The ImmunoStrip test, produced by Agdia Inc. (product number STX 06200/0050, to be used until March 2024), is intended for qualitative determination of Bt-Cry1Ab/Cry1Ac proteins in corn and cotton seed and leaf tissue.

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 were 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% (MIC₅₀) and 90% (MIC₉₀) 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.

To test whether the moult inhibition recorded in the field-collected population of *Ostrinia nubilalis* in the diagnostic-dose bioassays differed significantly from the expected 99% it was checked if their confidence intervals were covering the value for 99 % (1.47062891). If this is the case the MIC's do not differ significantly.

3 Results and Discussion

The stepwise approach followed to perform the bioassays is shown in Annex I.

3.1 Collection of ECB

The sample sites where ECB larvae were collected in 2022 are shown in Table 2, and the area is displayed on a map in Annex II. In total 45 oviposition cages were used. Of the 779 larvae of ECB collected in Northeast Iberia, 328 specimens survived the diapause period, reached the adult stage and mated, meaning that 42.1 % of the field collected larvae were represented in the bioassay (Table 3). Therefore, the detection limit for resistance allele frequency in 2022 was 5.52 % calculated based on the model developed by ANDOW & IVES (2002).

Table 2. *O. nubilalis* collection details in Spain for the 2022-2023 season.

Area	ID	Collection site ^a	Collected	Surface (ha) ^b	Distance to the nearest MON810 field (m) ^c	No of larvae sent ^d	GPS coordinates
refG		German reference	2005				
refES		Spanish reference	12.2015				
IbNE	Zone 1						
	ES.2022-Sarinena 2	ES-22200-Sarinena 2 (HU)	27/09/2022	9	ND	183	41.794075, 0.142136
	ES.2022-Sarinena 7	ES-22200-Sarinena 7 (HU)	27/09/2022	8.4	ND	38	41.782520, 0.124600
	ES.2022-Candasnos 1	ES-22591-Candasnos 1 (HU)	28/09/2022	24.1	ND	205	41.487451, 0.093589
	ES.2022-Candasnos 2	ES-22591-Candasnos 2 (HU)	28/09/2022	41.75	ND	19	41.491109, 0.057086
		Total				445	
	Zone 2						
	ES.2022-Girona 6	ES-17492-Girona 6 (GI)	28/09/2021	1.35	0	326	42.281226, 3.021848
	ES.2022-Girona 1,3,4.	ES-17142-Girona 1,3,4 (GI)	27/09/2021	5.3	Girona 1: 10, Girona 3,4: 0	8	42.054971, 3.056002
		Total				334	
		GRAND TOTAL				779	

^a Spanish provinces: HU = Huesca, GI = Girona; ^b data are approximate; ^c there could be other nearer fields that are not known by the technician and/or the farmer, ND no data;

^d number of larvae (counting was done by the sender) sent to Germany after removing those that were damaged or appeared to contain pathogens.

Table 3. Development of *O. nubilalis* collected in Spain for the 2022-2023 season.

Area	ID	No of larvae that reached the adult stage				Parasitized	
		N	%	♂♂ (n)	♀♀ (n)	Lv (n)	Lv (%)
IbNE	Zone 1						
	ES.2022-Sarinena 2	84	45.9	42	42	12	6.6
	ES.2022-Sarinena 7	6 ^a	15.8	3	3	5	13.2
	ES.2022-Candasnos 1	107	52.2	56	51	25	12.2
	ES.2022-Candasnos 2	5	26.3	1	4	2	10.5
	Total	202	45.4	102	100	44	9.9
	Zone 2						
	ES.2022-Girona 6	121	37.1	59	62	36	11.0
	ES.2022-Girona 1,3,4.	5	62.5	4	1	0	0.0
	Total	126	37.7	63	63	36	10.8
	Grand total	328	42.1	165	163	80	10.3

^a of the 38 larvae delivered 20 were infected with *Beauveria* and died

3.2 Susceptibility to Cry1Ab in the 2022-2023 campaign

To determine the susceptibility of the field collections to Cry1Ab, larval mortality and larval moult inhibition data at the discriminating concentration of Cry1Ab tested (determined to be 28.22 ng/cm²) were studied (Table 4).

Of the 1,536 larvae exposed to the discriminating concentration 123 larvae (8.01 %) died, 1,386 (90.23 %) survived but did not reach the 2nd larval stage, and 27 (1.76 %) reached the second larval stage and were used for a confirmatory experiment (Table 4). If the mean values of the two zones (z1 98.80 %, z2 99.28 %) are used for the calculation of the moulting inhibition, the resulting moulting inhibition is 99.04 %.

Table 4. Response of *O. nubilalis* larvae in the 2022-2023 season.

Zone	Coll. Site	Test*	Larvae used (n)	Dead	L1	L2**	L3	L4	MI (%)	MORT (%)
1	Sarinena 2	Cry1Ab	368	52	301	15	0	0	95.92	14.13
		Control	48	1	0	2	44	1	2.08	2.08
		neg control	48	5	0	0	43	0	10.42	10.42
		pos control	1800	1800	0	0	0	0	100.00	100.00
	Sarinena 7	Cry1Ab	80	5	75	0	0	0	100.00	6.25
		Control	28	0	0	0	28	0	0.00	0.00
		neg control	28	1	0	0	27	0	3.57	3.57
		pos control	230	230	0	0	0	0	100.00	100.00
	Candasnos 1	Cry1Ab	416	45	368	3	0	0	99.28	10.82
		Control	60	0	0	0	60	0	0.00	0.00
		neg control	60	1	0	0	59	0	1.67	1.67
		pos control	2235	2235	0	0	0	0	100.00	100.00
Candasnos 2	Cry1Ab	32	0	32	0	0	0	100.00	0.00	
	Control	8	1	0	0	7	0	12.50	12.50	
	neg control	8	0	0	0	8	0	0.00	0.00	
	pos control	200	200	0	0	0	0	100.00	100.00	
2	Girona 1,3,4	Cry1Ab	16	0	16	0	0	0	100.00	0.00
		Control	4	0	0	0	4	0	0.00	0.00
		neg control	4	0	0	0	4	0	0.00	0.00
		pos control	40	40	0	0	0	0	100.00	100.00

Table 4. continued

Zone	Coll. Site	Test*	Larvae used (n)	Dead	L1	L2**	L3	L4	MI [%]	MORT [%]
	Girona 6	Cry1Ab	624	21	594	9	0	0	98.56	3.37
		Control	72	1	0	0	70	1	1.39	1.39
		neg control	72	0	1	0	71	0	1.39	0.00
		pos control	2300	2300	0	0	0	0	100.00	100.00
Lab	Es Ref	Cry1Ab	160	49	111	0	0	0	100.00	30.63
		control	80	5	0	4	70	1	6.25	6.25
		neg control	80	8	0	0	72	0	10.00	10.00
		MON810	600	600	0	0	0	0	100.00	100.00
	G04	Cry1Ab	160	36	124	0	0	0	100.00	22.50
		control	80	0	1	1	77	0	1.25	0.00
		neg control	80	9	0	2	69	0	11.25	11.25
		MON810	600	600	0	0	0	0	100.00	100.00

* Bioassays: "Cry1Ab" = 28.22 ng/cm² Cry1Ab protein, "Control" = 50 mM bicarbonate buffer (pH 10.25). Total number of larvae tested: 8,781. Test duration: 7 days. **each larva that reached the 2nd larval stage in the Cry1Ab bioassay died within 7 days when fed with MON810 leaves

Of 220 larvae exposed to the control 3 larvae (1.36 %) died, all other larvae developed beyond first larval stage (L2 n=2 (0.91 %), L3 n=213 (96.82 %), L4 n=2 (0.91 %)). If the mean values of the two zones (z1 3.65 %, z2 0.69 %) are used for the calculation of the moulting inhibition, the resulting moulting inhibition is 2.17 %.

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 4). For the season reported here, 27 larvae developed to the second larval stage after 7 days of exposure to a dosage representing the diagnostic dose (28.22 ng/cm²). The confirmatory experiment conducted showed that each of these larvae died after feeding on *Bt* maize within 7 days. Applying the ImmunoStrip test, produced by Agdia Inc., it could be demonstrated that the *Bt* plants used contain *Bt* (Figure A4).

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

220 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 variety 'Golden Bantam' for seven days. Seven of the larvae died (3.18 %), 1 (0.45 %) developed to the second and 212 (96.36 %) developed to the third larval stage (Table 4).

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

6,805 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 4).

3.6 Historical susceptibility of corn borers to Cry1Ab

During 2008–2022, 89 samples of ECB from different areas were analyzed. The susceptibility of those from Iberia Northeast to Cry1Ab is shown in Table 5.

Table 5. Susceptibility of *O. nubilalis* neonates exposed to Cry1Ab as measured by the MIC or response to DD (RDD) for areas tested.

Area	Year	RDD (%)	MIC ₅₀ (95% CI) ^a	MIC ₉₀ (95% CI) ^a
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		
	2019 ⁵	99.74		
	2020 ⁵	89.61		
	2021 ⁶	98.33		
2022 ⁶	99.04			

MIC moultin inhibition concentrations, CI confidence interval, ^a ng Cry1Ab/cm²; ¹ batch 1 of Cry1Ab, ² batch 2 of Cry1Ab, ³ batch 2a of Cry1Ab, ⁴ batch 2b of Cry1Ab, ⁵ batch 2c of Cry1Ab, ⁶ batch 2d 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 June 2023) showed that the reference strain G.04 is not infected with microsporidia or with *Nosema* (Figure A3).

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 A3).

It was agreed that the reference strains will also be used as a negative control in the diagnostic concentration assays. The results are present in Table 4. Whereas strain G.04 suffered low mortality in the control (6.25 %), the strain ES.ref showed no mortality.

After exposure to Cry1Ab, no larvae reached the 2nd larval stage; therefore the MIC for both reference strains is 100%.

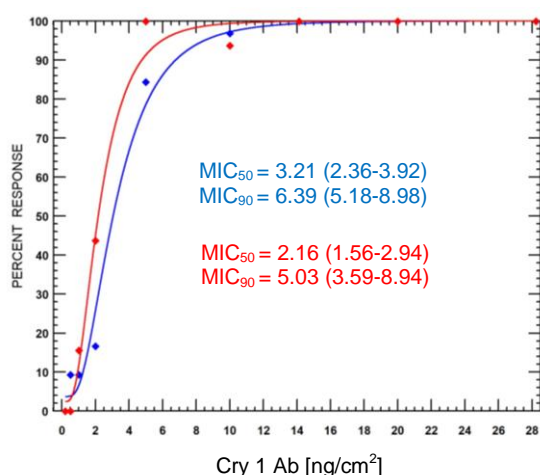
Fitted curves of susceptibility to the Cry1Ab protein of laboratory reference strains of *O. nubilalis* were generated taking into account the moultin inhibition concentration of neonate larvae after seven days feeding on treated diet. A dose-response relationship was calculated for the moultin inhibition of the reference strains ES.ref and G.04 (Table 6, Figure 7). Moultin inhibition concentrations at 50% (MIC₅₀) and 90% (MIC₉₀) for *O. nubilalis* kept as reference strains appear to continue to be well within the historical range for the populations tested (Table 5).

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 of MIC ratios (MICR) (ROBERTSON et al., 2007). The MIC₅₀ and MIC₉₀ values for the ECB reference strains from Germany (G04) and Spain (ES.ref) differ. While the MIC₅₀s differ significantly for the two reference strains (as indicated by the exclusion of 1 in the 95% confidence limits for the MICR (Figure 7), the differences between the two MIC₉₀s are not statistically confirmed. Both strains are clearly very susceptible to Cry1Ab.

Table 6. Results from Probit analyses for the ECB reference strains tested in the season 2022-2023.

Area	n	Slope \pm SE	χ^2	D.f.	MIC ₅₀ (95% CI) ^a	MIC ₉₀ (95% CI) ^a
refG	350	4.284 \pm 0.838	7.428	7	3.21 (2.36-3.92)	6.39 (5.18-8.98)
refES	352	3.489 \pm 0.423	16.296	7	2.16 (1.56-2.94)*	5.03 (3.59-8.94)

^a 50% and 90% moulting inhibition concentrations (MIC₅₀ and MIC₉₀) 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.



	MIC ratios	
	(MIC ₅₀)	Conf. limits
G.04 vs. ES.ref	1.485	(1.093-2.018)
	(MIC ₉₀)	
G.04 vs. ES.ref	1.269	(0.873-1.845)

Figure 7. Fitted curves of susceptibility as percentage moult inhibition after seven days feeding on treated diet of ECB reference strains to the batch 2d of protein Cry1Ab (PoloPlus Version: 1.0, LeOra Software 2002-2022). (Reference strain G.04: blue; reference strain ES.ref: red). According to the statistical analyses both curves do not have equal slopes and equal intercepts (P>0.05; χ^2 : 7.19, d.f.: 2; tail probability: 0.027)

4 Conclusions

In 2022-2023, ECB larvae from maize fields in two zones in Northeast Iberia were analyzed, along with two reference strains. ECB larvae were exposed to artificial diet treated with the discriminating concentration, and mortality and growth inhibition were evaluated after 7 days. Of the 1,536 larvae exposed to the discriminating concentration 123 larvae died, 1,386 survived but did not reach the 2nd larval stage, and 27 reached the 2nd 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 99.04 %. In the confirmatory experiment, all of these larvae died after feeding on *Bt* maize within seven days. The confirmatory plant assays showed that no insects were capable of surviving on MON 810 and therefore there was no indication of a change in susceptibility to MON 810.

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 [REDACTED] and [REDACTED] (CSIC, Madrid, ES).

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Annex I Flow-chart

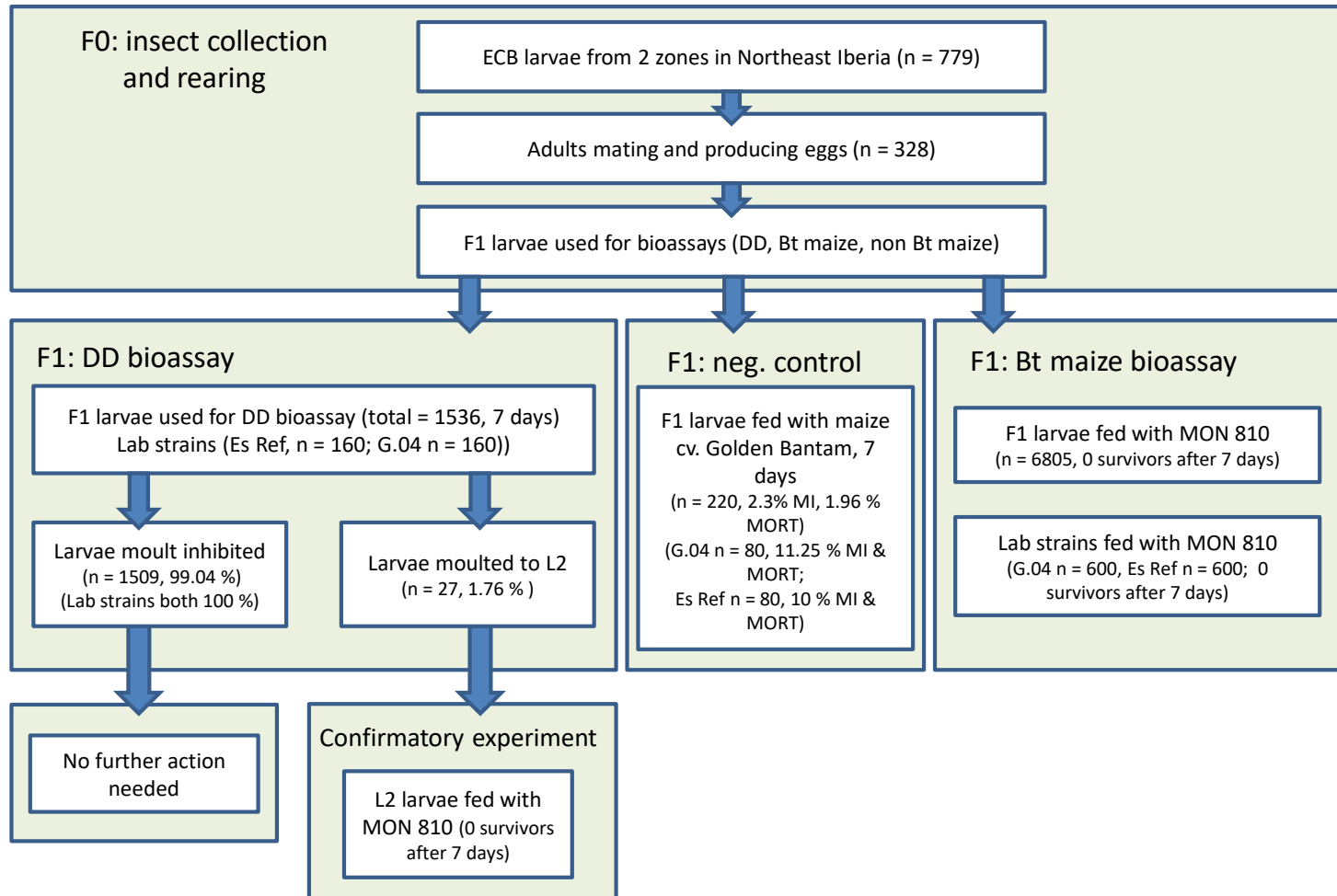


Figure A1. Flow-chart showing the activities with ECB in 2022-2023

Annex II
Area of collection activities for ECB in 2022

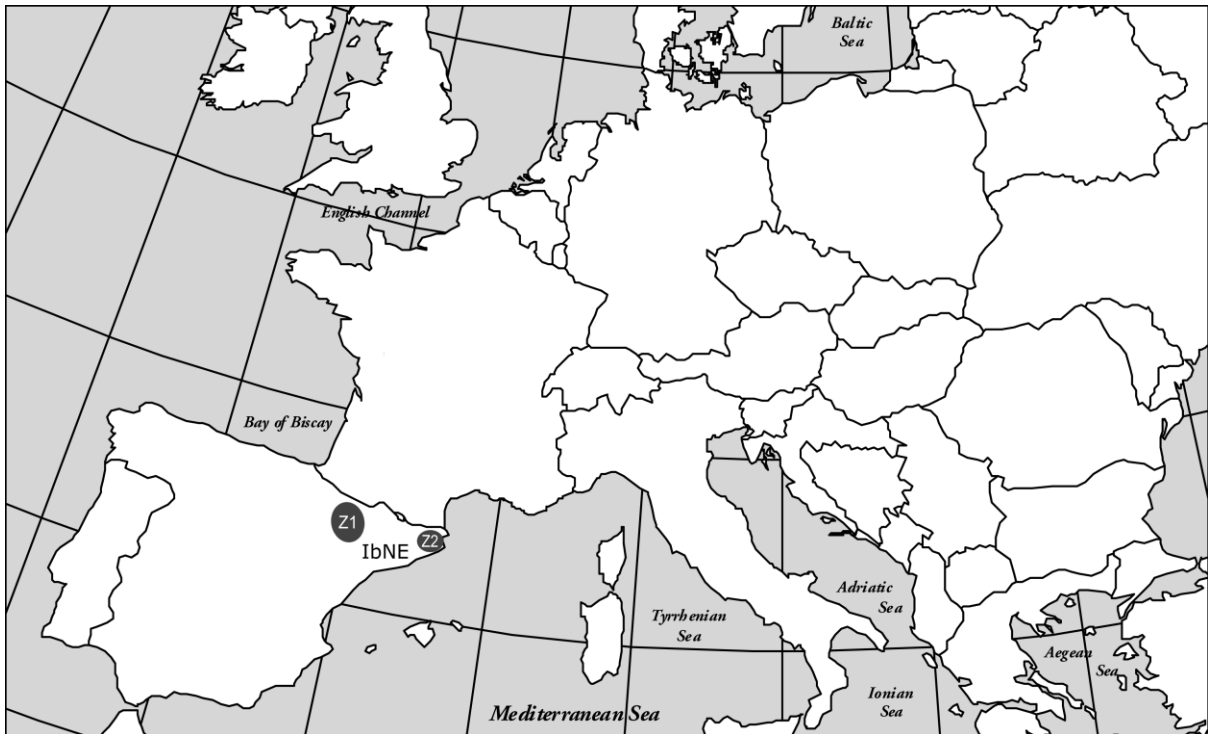


Figure A2. Area (with zone 1 (Z1) and zone 2 (Z2)) where ECB were sampled in 2022 (Iberia Northeast)

Annex III

Proof whether the moult inhibition recorded in the field-collected *O. nubilalis* in the diagnostic-dose bioassays differed significantly from the expected 99%

Table A1. Test whether there is a significant difference between the value for MIC 99 % (1.47062891) and the confidence intervals for MIC's of ECB sampled in 2022. (SD standard deviation, CI confidence interval)

Zone	Coll. Site	Test	MI [%]	divided by 100	arcsin	SD	N	Confidence	Mean	Lower CI	Upper CI	Rating
1	Sarinena 2	Cry1Ab	95.92	0.9592	1.367406847	0.09626179	4	0.09433482	1.49871021	1.404375394	1.593045036	1.47062891 falls within the CI, i.e. no significant difference to MIC 99 %.
	Sarinena 7	Cry1Ab	100.00	1	1.570796327							
	Candasnos 1	Cry1Ab	99.28	0.9928	1.485841358							
	Candasnos 2	Cry1Ab	100.00	1	1.570796327							
2	Girona 1,3,4	Cry1Ab	100.00	1	1.570796327	0.085057792	2	0.11788192	1.51065139	1.392769467	1.628533304	1.47062891 falls within the CI, i.e. no significant difference to MIC 99 %.
	Girona 6	Cry1Ab	98.56	0.9856	1.450506444							

Annex IV

Proof for stability and quality of the pest insect reference strains

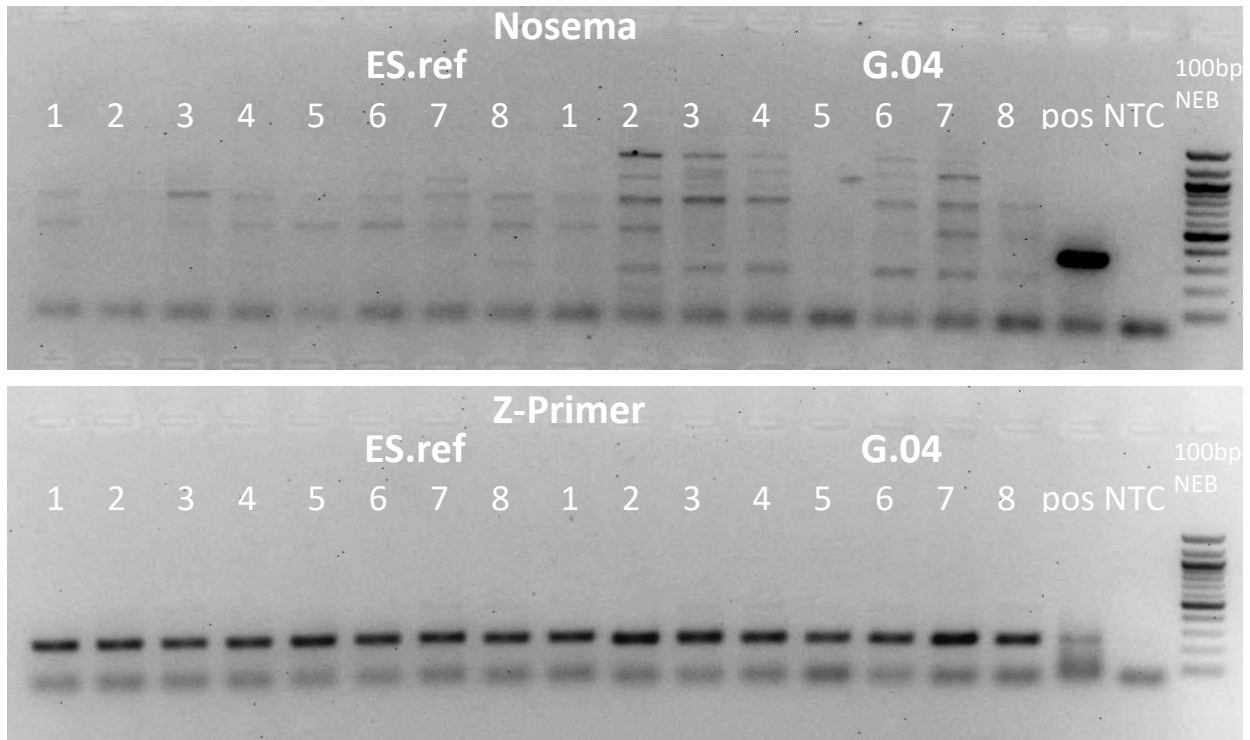


Figure A3. PCR analyses for checking if the reference strains of ECB (ES.ref (Spain)) and G.04 (Germany) are infected with *Nosema* (according to SA EGLITZ, 2004).

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

Annex V

Proof for validity of the bioassays with *Bt* corn



Figure A4. The ImmunoStrip test used to check whether the *Bt* plants used contain *Bt*. This test, produced by Agdia Inc., is intended for qualitative determination of Bt-Cry1Ab/Cry1Ac proteins in corn and cotton seed and leaf tissue. Here each plant tested is containing *Bt*.