Report

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

- Results for 2021-2022 -

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).

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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 [1] harmonised IRM plan in 2012, 2017 and 2019) 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 2021 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. 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 ± 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[2] 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 as also presented in previous reports (e.g., 2b vs. 2c), demonstrating the equivalence of the biological activity of protein batches 2c and 2d.

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 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% (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 1,652 larvae from north-Iberian collection sites were exposed to the discriminating concentration. In the control (50 mM bicarbonate buffer, pH 10.25) 280 larvae were tested. As requested by EFSA, the laboratory reference strains were included as negative controls for the DC 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

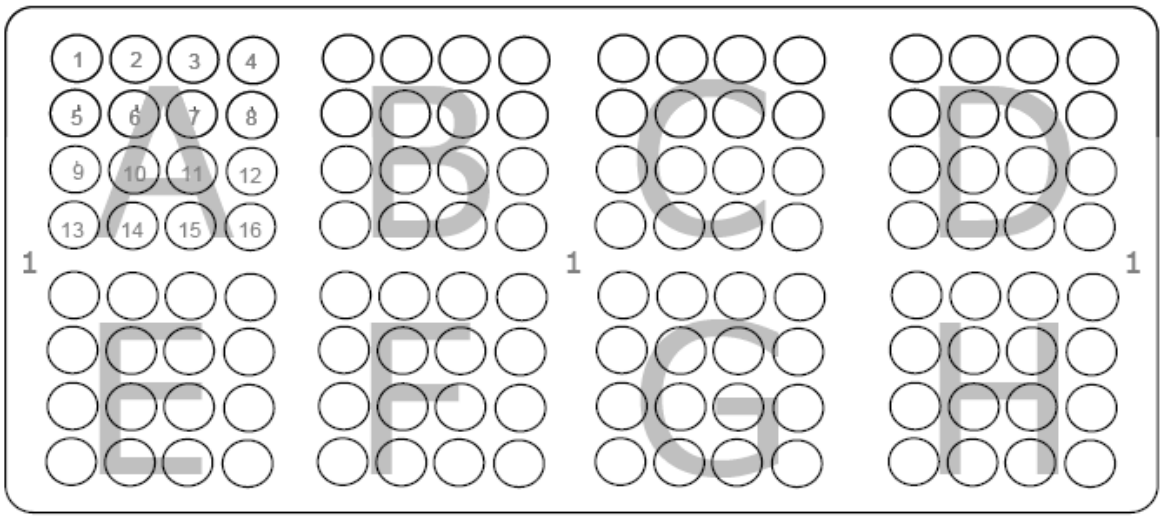


Figure 5. IDs of BAW128 trays (tray number, field letter, well number; *e.g.*: 1.A.13)

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 planned. 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 December 2021), 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% (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 sample sites where ECB larvae were collected in 2021 are shown in Table 2, and the area is displayed on a map in Annex I. In total 51 oviposition cages were used. Of the 807 larvae of ECB collected in the Ebro valley Spain, 308 specimens survived the diapause period, reached the adult stage and mated, meaning that 38.2 % of the field collected larvae were represented in the bioassay (Table 3). Therefore, the detection limit for resistance allele frequency in 2021 was 5.70 % calculated based on the model developed by ANDOW & IVES (2002).

Table 2. *O. nubilalis* collection details in Spain for the 2021-2022 season.

Area	ID	Collection site[a]	Collected	Surface (ha) [d]	Distance to the nearest MON810 field (m) [e]	No of larvae collected [f]	GPS coordinates
refG [b]	G.04	German reference	2005				
refES [c]	ES.ref	Spanish reference	12.2015				
IbNE	Zone 1						
	ES.2021-Sarinena 3	ES-22200 Sarinena 3 (HU)	26/09/2021	35	ND	27	41.799982, 0.195974
	ES.2021-Sarinena 9	ES-22200 Sarinena 9 (HU)	26/09/2021	48	ND	277	41.838243, 0.134516
		Total				**304**	
	Zone 2						
	ES.2021-Candasnos 1	ES-22591 Candasnos 1 (HU)	28/09/2021	4.5	8	110	41.519798, 0.062997
	ES.2021-Candasnos 2	ES-22591 Candasnos 2 (HU)	28/09/2021	11.3	260	22	41.515229, 0.03243
	ES.2021-Candasnos 3	ES-22591 Candasnos 3 (HU)	28/09/2021	11.0	500	109	41.541163, 0.048282
	ES.2021-Candasnos 4	ES-22591 Candasnos 4 (HU)	27/09/2021	22.5	300	50	41.487854, 0.093098
	ES.2021-Candasnos 5	ES-22591 Candasnos 5 (HU)	29/09/2021	84.2	100	212	41.490073, 0.094604
		Total				**503**	
		GRAND TOTAL				**807**	

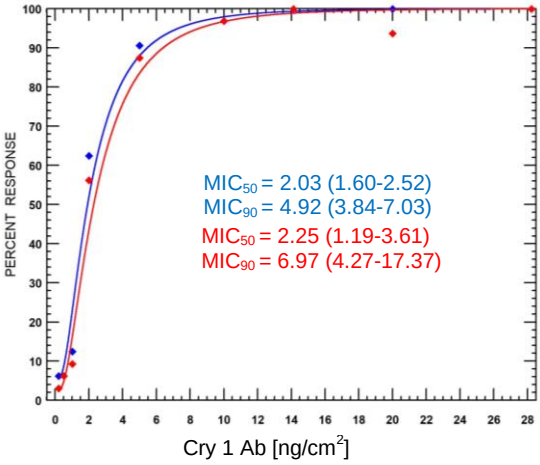
[a] Spanish provinces: HU = Huesca; [b] reference strain Germany; [c] reference strain Spain; [d] the area of the whole field, even though larvae were collected on the refuge area of the field, data are approximate; [e] there could be other nearer fields that are not known by the technician and/or the farmer, ND no data; [f] larvae sent to Germany after removing those that were damaged or seemed to have some pathogens

Table 3. Development of *O. nubilalis* collected in Spain for the 2021-2022 season.

Area	ID	No of larvae that reached the adult stage				Parasitized	
		male	female	total	%	n	%
IbNE	Zone 1						
	ES.2021-Sarinena 3	8	15	23	85.2	2	7.4
	ES.2021-Sarinena 9	54	39	93	33.6	110	39.7
	Total	**62**	**54**	116	**38.2**	**112**	**36.8**
	Zone 2						
	ES.2021-Candasnos 1	20	20	40	36.4	11	10.0
	ES.2021-Candasnos 2	2	8	10	45.5	7	31.8
	ES.2021-Candasnos 3	16	12	28	25.7	10	9.2
	ES.2021-Candasnos 4	23	19	42	84.0	6	12.0
	ES.2021-Candasnos 5	35	37	72	34.0	14	6.6
	Total	**96**	**96**	192	**38.2**	**48**	**9.5**
	GRAND TOTAL	**158**	**150**	**308**	**38.2**	**160**	**19.8**

3.2 Susceptibility to Cry1Ab in the 2021-2022 campaign

To analyze if the Cry1Ab protein batches 2c and 2d differed in efficacy a bridging experiment was done with larvae of strain Es.ref, applying the same method as for the bioassays with the reference strains. The proteins of both batches did not significantly differ in their efficacy (Figure 6). The same was found in the bridging experiments presented in the 2019-2020 campain for Cry1Ab protein batches 2b and 2c.



	MIC ratios	
	($MICR_{50}$)	Conf. limits
Batch 2c vs. 2d	0.903	(0.651-1.253)
	($MICR_{90}$)	
Batch 2c vs. 2d	0.705	(0.466-1.066)

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 Version: 1.0, LeOra Software 2002-2022). According to the statistical analyses both curves have equal slopes and equal intercepts (P>0.05; χ^2: 2.97, d.f.: 2; tail probability: 0.227)

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/cm^2) were studied (Table 4).

Of the 1,652 larvae exposed to the discriminating concentration 57 larvae died, 1595 survived but did not reach the 2nd larval stage, and 21 reached the second larval stage and were used for a confirmatory experiment (Table 4). The resulting moulting inhibition was 98.33 %.

Table 4. Response of *O. nubilalis* larvae in the 2021-2022 season.

Coll. Site	Test*	Larvae used (n)	Dead	L1	L2**	L3	L4	MI [%]	MORT [%]
Zone 1	Cry1Ab	576	45	528	3	0	0	98.34	5.76
	Control	76	1	0	2	70	3	0.78	0.78
	non-GM maize	76	0	0	0	76	0	0.00	0.00
	MON 810 leaves	2500	2500	0	0	0	0	100.00	100.00
Zone 2	Cry1Ab	1076	12	1046	18	0	0	98.33	1.12
	Control	204	1	0	2	173	28	0.49	0.49
	non-GM maize	203	1	0	7	195	0	0.49	0.49
	MON 810 leaves	4400	4400	0	0	0	0	100.00	100.00
Total	Cry1Ab	1652	57	1574	21	0	0	98.33	3.44
	Control	280	2	0	4	243	31	0.64	0.64
	non-GM maize	279	1	0	7	271	0	0.25	0.25
	MON 810 leaves	6900	6900	0	0	0	0	100.00	100.00
Es Ref	Cry1Ab	128	9	119	0	0	0	100.00	7.03
	control	64	0	0	0	64	0	0.00	0.00
G04	Cry1Ab	128	8	120	0	0	0	100.00	6.25
	control	64	0	0	2	62	0	0.00	0.00

* Bioassays: "Cry1Ab" = 28.22 ng/cm^2 Cry1Ab protein, "Control" = 50 mM bicarbonate buffer (pH 10.25). Total number of larvae tested: 9111. Test duration: 7 days. **all larvae that reached the 2nd larval stage in the Cry1Ab bioassay died within 7 days when fed with MON810 leaves

Of 280 larvae exposed to the control 2 larvae died, all other larvae developed beyond first larval stage (L2 n=4 (1.43 %), L3 n=243 (86.79 %), L4 n=31 (11.07 %)). The resulting moulting inhibition was 0.64 %.

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, 21 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)

279 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. One of the larvae died (0.36 %), 7 (2.51 %) developed to the second and 271 (97.13 %) developed to the third larval stage (Table 4).

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

6,900 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 2008–2018, 63 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 DC (RDC) for areas tested.

Area	Year	RDC (%)	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		

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, [5] batch 2c of Cry1Ab, [6] batch 2d of Cry1Ab

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

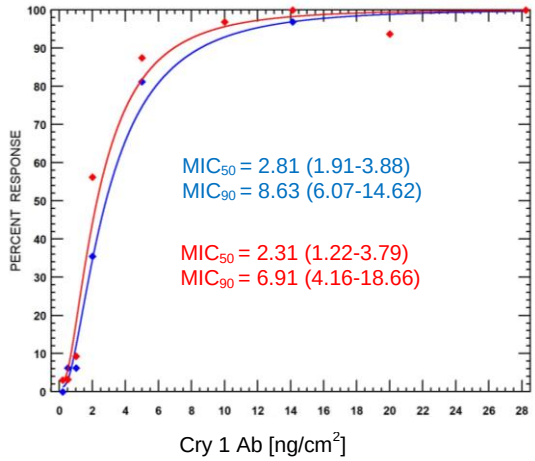
The reference strain G.04 was kept in the laboratory in sub-strains since 2011 and checked regularly for performance (size of adults, size of egg masses, and development of larvae). In 2011, by performing a PCR based method (SAEGLITZ, 2004), infection with *Nosema* was identified for some individuals 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 March 2022) 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).

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. While both strains suffered no mortality in the control, less than 8% of larvae died after exposure to Cry1Ab.

After exposure to this protein, 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 moulting inhibition concentration of neonate larvae after seven days feeding on treated diet (Figure 7). 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	1.218	(0.881-1.684)
	($MICR_{90}$)	
G.04 vs. ES.ref	1.249	(0.836-1.865)

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 have equal slopes and equal intercepts (P>0.05; χ^2: 1.77, d.f.: 2; tail probability: 0.412)

A dose-response relationship was calculated for the moulting inhibition of the reference strains ES.ref and G.04 (Table 6). 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 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 7) as indicated by the inclusion of 1 in their MICR 95% confidence limits.

Table 6. Results from Probit analysis for the ECB reference strains tested in the season 2021-2022.

Area	n	Slope ± SE	χ^2	D.f.	MIC_{50} (95% CI) [a]	MIC_{90} (95% CI) [a]
refG	351	2.632 ± 0.281	10.709	7	2.81 (1.91-3.88)	8.63 (6.07-14.62)
refES	350	2.692 ± 0.292	23.709	7	2.31 (1.22-3.79)*	6.91 (4.16-18.66)*

[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 not significantly different (P > 0.05) to the reference strain G.04.

4 Conclusions

In 2021-2022, ECB larvae from one area with two 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 1,652 larvae exposed to the discriminating concentration 57 larvae died, 1595 survived but did not reach the 2nd larval stage, and 21 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 98.33 %. 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

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Annex I
Flow-chart

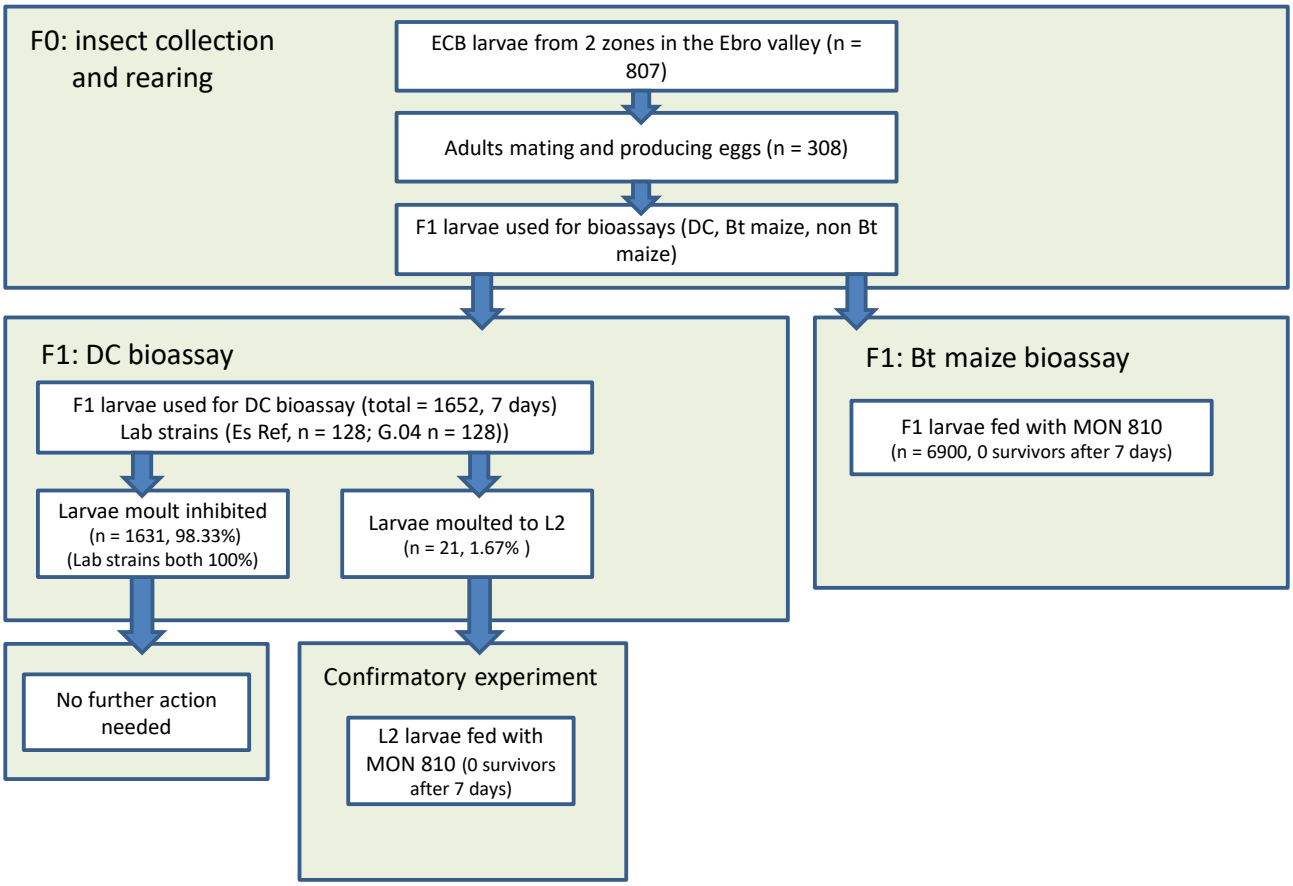

Figure A1. Flow-chart showing the activities with ECB in 2021-2022

Annex II
Areas of collection activities for ECB in 2021

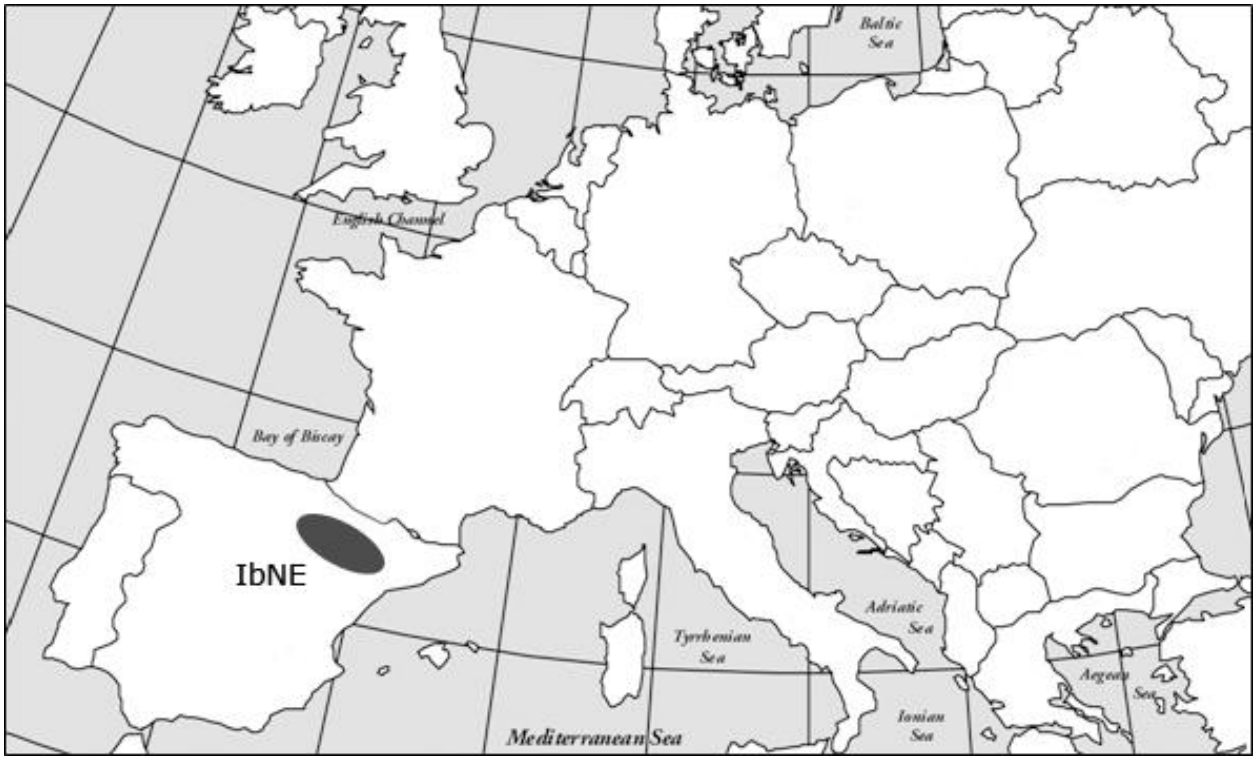

Figure A2. Area where ECB were sampled in 2021 (Iberia Northeast)

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

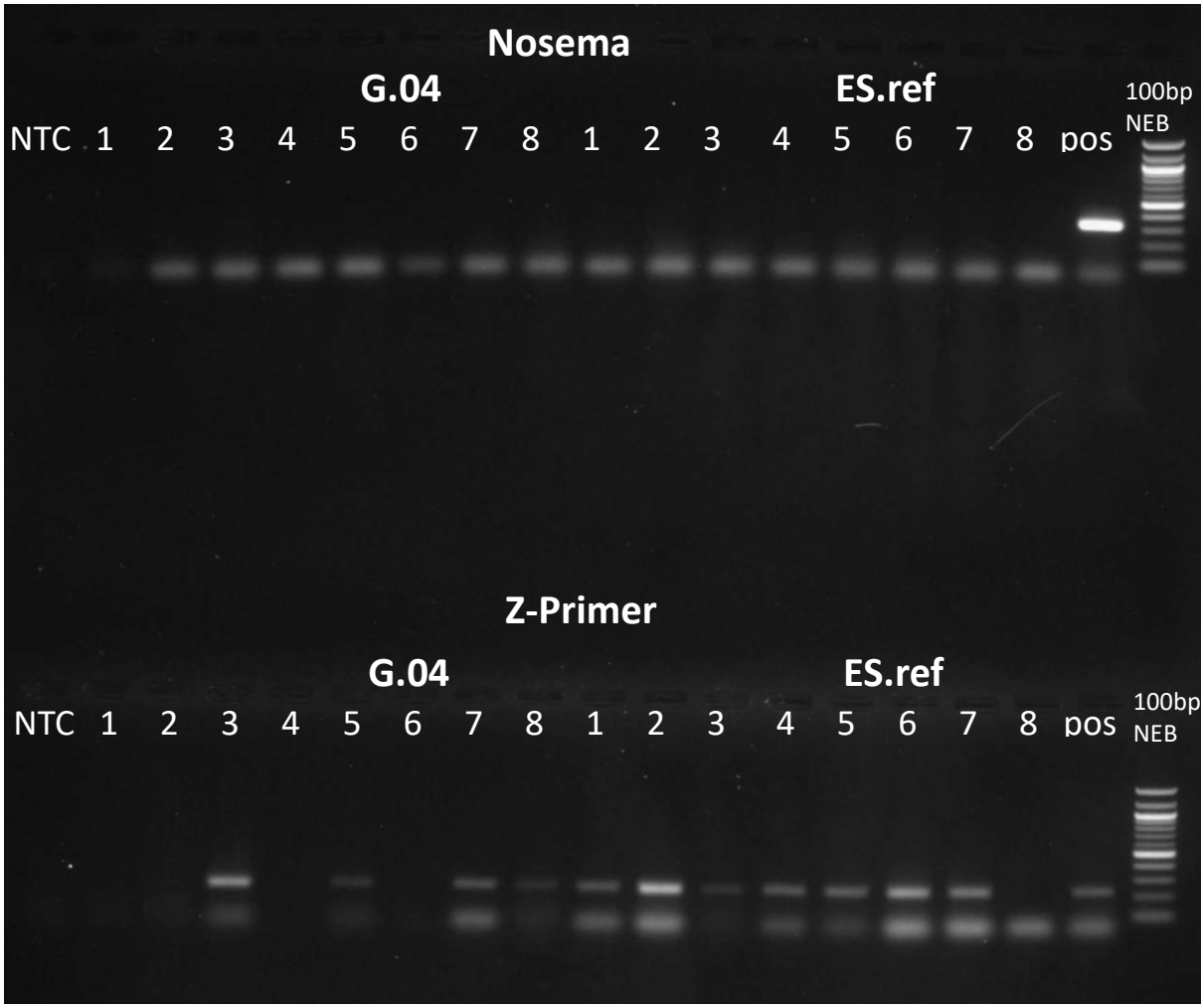

Figure A3. PCR analyses for checking if the reference strains of G.04 (Germany) and ECB (ES.ref (Spain)) are infected with *Nosema* (according to SAEGLITZ, 2004).

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