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ORIGINAL CONTRIBUTION

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Ten years of MON 810 resistance monitoring of field populations of *Ostrinia nubilalis* in Europe

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Abstract

From 2005 to 2015, *Ostrinia nubilalis* were collected in the most important maize-growing areas in Europe where MON 810 was cultivated. The susceptibility of these *O. nubilalis* collections to the Cry1Ab protein was determined using overlay bioassays and compared to that of reference (control) strains. Larvae that died or did not moult after 7 days were used to calculate a moulting inhibition concentration (MIC). Two different batches of Cry1Ab protein were used over the course of this study. Between 2005 and 2015, 145 collections of *O. nubilalis* from 14 areas were analysed. The Cry1Ab susceptibility of populations from different geographic regions differed only slightly across years. The greatest variability in the MIC_{50} for field samples collected from 2005 to 2011 and tested with batch 1 was 6.6-fold in 2006. For field-collected *O. nubilalis*, the difference between MIC_{50} values of the most susceptible and most tolerant samples was 13.1-fold for this period. For samples collected in 2012–2015 and tested with batch 2, the greatest variability was 4.1-fold in 2014. A diagnostic concentration (MIC_{99}) was calculated for batch 1 (48 ng/cm^2) using the results from all the collections in 2005–2012. Bridging experiments indicated that the diagnostic concentration for batch 2 was 28 ng/cm^2. From 2006 onwards, no *O. nubilalis* reached the 2nd larval stage when the diagnostic concentration of either batch of Cry1Ab was used. Only one insect collected from Romania in 2012 and two insects collected as reference strain from Spain in 2015 survived exposure to a dosage of 20 ng/cm^2, and none of these larvae survived on MON 810 maize. Our results indicate that there has been no significant change in susceptibility to Cry1Ab in European populations of *O. nubilalis* over the period 2005–2015.

KEYWORDS

Cry1Ab protein, diagnostic concentration, European corn borer, moulting inhibition concentration

1 | INTRODUCTION

It is likely the European corn borer, *Ostrinia nubilalis* (Hübner 1796), originated on the Asian steppes (Zwölfer, 1928) and now is native to southern Europe (Beck, 1987). It was introduced into North America between 1909 and 1914 (Showers, 1993; Smith, 1920) and since has rapidly spread across North America (Caffrey & Worthley, 1927; Hudon & LeRoux, 1986; Roelofs, Du, Tang, Robbins, & Eckenrode, 1985). Apart from maize, more than 200 weeds and cultivated plants are recorded as host plants for *O. nubilalis* (Hodgson, 1928; Ponsard et al., 2004). This species is one of the most damaging pests of maize in North America and Europe and was therefore a major target pest for control using

genetically modified maize expressing *Bacillus thuringiensis* (*Bt*) proteins (*Bt* maize). For example, *Bt* maize containing the event MON 810 expresses the Cry1Ab protein derived from *B. thuringiensis* subsp. *kurstaki*, which confers protection against certain lepidopteran insect pests such as *O. nubilalis* and the Mediterranean corn borer *Sesamia nonagrioides*.

The development of resistance in targeted lepidopteran pests is a potential concern for the deployment of *Bt* maize that are widely cultivated and very effective. To date, however, there have been only two lepidopteran species confirmed to have developed field-evolved resistance to *Bt* maize. One of these, the African stem borer (*Busseola fusca*), developed resistance to Cry1Ab (MON 810) maize in South Africa (Van den Berg, Hilbeck, & Bøhn, 2013; Van Rensburg, 2007). The other, fall armyworm (*Spodoptera frugiperda*), developed resistance to Cry1F in Puerto Rico and Brazil (Farias et al., 2014; Storer et al., 2010) and to Cry1Ab in Brazil (Omoto et al., 2016). Some of the factors likely to have contributed to these resistance cases include less than high dose control of the target pest, high product adoption, low alternative host availability and low refuge compliance (Castañera, Farinós, Ortego, & Andow, 2016; Storer et al., 2010). There have been no reports of resistance to any Cry protein in *O. nubilalis* from Europe or North America.

To maintain the benefits of MON 810 maize varieties, insect resistance management (IRM) programmes have been implemented in Europe and North America where MON 810 varieties are grown commercially. These programmes include insect resistance monitoring as one element. Baseline susceptibility to the Cry1Ab protein was established for *O. nubilalis* collected in different maize-growing areas in Spain (Farinós, de la Poza, Hernández-Crespo, Ortego, & Castañera, 2004; González-Núñez, Ortego, & Castañera, 2000), Germany (Saeglitz et al., 2006) and the USA (Marçon, Siegfried, Spencer, & Hutchison, 2000; Marçon, Young, Steffey, & Seigfried, 1999). Annual monitoring studies have been (and continue to be) conducted for both *O. nubilalis* and *S. nonagrioides* in Spain (Castañera et al., 2016; Farinós et al., 2004, 2011) and for *O. nubilalis* in the USA (Siegfried et al., 2007). In Europe, this monitoring programme follows the directives of the working group on IRM submitted to the Appropriate Authorities of the Member States and the European Commission (available since 2003 but published in 2007 [Alcalde et al., 2007] and subsequently updated as the EuropaBio harmonized IRM plan in September 2012 [Anonymous 2012]). The objective is to detect any changes in target insect susceptibility relative to the baseline that could result in the decreased efficacy of MON 810 maize varieties, enabling the implementation of additional risk mitigation measures.

In this study, we review the results of the Cry1Ab resistance monitoring programme for *O. nubilalis* in maize-growing areas in Europe during the period 2005–2015, including the development and implementation of a diagnostic concentration (DC) approach for susceptibility testing.

2 | MATERIALS AND METHODS

2.1 | Field collection and culture of *O. nubilalis*

From 2005 to 2015, *O. nubilalis* were collected in the most important European maize-growing areas where MON 810 varieties were cultivated (Figure S1). This monitoring programme ceased in countries where MON 810 maize is not grown anymore. Hence, beginning in 2013 *O. nubilalis* samples were only collected on the Iberian Peninsula. For each area, up to three different collection sites separated by at least 50 km were chosen. In refuges and non-*Bt* maize fields adjacent to *Bt* maize, *O. nubilalis* were mainly collected as larvae by dissecting maize stalks but also as adults using light traps or collecting egg masses laid on leaves. If more than one larva per stalk was found, only one was taken to avoid collecting siblings. At least 300 healthy larvae were collected from each area sampled. In the rare cases, when egg masses were collected, only one larva per mass (containing 20–30 eggs) was taken.

Field-collected insects from different sites within the area studied were reared separately at 25 ± 2°C, 90% ± 2% RH and a photoperiod of 20:4 hr (L:D) to avoid cross-contamination with diseases like *Beauveria* sp. or *Nosema* sp. If the larvae did not pupate after a period of 2 weeks, they were assumed to have entered diapause and were transferred to another chamber maintained at 8 ± 2°C, 70% ± 5% RH and a photoperiod of 0:24 hr (L:D) until April. Larvae surviving the diapause period were transferred to fresh containers and placed in incubators with a humidity of 90% RH and a photoperiod of 20:4 hr (L:D). The temperature was raised gradually from 15 to 25°C over a period of 10 days and then kept at 25°C until adult emergence. The adults were transferred to tubular oviposition cages and fed with 15% honey water to increase their fecundity (Leahy & Andow, 1994). The insides of the cages were covered with filter paper (oviposition medium) that was changed twice a week. Egg masses were cut out and transferred to Petri dishes with moistened filter paper. If necessary, egg masses were stored for up to 7 days at 8 ± 2°C. Beginning in 2016, incubating egg masses were first heat-treated at 43°C for 40 min to reduce the possibility of *Nosema* infections (Showers, Hellmich, Derrick-Robinson, & Hendrix, 2001) and then placed in an incubator with a daily cycle of 20 hr at 25 ± 2°C, 4 hr at 20 ± 2°C, 90% RH and a photoperiod of 20:4 hr (L:D) (Guthrie, Robbins, & Jarvis, 1985). *O. nubilalis* sampled as adults were kept under the same conditions as described above. *O. nubilalis* sampled as egg masses were treated similarly to egg masses produced by adults reared in the laboratory.

2.2 | Laboratory reference strains

A laboratory reference strain (G.04) collected in 2002 in a field at Niedernberg, Germany, and kept in culture since then by G. Langenbruch (Darmstadt, Germany), was maintained under the same conditions as described above at Bio-Test Labor GmbH Sagerheide (BTL; Sanitz/Gr. Lüsewitz, Germany) beginning in 2005. In 2015, a second reference strain (ES.ref) of *O. nubilalis* was collected in an area in Northwest Spain (Galicia) where *Bt* maize has never been cultivated and established at BTL.

Long-term rearing of a strain can cause poor performance (Moar et al., 2008), so the reference strain G.04 was kept as two substrains and checked regularly for performance (size of adults, size of egg masses and development of larvae). No reduction in any of these three performance traits was observed. In 2011, analysis using a PCR-based

method (Saeglitz et al., 2006) revealed that some individuals in one substrain were infected with *Nosema*, so that this substrain was terminated and the remaining substrain was used continuously since then. Individuals of this substrain produce good-quality egg masses and normal-sized adults, and according to periodic PCR analyses, the substrain is not infected with microsporidia and especially not with *Nosema*. The strain ES.ref, established as a second reference strain using *O. nubilalis* collected in 2015 in fields with non-*Bt* maize located in Northwest Spain, was also checked for infection with *Nosema*. Using the PCR method, no individuals were found to be infected with *Nosema* or other microsporidia.

2.3 | Cry1Ab protein

Two batches of Cry1Ab protein provided by Monsanto were used for the MON 810 monitoring. Batch 1 (2 mg Cry1Ab/ml in 25 mM bicarbonate buffer, pH 10.5) was provided in 2005 and used from 2005 to 2011 (and in very few cases also in 2012). A second batch (1.64 mg Cry1Ab/ml in 50 mM bicarbonate buffer, pH 10.25) was used from 2012 onward. This batch was resupplied in 2014 as batch 2a. To avoid multiple freezing and thawing and prevent protein degradation, each Cry1Ab batch was divided into aliquots at the start of the procedure and stored at −80°C until used. Test concentrations were prepared with the same buffers in which the different toxin batches were diluted. Bridging experiments were carried out in 2012 to determine whether batches 1 vs. 2 (using reference strain G.04 and samples from IbC and IbNE) differed in their efficacy.

2.4 | Susceptibility bioassays

The bioassays were carried out in 128-well trays (BAW128; Bio-Serv, Frenchtown, NJ, USA) using the overlay treatment method. In each cell, 1 ml of artificial diet (Table S1) was dispensed; after it solidified, 100 µl of Cry1Ab protein solution was applied to the surface. Egg masses from each of the locations sampled were incubated, and neonate larvae, within 12 hr of hatching, were transferred to the cells (one per cell), which were then covered with a lid (BACV16; Bio-Serv). From 2012, the protocol of this bioassay was modified by combining all sites in an area into one bioassay population. Adults rather than larvae were combined to minimize the spread of infection between insects from different sites.

Eight concentrations (0.5–80 ng Cry1Ab/cm²) (batch 1) or nine concentrations (0.2–28.22 ng Cry1Ab/cm²) (batch 2) plus a control (bicarbonate buffer) were tested for each population. Until 2013, for each area three collection sites with 16 larvae were tested using each concentration of Cry1Ab batch 1 and 32 larvae were tested for the non-*Bt* control; in cases where batch 2 was used, 32 larvae were tested for each Cry1Ab concentration and 64 larvae were tested for the control. Beginning in 2014, three replicates were prepared for each concentration and the control. Each replicate consisted of 32 larvae per concentration (64 for controls), giving a total of 96 larvae for each concentration tested (192 for controls). For each replicate, neonate larvae from different oviposition cages were used. The susceptibility of the reference strains of *O. nubilalis* to Cry1Ab was assessed using the same stock solution.

Each assay was carried out at 25°C, 70% RH and a photoperiod of 0:24 hr (L:D). After 7 days, larval mortality and developmental stage were recorded. Larvae that had not grown beyond the first instar would not survive under field conditions (e.g., Siegfried, Spencer, & Nearman, 2000), so the criterion for mortality used in this study included both death and inhibition of moulting and was used to calculate "moulting inhibition concentrations" (MICs). The Cry1Ab concentration that resulted in death or moulting inhibition of 99% of the larvae (MIC_{99}) was used as a DC. MIC_{99} was calculated for batch 1 using the pooled results of all the experiments with *O. nubilalis* collected from 2005 to 2012 in the field in the Czech Republic, France, Germany, Italy, Panonia, Poland, Portugal, Romania and Spain, representing a total of 11,502 larvae.

2.5 | Statistical analyses

All statistical analyses were carried out using the computer program SYSTAT, Version 11.0 (Systat Software, Inc., San Jose, CA, USA) except for the analysis of the concentration response, for which PoloPlus 1.0 was used (LeOra Software, Berkley, CA, USA). The results for growth inhibition at different concentrations of Cry1Ab were adjusted by Probit weighted regression lines, and MIC_{50} and MIC_{90}-values were calculated for each collection along with their 95% confidence limits using PoloPlus 1.0. Mortality of *O. nubilalis* in the control was required to be below 20% for the corresponding bioassay to be used in the analyses.

The measure of how well the data (response of *O. nubilalis* to different concentrations of protein) fitted the assumptions of the Probit model (goodness-of-fit) was assessed by comparing the responses predicted by the Probit model to the observed responses (χ^2 test).

Fitted curves of susceptibility to the Cry1Ab protein of reference strains and field collections of *O. nubilalis* were generated for the MIC of neonate larvae after 7 days of feeding on treated diet. The significance of differences in the susceptibility of the reference strains and field-collected insects was tested by determining the 95% confidence limits (CL) of the MIC ratios (MICR) (Robertson & Preisler, 1992). Concentrations are significantly different ($p < .05$) if the MICR 95% confidence limits do not include 1.

3 | RESULTS

3.1 | Susceptibility to Cry1Ab in the years 2005–2015

During 2005–2015, 145 samples (including assays without a concentration–response relationship) of *O. nubilalis* collected from maize fields in 14 areas in the Czech Republic, France, Germany, Italy, Hungary, Slovakia, Poland, Portugal, Romania and Spain were analysed (Table 1). For *O. nubilalis* collected in the field in the years 2005–2011 and tested using Cry1Ab batch 1, the difference between MIC_{50}-values of the most susceptible and the most tolerant samples

TABLE 1 Susceptibility of *Ostrinia nubilalis* neonates exposed to Cry1Ab measured over time for the areas included in this study. Collection sites were pooled based on their geographic and climatic similarity

Area	Year[a]	Slope ± *SE*	χ^2	*df*	MIC_{50} (95% CL)[b]	MIC_{90} (95% CL)[b]
Czech Republic/Moravia	2005[1]	4.149 ± 0.63	23.192	30	7.59 (6.30–8.72)	15.47 (13.16–19.89)
	2008[1]	3.561 ± 0.654	70.888	21	6.64 (2.68–9.81)	15.20 (10.21–67.19)
	2009[1]	4.37 ± 1.22	0.22	6	9.00 (5.74–12.35)	17.67 (12.80–37.88)
	2011[1]	5.484 ± 1.44	14.576	14	1.28 (0.89–1.54)	2.19 (1.78–3.86)
	2012[2]	3.567 ± 0.666	1.267	6	1.83 (1.40–2.43)	4.19 (3.02–7.61)
Southwest	2005[1]	1.962 ± 0.322	67.948	22	11.21 (1.89–23.53)	50.43 (24.06–200.47)
France	2006[1]	5.282 ± 0.906	11.368	34	3.14 (2.58–3.61)	5.49 (4.74–6.91)
	2007[1]	2.732 ± 0.740	4.931	6	9.08 (4.75–12.92)	26.74 (18.13–68.38)
	2008[1]	3.698 ± 0.588	12.962	14	4.82 (3.89–5.88)	10.70 (8.37–16.01)
West France	2006[1]	8.681 ± 1.559	53.392	30	18.48 (15.53–21.67)	25.97 (22.06–37.29)
Northern Germany/Southwest Poland	2005[1]	2.996 ± 0.420	22.015	16	13.33 (9.45–17.64)	35.70 (25.97–60.49)
	2006[1]	3.474 ± 0.858	0.101	6	2.82 (1.87–3.70)	6.60 (4.84–13.18)
	2007[1]	3.910 ± 0.450	74.508	22	5.58 (4.04–7.53)	11.87 (8.58–22.98)
	2008[1]	3.316 ± 0.359	194.810	22	3.80 (1.82–7.28)	9.26 (5.35–61.55)
	2011[1]	2.609 ± 0.39	11.877	6	7.93 (4.41–15.93)	24.57 (12.99–117.36)
Southern Germany/East France	2005[1]	ncr[c]				
	2006[1]	2.064 ± 0.248	65.733	22	4.79 (2.42–7.43)	20.00 (12.44–48.41)
	2007[1]	3.120 ± 0.312	34.567	30	5.78 (4.84–6.79)	14.88 (12.11–19.80)
Northwest Italy	2007[1]	3.287 ± 0.67	3.733	6	2.81 (2.16–3.59)	6.90 (5.04–13.01)
Northeast Italy	2008[1]	3.663 ± 0.335	41.878	38	9.43 (8.21–10.78)	21.10 (17.76–26.58)
Pannonian area	2006[1]	2.695 ± 0.516	32.750	22	6.13 (2.83–8.76)	18.33 (13.26–32.59)
	2008[1]	3.271 ± 0.467	7.634	14	4.01 (3.24–4.89)	9.87 (7.67–14.62)
	2010[1]	3.343 ± 0.441	51.431	22	7.81 (5.47–10.38)	18.88 (13.66–35.26)
	2012[2]	4.958 ± 1.203	7.539	6	4.96 (2.66–6.42)	8.99 (6.89–21.02)
Southeast Poland	2007[1]	3.191 ± 0.524	13.342	6	6.29 (3.49–12.48)	15.86 (8.96–86.93)
	2008[1]	ncr[c]				
East Romania	2008[1]	3.00 ± 0.48	6.00	6	14.29 (10.56–19.19)	38.18 (27.16–65.65)
	2009[1]	2.762 ± 0.29	45.227	22	9.75 (7.07–13.03)	28.38 (20.20–48.72)
	2012[2]	3.262 ± 0.239	661.69	54	3.00 (1.76–4.61)	7.42 (4.80–19.78)
West Romania	2008[1]	3.104 ± 0.347	21.309	22	8.53 (7.06–10.19)	22.07 (17.67–30.02)
	2009[1]	2.732 ± 0.252	47.992	22	4.60 (3.53–6.08)	13.55 (9.59–23.42)
	2012[2]	2.871 ± 0.296	63.376	30	2.90 (2.17–3.70)	8.11 (6.07–12.77)
Central Iberia	2009[1]	2.179 ± 0.214	77.723	30	3.09 (2.03–4.33)	11.98 (8.12–22.31)
	2011[2]	3.113 ± 0.243	120.85	46	1.56 (1.27–1.91)	4.04 (3.12–5.91)
	2013[2]	3.023 ± 0.226	19.007	16	2.40 (2.04–2.83)	6.38 (5.18–8.34)
	2015[2]	5.04 ± 0.522	29.708	25	1.88 (1.68–2.11)	3.38 (2.91–4.21)
Northeast Iberia	2008[1]	2.411 ± 0.265	24.643	14	7.03 (4.89–10.03)	23.91 (15.76–46.84)
	2009[1]	3.887 ± 0.458	30.239	22	6.40 (5.32–7.75)	13.68 (10.77–20.02)
	2011[2]	3.463 ± 0.294	69.585	46	1.79 (1.54–2.07)	4.19 (3.45–5.48)
	2013[2]	3.775 ± 0.262	92.406	24	2.40 (1.92–3.00)	5.24 (4.04–7.80)
	2015[2]	3.144 ± 0.221	52.973	25	2.12 (1.75–2.55)	5.43 (4.36–7.29)
Southwest Iberia	2008[1]	3.078 ± 0.301	78.879	46	3.54 (2.89–4.20)	9.24 (7.46–12.70)
	2010[1]	3.665 ± 0.377	71.045	54	5.19 (4.49–5.91)	11.61 (9.71–15.06)
	2012[2]	3.902 ± 0.285	189.77	28	4.08 (2.99–5.50)	8.69 (6.30–15.56)
	2014[2]	2.798 ± 0.24	84.072	25	1.32 (0.94–1.74)	3.80 (2.78–6.21)

[a]Superscript in year of collection indicates Cry1Ab batch used (1 or 2).
[b]MIC values given in ng Cry1Ab/cm^2. CL, confidence limits.
[c]ncr, no concentration response.

was 13.1-fold for all years. Samples were pooled to correspond to homogeneous regions based on knowledge of insect biology and geography (Alcalde et al., 2007). Susceptibility of *O. nubilalis* collected in different geographic regions and exposed to purified Cry1Ab protein differed only slightly. Although variation in susceptibility to Cry1Ab was found among the populations pooled according to geographic and climatic conditions, the magnitude of the variation in MIC_{50} was small. The MIC_{50}-values varied by 1.8-fold, 6.6-fold, 2.6-fold, 4.2-fold, 3.2-fold, 2.0-fold and 5.1-fold for *O. nubilalis* collected in 2005, 2006, 2007, 2008, 2009, 2010 and 2011, respectively. The MIC_{50}-values for samples collected in the field in the years 2012–2015 and tested using Cry1Ab batch 2 varied by 1.4-fold, 1.0-fold, 4.1-fold and 1.6-fold for *O. nubilalis* collected in 2012, 2013, 2014 and 2015, respectively. The susceptibility of the field populations to batches 1 and 2 was also similar to that of the reference strains. There was no shift towards decreased susceptibility over time in the field-collected strains (Table 1).

It seems that the susceptibility of larvae from G.04 declined during the bioassays from 2006 to 2012 (Figure 1). As no other change in health or performance was observed in this strain, it is possible that the Cry1Ab protein in batch 1 degraded over time. A concentration–response relationship could be identified for moulting inhibition of the reference strain ES.ref only if an outlier value for two larvae that moulted to the second instar after being exposed to 20 ng/cm^2 was not included in the calculation. When the outlier was excluded, the Spanish reference strain (ES.ref: MIC_{50} 1.82 [1.53–2.16], MIC_{90} 2.95 [2.43–4.54]) was more susceptible than the other reference strain (G.04: MIC_{50} 4.03 [2.85–4.86], MIC_{90} 7.03 [5.83–9.91]) (Figure 2).

In Iberia, the only area in Europe where MON 810 varieties of maize are significantly cultivated, *O. nubilalis* remains highly susceptible to Cry1Ab protein. *O. nubilalis* sampled in Southwest Iberia in 2014 were even more susceptible than those collected in this region

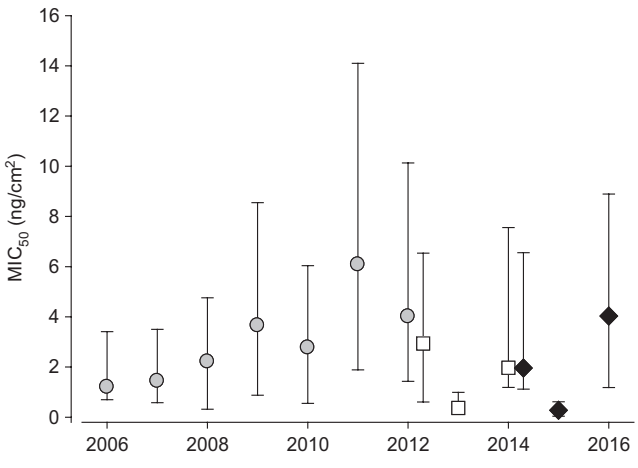

FIGURE 1 Moulting inhibition concentration (MIC_{50})-values (±95% confidence limits) of *Ostrinia nubilalis* (reference strain G.04) recorded after feeding for 7 days on a diet treated with batch 1 (grey circles), batch 2 (white squares) and batch 2a (black diamonds) of protein Cry1Ab. Batch 2a was a resupply of batch 2 and was shown by a bridging experiment to have an equivalent diagnostic concentration (data not shown)

in 2012 (Table 1). Whereas the MIC_{50} and MIC_{90} values for *O. nubilalis* collected in 2015 in fields of IbC were significantly less than that of the reference strain G.04 in susceptibility to Cry1Ab (Figure 2), there was a significant difference between MIC_{50} of larvae of G.04 and IbNE, but not between MIC_{90} values. Different results were achieved if the MIC_{50} and MIC_{90} values for the Spanish field populations and reference strain from Spain (ES.ref) were compared (Figure 2 and Table S2). The MIC_{50} values were not significantly different, but the MIC_{90} value from IbNE differed significantly from the reference strain ES.ref, whereas IbC did not. Overall, the responses of the field-collected IbC and IbNE populations fell between those of the two reference strains.

3.2 | Diagnostic concentration

Using the MIC_{99}, the DC for *O. nubilalis* larvae from Europe was 48.22 ng/cm^2 for batch 1. In separated bioassays used as bridging experiments, larvae of *O. nubilalis* (reference strain G.04, IbC and IbNE) were exposed to Cry1Ab batch 1 and batch 2 each with eight concentrations (0.5–80 ng/cm² Cry1Ab). According to these bioassays, batch 2 induced a stronger response. The quotient between MIC_{99} (batch 1) divided by MIC_{99} (batch 2) is 1.709. The DC_{batch1} (48.22 ng/cm^2) divided by 1.709 is resulting in a lower DC of 28.22 ng/cm^2.

3.3 | Confirmatory experiments

In 2005, one larva reached the 2nd instar at the highest concentration. It was decided then that in future bioassays all larvae of *O. nubilalis* that survived at the highest concentration (50%–100% of the DC, depending on the year) would be placed in plastic boxes and provided ad libitum with newly detached leaves from MON 810 maize, and any survivors recorded. For the seasons reported here, except for 2005, no larvae reached the 2nd instar when the diagnostic concentration of either batch of Cry1Ab was used after 7 days, and therefore, this additional testing was not necessary. For the *O. nubilalis* collected 2014 in Spain, two larvae survived 7 days of exposure to a concentration of 50% of the DC (14.11 ng/cm^2). One insect collected from Romania in 2012 and two insects collected as reference strain from Spain in 2015 survived exposure to a dosage of 20 ng/cm^2. When these five larvae were fed MON 810 maize, all of them died within 2 days.

4 | DISCUSSION

Many studies have examined the effect of *Bt* proteins on different pests of maize (Chaufaux, Seguin, Swanson, Bourguet, & Siegfried, 2001; Pereira, Lang, Storer, & Siegfried, 2008; Petzold-Maxwell et al., 2014; Priesnitz, Vaasen, & Gathmann, 2016). Studies analysing economically important European pest species are rare because only in Spain is *Bt* maize a sufficiently important crop (James, 2014). The data from the present study indicate that, despite use of Cry1Ab maize event MON 810 in Europe over a 10-year period, there is no evidence of any change in susceptibility of *O. nubilalis*. There was

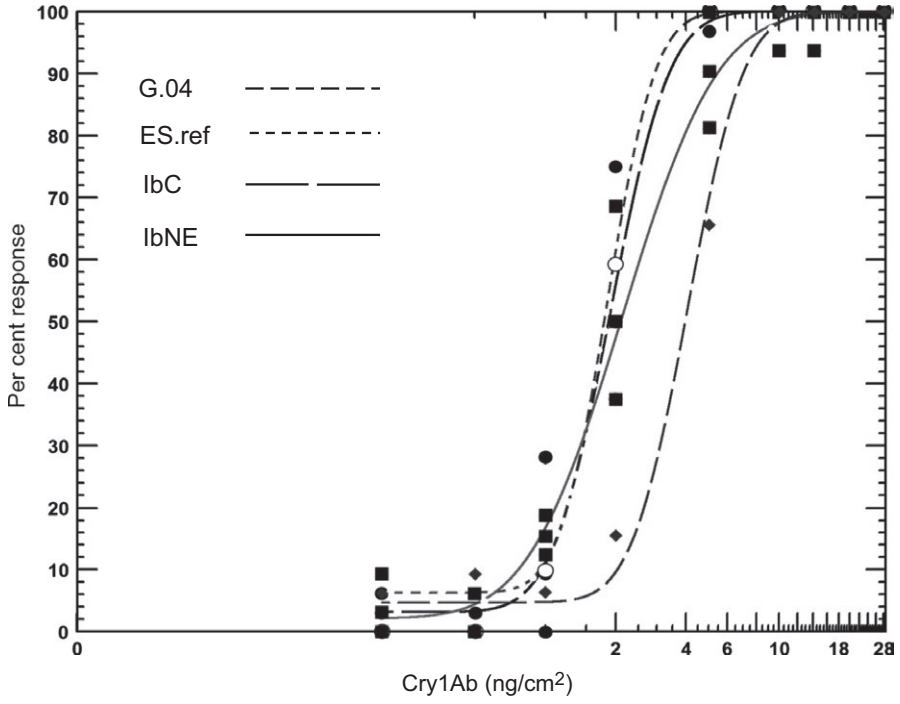

FIGURE 2 Fitted curves of susceptibility of two reference strains of *Ostrinia nubilalis* (G.04 collected in 2002 (black diamonds), ES.ref collected in 2015 (white circles)) tested in 2016 and field samples (collected in 2015: Iberia Central [IbC] (black circles) and Iberia Northeast [IbNE]) (black squares) measured over 7 days as the percentage that did not moult when fed a diet treated with batch 2 of protein Cry1Ab. (see Table S2)

little variability among regions over the course of the study, and comparisons in 2015 showed similar susceptibility of samples collected in Iberia (where MON 810 has been grown the longest) and reference strains. These results are similar to those from the USA, where more than 10 years of monitoring of field-collected populations found no change in Cry1Ab susceptibility of *O. nubilalis* (Siegfried et al., 2007). Similar levels of variability were also observed in a study that included populations of different voltine ecotypes and pheromone strains (Marçon, Taylor, Mason, Hellmich, & Siegfried, 1999). For the current study, the pheromone races were not distinguished.

In the first year of the present study (2005), some larvae survived at the highest tested Cry1Ab dose, leading us to implement a confirmatory testing plan in subsequent bioassays. In only one subsequent year (2015) did any larvae survive the highest or the DC, and none of those survived the confirmatory test on *Bt* maize leaves. Similar results have been reported in other world areas. In the USA, Siegfried et al. (2007) tested more than 150 US field-collected *O. nubilalis* populations and only one showed <99% mortality at the diagnostic Cry1Ab concentration. In subsequent testing, the larvae from this exceptional population did not survive on vegetative-stage *Bt* maize (Crespo et al., 2009; Siegfried et al., 2007). In a study of Asian corn borer (*Ostrinia furnacalis*) in the Philippines, some larvae survived on the DC of Cry1Ab but not on *Bt* corn leaf tissue (MON 810) (Alcantara, Estrada, Alpuerto, & Head, 2011). These studies indicate that survival, if any, on the chosen DC should be further evaluated to assess its significance relative to product performance in the field (Siegfried et al., 2007).

As recently reported (Castañera et al., 2016), monitoring of *S. nonagrioides* also has revealed no evidence for the development of Cry1Ab resistance in Spain over 16 years of *Bt* maize use. Castañera et al. (2016) speculate that the compliance with refuge requirements in NE Spain, which is currently >90%, will contribute to product longevity. The present study includes data from areas throughout Europe and similarly shows no evidence of resistance to MON 810 maize. In addition to the factors already mentioned, the low adoption of *Bt* maize in the EU, outside of Spain, may have contributed to the observed lack of resistance.

To be suited for long-term, broad-area monitoring studies, the tools and methods used must make efficient use of protein and insect samples, as well as enabling comparisons across years. Regarding the assay method itself, two different bioassays, the incorporation method and the overlay or surface treatment method, have been commonly used. An advantage of the overlay treatment is the lower amount of protein required for each test. Saeglitz et al. (2006) argue that, compared with the overlay treatment, the incorporation method results in a more homogenous distribution of the protein solution in the diet and thus more consistent results. However, the incorporation method also is more time-consuming in terms of preparation and requires larger amounts of protein (Meise, 2003; Saeglitz, 2004; Siegfried et al., 2007). For these reasons, the majority of the susceptibility data published (as in this study) are based on the overlay treatment.

Comparisons of baseline susceptibility data from bioassays using the same method but with different batches of protein are difficult because susceptibility values may change with the batch. These differences may be erroneously interpreted as resistance evolution. The greatest variability in the MIC_{50} for field samples collected from 2005 to 2011 and tested with batch 1 was 6.6-fold in 2006. For field-collected *O. nubilalis*, the difference between MIC_{50} values of the most susceptible and most tolerant samples was 13.1-fold for this period. For samples collected in 2012–2015 and tested with batch 2, the greatest variability was 4.1-fold in 2014. For American *O. nubilalis* populations, LC_{50} values ranged from 4.11 to 11.95 ng/cm^2 for bioassays performed with native purified Cry1Ab protein (Siegfried, Zoerb, & Spencer, 2001). Previously reported LC_{50}-values for *O. nubilalis* of

Spanish origins varied from 3–4 ng/cm^2 (Farinós et al., 2004) to 104–109 ng/cm^2 (González-Núñez et al., 2000). Saeglitz et al. (2006) argue that the same protein batch should be used for baseline susceptibility bioassays and subsequent monitoring tests, but this requires producing and storing sufficient amounts of protein for long-term susceptibility monitoring all at one time. Cry1Ab protein activity can decline up to 11-fold after 2 years' storage at −20°C, but when stored at −80°C, the protein activity remains relatively stable for at least 30 months (Nguyen & Jehle, 2009). Because it is not possible to use a single protein standard indefinitely, every new protein batch has to be compared in bridging experiments with the protein standard used before.

The use of DCs, rather than concentration–response curves, provides several benefits including use of less protein, fewer total insects and greater sensitivity (more insects tested at the most informative concentration level) (Siegfried et al., 2007; Sims, Greenplate, Stone, Caprio, & Gould, 1996). Consequently, resistance in monitoring for Europe is being transitioned to a DC strategy using the values developed in this study.

The reference strains were established as a quality control for the bioassays and were used in protein bridging studies. Because rearing of insects for long periods could result in inbreeding effects, and the reference strain G.04 has been kept in culture since 2002, we considered whether this strain should be refreshed with new field-collected insects, but concluded that this could alter its response to Cry1Ab. Instead, a new reference strain (ES.ref) was established. However, bioassays showed that the reference strain G.04 did not shift in susceptibility during this decade-long study.

Previous studies on the baseline susceptibility of European corn borer revealed some variability (Marçon, Young et al., 1999; Siegfried, Marçon, Witkowski, Wright, & Warren, 1995), which indicated a potential for resistance development (Rossiter, Yendol, & Dubois, 1990). In addition, variation in susceptibility among *O. nubilalis* from different regions makes identifying and differentiating between natural variation and low levels of resistance challenging (Glare & O'Callaghan, 2000; Koziel et al., 1993). Thus, continuous monitoring using robust methods is necessary to ensure that any resistance that does evolve is detected and addressed promptly (Siegfried et al., 2007; Sivasupramaniam, Head, English, Li, & Vaughn, 2007).

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AUTHOR CONTRIBUTION

TT, CB and KG conceived research. CB and KG conducted experiments. FO and GF contributed samples. TT, CB and KG analysed data and conducted statistical analysis. TT and CB wrote the manuscript. TT and KG secured funding. All authors read and approved the manuscript.

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