Appendix 8. Insect Resistance Monitoring in European populations of *Ostrinia nubilalis* (ECB): 2011 Season

Report

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

- Results for 2005-2012 -

Date

27/06/2012

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1. Introduction

The European corn borer (ECB), *Ostrinia 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 ECB has rapidly spread across North America (CAFFREY and 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 ECB (HODGSON, 1928; PONSARD et al., 2004). Now ECB is one of the most damaging pests of maize in North America and Europe and a major target pest for control with transgenic maize expressing *Bacillus thuringiensis* (Bt) toxins.

In order to maintain the benefits obtained from growing Bt maize varieties, Monsanto established an insect monitoring program across Europe and in particular in areas where commercial activity of MON 810 genetically modified maize is occurring or planned. An important need prior to the growing of Bt maize varieties consists of establishing the baseline susceptibility of field populations of ECB to the Bt Cry1Ab protein, which is the active ingredient in MON 810. Then, every two years, a routine monitoring program will survey and quantify any potential change in susceptibility in ECB field populations exposed to Bt maize cultivation. This program will enable early detection of resistance in ECB if it occurs, and this will allow the proposal and implementation of additional risk mitigation measures. For efficiency, the baseline studies and monitoring of resistance have been focused in areas where the potential resistance risk is relatively high because the introduction of Bt maize is relatively high (or expected to be), and local entomologists recognize ECB to be abundant.

Previous baseline susceptibility studies. Baseline susceptibility to Cry1Ab protein has been established for ECB populations collected in different maize areas in Spain (GONZALEZ-NUNEZ et al., 2000, FARINÓS et al., 2004), Germany (SAEGLITZ et al., 2006) and the United States (MARÇON et al., 1999a and 2000). The methodology for those studies involved applying a solution with the appropriate Cry1Ab protein concentrations on the surface of artificial diet (GONZALEZ-NUNEZ et al., 2000).

2. Materials and Methods

2.1. Insect Collection

For the current study, ECB were collected from 2005–2011 in major European maize growing regions (also referred as populations): Czech Republic/Moravia (CZ), Southwest and West France (Fsw, Fw), Northern Germany/Southwest Poland (GnPLw), Southern Germany and East France (GsFe), Northern Italy (ITne, ITnw), the Panonian region (PAN, Western Slovakia and North West Hungary), Southeast Poland (PLse), South Portugal (Ps), Romania (ROe, ROw) and Spain (ESne, ESc, ESsw; see Fig. 1). For each region, up to three sampling sites separated by at least 50 to 100 km were chosen. ECB were collected as adults, larvae or egg masses in naturally infested fields. Larvae were collected by dissecting corn stalks a few days before harvesting or in spring after diapause. If more than one larva per stalk was found, only one was taken to avoid collecting siblings (Fig. 2). For each sampling site, the aim was to collect at least 300 larvae (Table 1).

To collect egg masses, light traps and large cages (2 x 2 x 2 m) were used in corn fields. Adult ECB were attracted by the light during flying season and caught alive in the cages. Every two to three days, egg masses oviposited on the corn plants in the cage were cut off and transferred to the laboratory. The number of collected egg masses varied between 35 and 815.

This insect collection and population establishment scheme is in compliance with the industry IRM (Insect Resistance Management) working group guidelines proposed to the competent authority (EU Commission) (available since 2003 but published in 2007; ALCALDE et al., 2007).

2.2. Insect Culture

Field-collected ECB larvae were placed in plastic boxes containing corrugated cardboard and maintained in a growth chamber at 25°C, 90% RH and a photoperiod of 20:4h (L:D) on an agar-based wheat germ diet (Table 2). If the larvae did not pupate after a period of two weeks, they were assumed to have entered diapause and were transferred to another climatic chamber maintained at 8 ± 2°C, 70 ± 5% RH, and a photoperiod of 0:24h (L:D) (Fig. 3). All ECB collected as larvae (L3–L5) entered diapause after collection. The mass of larvae varied between 61–165 mg at the beginning of diapause.

In early 2006 it was tested if diapause can be broken artificially. After three month in diapause conditions a portion of the larvae collected near Heinersdorf/Oderbruch, Germany (G.03) were placed at 25 ± 2°C, 80 ± 5% RH, and a photoperiod of 24:0h (L:D). The cardboard was moistened daily until pupation (Fig. 4). The emergence of these adults occurred over a period of nearly two months at a very low level. This complicated the production of a sufficient number of egg masses to be used in subsequent bio assays. To achieve a more synchronized adult eclosion it was therefore decided to allow the completion of diapause until May (as in nature) as standard practice.

Larvae surviving the diapause period were transferred to fresh containers to prevent contamination by dead larvae. To increase the temperature step-wise, these containers were maintained for 5d at 15 ± 2°C, for the next 5d at 20 ± 2°C, and thereafter at 25 ± 2°C. At all temperature steps, larvae were maintained at 90 ± 5% RH and a photoperiod of 24:0h (L:D). During this time, additional water for drinking was added. Any food supplied was not taken by the larvae. To transfer emerged adults from diapause boxes into oviposition cages CO_2 was used.

Egg masses were obtained by confining batches of up to ten pairs of adults in cylindrical plastic tubes (∅ 11 cm, Fig. 5). Adults were fed with 15 % honey-water to increase fecundity and egg laying (LEAHY & ANDOW, 1994). The inside of the tubes were covered with filter paper as oviposition medium. These paper sheets were changed twice a week. 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. Oviposition cages and incubated egg masses were maintained in an environmental chamber for 20 h at 25 ± 2°C, 4 h at 20 ± 2°C, 90 ± 5% RH and a photoperiod of 20:4h (L:D) (GUTHRIE et al., 1985).

2.3. Bioassays

Two batches of Cry1Ab toxin have been used. The first batch was provided by Monsanto and was stored at -80°C until used (NBR: 7553190, 05/23/2005; concentration 2.0 mg/ml in 25 mM bicarbonate buffer, pH 10.5). To prepare the test concentrations, a bicarbonate buffer (25 mmol/l) with pH 10.5 was used. The second batch was also provided by Monsanto and was stored at -80°C until used (NBR: 11247229, 31/01/2012; concentration 1.64 mg/ml in 50 mM bicarbonate buffer,

pH 10.25). To prepare the test concentrations, a bicarbonate buffer (50 mmol/l) with pH 10.25 was used. To analyse if the two batches differ in efficacy a bridging experiment was done. The bioassays were performed in 128 well trays (Bio-Ba-128, Color-Dec, Italy). In each cell 1 ml of artificial diet was dispensed. After the diet solidified, 100 µl of toxin solution was applied to the surface and allowed to dry over night at room temperature. To avoid contaminations the trays were covered with a sheet of filter paper. Egg masses of each sampling location (field-collected or offspring of collected larvae) were incubated and neonate larvae, within 12 h after hatching, were transferred to the cells. If the number of collected egg masses was too low to perform an assay, ECB were reared for one generation and the resulting offspring were tested. A single neonate was placed in each cell and confined with a cover (Bio-Cv-16, Color-Dec Italy) (Fig. 6). Eight concentrations and a control (bicarbonate buffer) were tested for each population. Those tested with the first batch were exposed to 0.5–256.0 ng Cry1Ab/cm^2 and those tested with the second batch to 0.2–40.0 ng Cry1Ab/cm^2. Each concentration was tested with at least 16 larvae per sampling site. As for each region (population), ECB from up to three sampling sites separated by at least 50 to 100 km were analysed, a total of 3 (replicates) times 16 larvae was used for each concentration and each population. 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 were considered to be dead because larvae unable to moult under field conditions would not survive (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 programme SYSTAT, Version 10.0, except for concentration-response analysis where PoloPlus 1.0 was used (LeOra Software Company). The measure of how well the data (response of ECB to different concentrations of toxin) 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, lines are 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.

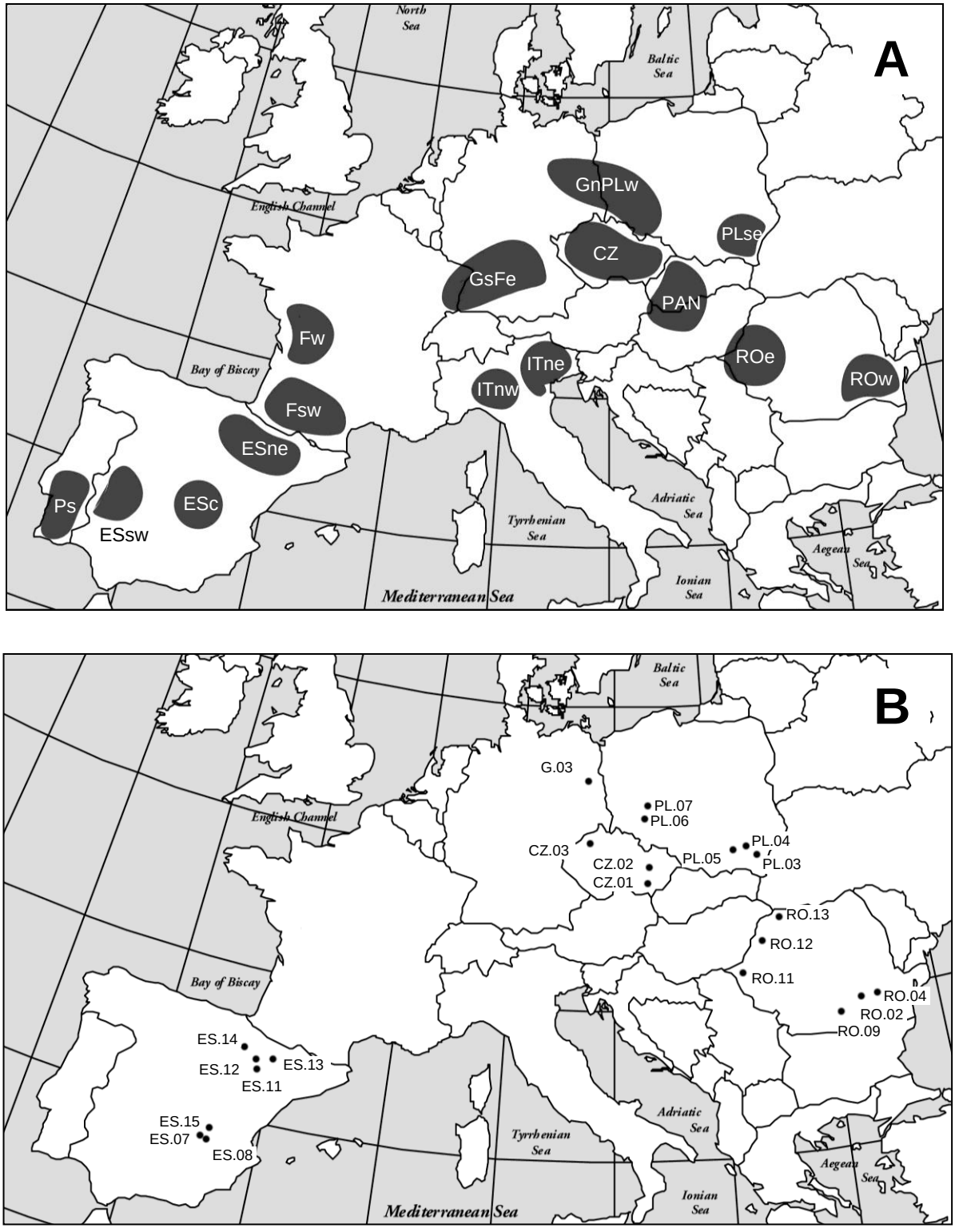

Fig. 1: ECB populations considered in Europe (**A**) and sites sampled 2011 (**B**)

Fig. 2: Dissected maize stalk with larvae.

Fig. 3: Growth chamber with plastic boxes containing diapausing ECB larvae

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Fig. 4: Corrugated cardboard with pupae.

Fig. 5: Oviposition cages for adult ECB.

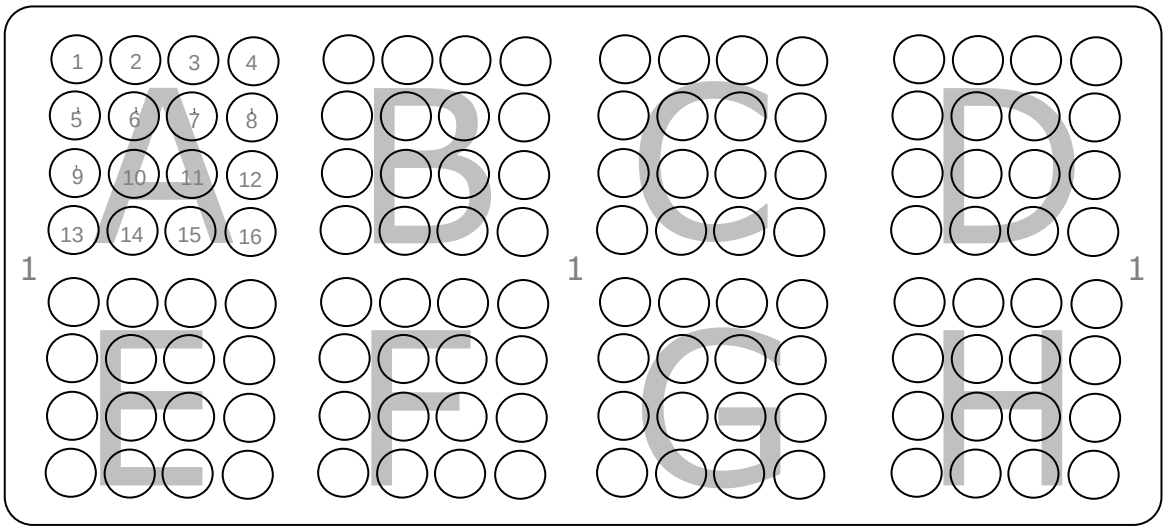

Fig. 6: IDs of Bio-Ba-128 trays (tray number, field letter, well number; i.e.: 1.A.13)

Table 1: Source description of ECB collections used to establish baseline susceptibility to the Cry1Ab toxin.

(in prep. – in preparation, results will be reported 2013; new data are marked **bold**).

Country	Collection site	ID	Population	Collected	Eggs	Larvae	Adults	Tested
Czech Republic	Čejč	CZ 01	CZ	2005		x		2006
	Velké Bílovice	CZ 01	CZ	2009		x		2009
	Tvrdonice	**CZ 01**	**CZ**	**2011**		**x**		**2011**
	Ivanovice na Hané	CZ 02	CZ	2005		x		2006
		CZ 02	CZ	2008	x			2008
		CZ 02	**CZ**	**2011**		**x**		**2012**
	Klapy	CZ 03	CZ	2005		x		2006
		CZ 03	CZ	2007		x		dead
		CZ 03	CZ	2008	x			2008
		CZ 03	**CZ**	**2011**		**x**		**2011**
France	Wiwersheim	F 01	GsFe	2005	x			dead
		F 01	GsFe	2006		x		2007
		F 01	GsFe	2007	x			2007
	Miradoux dans le Gers	F 02	Fsw	2005		x		2006
		F 02	Fsw	2008		x		2009
	Berat en Haute-Garonne	F 03	Fsw	2005		x		2006
	Lezat Sur Leze en Ariege	F 04	Fsw	2005		x		2006
	Savigny	F 05	Fw	2006	x			2006
	Pamproux	F 06	Fw	2006	x			2006
	Taizé-Aizié	F 07	Fw	2006	x			2006
	Saint Barthelemy	F 08	Fsw	2006		x		2007
		F 08	Fsw	2007		x		2008
		F 08	Fsw	2008		x		dead
	Auriac	F 09	Fsw	2006		x		2007
	Solomiac (Tarn et	F 10	Fsw	2006		x		2007
	Frégouville	F 11	Fsw	2007		x		dead
	Lavaur	F 12	Fsw	2007		x		dead
	Orgueil	F 13	Fsw	2008		x		2009
	Saint Bonnet sur-Gironde	F 14	Fw	2008		x		dead
	Lusignan	F 15	Fw	2008		x		dead
	Thurageau	F 16	Fw	2008		x		dead
Germany	Herbolzheim	G 01	GsFe	2005	x	x		2006
		G 01	GsFe	2007		x		dead
	Sugenheim	G 02	GsFe	2005		x		2006
	Heinersdorf	G 03	GnPL	2005		x		2006
		G 03	GnPL	2008	x			2008
		G 03	**GnPL**	**2011**	**x**			**2011**
	Niedernberg	G 04	lab	2005	x			2006
	(reference-strain)	G 04	lab	2005	x			2007
		G 04	lab	2005	x			2008
		G 04	**lab**	**2005**	**x**			**2012**
	Ansbach	G 05	GsFe	2006	x			2006
	Keindorf	G 06	GnPL	2006		x		2007
		G 06	GnPL	2008		x		dead
	Altreetz	G 07	GnPL	2007	x			2007
		G 07	GnPL	2008	x			2008
	Ulsenheim/Uffenheim	G 09	GsFe	2007	x			2007
	Kitzingen	G 10	GsFe	2007		x		2008
Hungary	Bana/Gulyaré	HU 01	PAN	2006		x		2006
		HU 02	PAN	2006		x		dead

Table 1: continued

Country	Collection site	Site ID	Population	Collected	Eggs	Larvae	Adults	Tested
Italy	Cicignolo	IT 01	ITnw	2007		x		dead
	Ghisalba	IT 02	ITnw	2007		x		dead
	Belfiore	IT 03	ITnw	2007		x		dead
	Fiume Veneto	IT 04	ITne	2007		x		dead
	Gonars	IT 05	ITne	2007		x		dead
	Gonars	IT 06	ITne	2007		x		dead
	Padova	IT 07	ITne	2008		x		2008
	Vicenza	IT 08	ITne	2008		x		2008
	Fossalta di Piave	IT 09	ITne	2008		x		2008
Poland	Rzeszow	PL 01	PLse	2005			x	dead
	Rynakowice	PL 02	GnPLw	2006		x		dead
		PL 02	GnPLw	2007		x		2008
		PL 02	GnPLw	2008	x			2008
	Krzeczowice	PL 03	PLse	2006		x		dead
		PL 03	PLse	2007		x		2008
		PL 03	PLse	2008	x			2008
		PL 03	**PLse**	**2011**		**x**		**dead***
		PL 04	**PLse**	**2011**		**x**		**dead***
		PL 05	**PLse**	**2011**		**x**		**dead***
		PL 06	**GnPLw**	**2011**		**x**		**dead***
		PL 07	**GnPLw**	**2011**		**x**		**dead***
Portugal	Mix of three sites	P 01	Ps	2008	x			2009
	Alvalade do Sado	P 02	Ps	2010		x		2011
Slovakia	Levice	SK 01	PAN	2006	x			2006
		SK 01	PAN	2008	x			2008
	Nemčiňany	SK 01	PAN	2010	x			2010
	Vrbové	SK 02	PAN	2006	x			2006
	Trnava	SK 03	PAN	2008	x			2008
	Dolná Krupá	SK 03	PAN	2010	x			2010
	Mojzesovo	SK 04	PAN	2010	x			2010
Romania	Simand	RO 01	ROw	2007		x		dead
	Fundulea	RO 02	ROe	2007		x		dead
		RO 02	ROe	2008		x		2009
		RO 02	**ROe**	**2011**		**x**		**dead***
	Chirnogi	RO 03	ROe	2008		x		dead
	Cascioarele	RO 03	ROe	2009		x		2010
	Dalga	RO 04	ROe	2008		x		dead
		RO 04	**ROe**	**2011**		**x**		**dead***
	Paulis	RO 05	ROw	2008		x		2009
	Lipava	RO 05	ROw	2009		x		2010
	Buteni	RO 06	ROw	2008		x		2009
	Sandra	RO 07	ROw	2008		x		2009
	Carpinis	RO 07	ROw	2009		x		2010
	Berceni	RO 08	ROe	2009		x		2010
	Tudor Vladimirescu	RO 09	ROe	2009		x		2010
	Troianul	**RO 09**	**ROw**	**2011**		**x**		**dead***
	Nadlac	RO 10	ROw	2009		x		2010
	Lovrin	**RO 11**	**ROw**	**2011**		**x**		**in prep.**
	Pir	**RO 12**	**ROw**	**2011**		**x**		**in prep.**
	Livada	**RO 13**	**ROw**	**2011**		**x**		**in prep.**

Table 1: continued

Country	Collection site	Site ID	Population	Collected	Eggs	Larvae	Adults	Tested
Spain	Hernán Cortés	ES 01	ESsw	2008		x		2009
	Don Benito	ES 02	ESsw	2008		x		2009
		ES 02	ESsw	2010		x		2011
	Obando	ES 03	ESsw	2008		x		2009
	Gimenells	ES 04	ESne	2008		x		2009
	Candasnos	ES 05	ESne	2008		x		dead
		ES 05	ESne	2009		x		2010
	Erla/ Santa Anastasia	ES 06	ESne	2008		x		2009
	Ejea de los Caballeros	ES 06	ESne	2009		x		2010
	Barrax/Aguas Nuevas	ES 07	ESc	2008		x		dead
	Aguas Nuevas	ES 07	ESc	2009		x		2010
	Barrax	**ES 07**	**ESc**	**2011**		**x**		**2012**
	El Salobral	ES 08	ESc	2009		x		2010
		ES 08	**ESc**	**2011**		**x**		**2012**
	Bujaraloz	ES 09	ESc	2009		x		2010
	Porzuna	ES 10	ESsw	2010		x		2011
	Alfamén/Agramonte	**ES 11**	**ESne**	**2011**		**x**		**2012**
	Alagón/Gallur	**ES 12**	**ESne**	**2011**		**x**		**2012**
	Sariñena	**ES 13**	**ESne**	**2011**		**x**		**2012**
	Valtierra	**ES 14**	**ESne**	**2011**		**x**		**2012**
	Motilleja	**ES 15**	**ESc**	**2011**		**x**		**2012**

*strains lost by severe infection with Beauveria, will be re-collected in the next season

Table 2: Artificial diet for rearing and testing ECB larvae.

Water	680 ml
Benzoic acid [2]	1 g
Sorbic acid [1]	1 g
Nipagin (methyl-paraben)[1]	1 g
Agar-agar *[2]	16 g
Maize powder [3]	112 g
Wheatgerm[4]	28 g
Brewer's yeast [5]	30 g
Fumidil B [6]	1 g
Ascorbic acid [1]	3 g
Vanderzant vitmain mix [1]	2 g

* To prepare diet for Cry1Ab bioassays the amount of agar was halved to 8 g.

1. BioServ; One 8th Street, NJ 08825 Frenchtown, USA: Prod.No. 6030 (ascorbic acid); Prod.No. 6967 (sorbic acid); Prod.No. F8045 (vitamin mix); Prod.No. 7685 (nipagin, methyl-paraben)
2. Carl Roth GmbH & Co. KG; Schoemperlenstr. 3–5, 76185 Karlsruhe, Germany: Art.No. 5210.2 (Agar-Agar, Kobe 1 pulv.); Art.No. 5781.1 (Benzoic acid, ≥ 99.5 %)
3. Gut & Gerne, BZ Bio-Zentrale GmbH; 94166 Stubenberg, Germany
4. Frießinger Mühle GmbH; 74206 Bad Wimpfen, Germany
5. Biolabor GmbH & Co.KG; PF 15 01 31, 28091 Bremen, Germany
6. CEVA Salud Animal, S.A., c. Carabela La Nina, 12 5^{a} planta, 08017 Barcelona, Spain

When lines are neither equal nor parallel, neither their intercepts nor their slopes are equal, meaning the populations being compared differ in susceptibility and response characteristics. Ratios of activity at a response level such as 50 or 90% provide a means to estimate the relative susceptibility of populations of ECB to Cry1Ab. To provide an estimate of the variability involved in these ratios, 95% confidence intervals are calculated for each ratio. To determine whether the response of one group differs significantly from the other, their 95% confidence limits are compared. If the limits overlap, then the lethal concentrations do not differ significantly (at an error rate of 5%).

3. Results and Discussion

Bioassays estimating parameters such as the LC_{50} or LC_{90} are recommended as part of resistance management strategies (SIMS et al., 1996). It is most convenient to conduct the bioassays with purified toxin comparable to that produced by the Bt plant.

Using artificial diet, the toxin can be provided as a surface treatment (GOULD et al., 1997; HILBECK et al., 1998) or mixture (SAEGLITZ et al., 2006).

Susceptibility data for ECB collected in different geographic regions and exposed to purified Cry1Ab toxin differ only slightly. The lowest MIC_{50} value (1.20 ng/cm^2) was found for a colony collected 2002 in Niedernberg, Germany (G 04) and kept since then as laboratory strain. Repeated analysis showed small MIC variability for this strain within a season and between years. The differences between the smallest and greatest MIC_{50} calculated were 5.2-fold and for MIC_{90} 4.4-fold. For the field-collected ECB, the differences between MIC_{50} values of the most susceptible and the most tolerant field-collected samples were 13.1-fold for all years.

Results for populations pooled according to geographic and climatic conditions are presented in Table 3, Figure 7 and appendix Figures A01-05. Populations pooled correspond to homogeneous regions based on available knowledge of insect biology and geography. This approach follows the IRM industry working group guidelines available since 2003 but published in 2007 (ALCALDE et al., 2007).

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 differed 1.8-fold, 6.6-fold, 2.6-fold, 4.2-fold, 3.2-fold, 2.04-fold and 5.1-fold for ECB collected in 2005, 2006, 2007, 2008, 2009, 2010 and 2011,

respectively (Tab. 3). A similar degree of variability was reported for ECB susceptibility to Cry1Ab for populations from three broad geographic areas in the US, chosen based on market penetration for Bt corn (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 et al., 1999b). For the current study, the pheromone races were not distinguished.

The two batches of cry1Ab used differ in their efficacy. In a bridging experiment with the lab strain G.04 the second batch induced higher mortality to ECB than the first batch (appendix Figure A01). The MIC_{50} and MIC_{90} values differed 1.4-fold, and 1.6-fold, respectively. According to a comparison using data of bridging experiments with the lab strain G.04 and both populations from Spain (Esc with ES.07, ES.08 and ES.15, and ESne with ES.11-15) the MIC_{50} and MIC_{90} values differed 1.6-fold, and 1.9-fold, respectively (appendix Figure A06).

Table 3. Susceptibility of ECB neonates exposed to Cry1Ab as measured by the MIC. Collections were pooled according to geographic and climatic similarity. ([a] ng Cry1Ab/cm^2; MIC moulting inhibition concentrations, CI confidence interval, * no concentration response, new data marked **bold**).

Population	MIC_{50} (95% CI)[a]	MIC_{90} (95% CI)[a]
Czech Republic		
2005 (CZ 01, CZ 02, CZ 03)	7.59 (6.30–8.72)	15.47 (13.16–19.89)
2008 (CZ 02, CZ 03)	*	
2009 (CZ 01)	8.99 (5.74–12.35)	17.67 (12.80–37.88)
2011 (CZ 01, CZ.02 CZ 03)3	**1.45 (0.27–3.08)**	**8.39 (3.92–53.57)**
France Southwest		
2005 (F 02, F 03, F 04)	11.13 (1.38–25.78)	56.98 (24.50–296.54)
2006 (F 08, F 09, F 10)	3.14 (2.58–3.61)	5.49 (4.74–6.91)
2007 (F 08)	9.08 (4.75–12.92)	26.74 (18.13–68.38)
2008 (F 02, F 13)	4.81 (3.89–5.88)	10.70 (8.37–16.01)
France West		
2006 (F 05, F 06, F 07)	18.48 (15.53–21.67)	25.97 (22.06–37.29)
Germany North/Poland West		
2005 (G 03)	13.33 (9.45–17.64)	35.70 (25.97–60.49)
2006 (G 06)	2.82 (1.87–3.70)	6.60 (4.84–13.18)
2007 (G 07, PL 02)	5.58 (4.04–7.53)	11.87 (8.58–22.98)
2008 (G 03, G 07, PL 02)	3.80 (1.82–7.28)	9.26 (5.34–61.55)
2011 (G.03)	**7.93 (4.41–15.93)**	**24.57 (12.99–117.36)**
Germany South/France East		
2005 (G 01, G.02)	*	
2006 (F 01, G 05)	4.79 (2.42–7.43)	20.00 (12.44–48.41)
2007 (F 01, G 09, G 10)	5.78 (4.84–6.79)	14.88 (12.11–19.80)
Italy Northeast		
2008 (IT 07, IT 08, IT 09)	9.47 (8.26–10.80)	21.10 (17.81–26.44)
Panonia		
2006 (HU 01, SR 01, SR 02)	6.13 (2.83–8.76)	18.33 (13.33–32.59)
2008 (SK 01, SK 03)	4.10 (3.24–4.89)	9.87 (7.67–14.62)
2010 (SK 01, SK 03, SK 04)	7.81 (5.47–10.38)	18.88 (13.66–35.26)
Poland Southeast		
2007 (PL 03)	6.29 (3.49–12.48)	15.86 (8.96–86.93)
2008 (PL 03)	*	
Portugal South		
2008 (P 01)[1]	3.66 (2.46–4.78)	11.90 (8.80–20.04)
2010 (P 02)[2]	4.37 (3.51–5.25)	11.81 (9.36–16.84)

[1] Pooled bioassay of three sites, [2] Replicated three times, [3] This data haven't been available when the 2010-report was submitted and are thus reported here, [4] second cry1Ab batch.

Table 3 continued

Population	MIC50 (95% CI)[a]	MIC90 (95% CI)[a]
Romania East		
2008 (RO 02)	14.29 (10.56–19.19)	38.18 (27.16–65.65)
2009 (RO 03, RO 08, RO 09)	9.75 (7.07–13.03)	28.38 (20.20–48.72)
Romania West[3]		
2008 (RO 05, RO 06, RO 07)	8.53 (7.06–10.19)	22.07 (17.67–30.12)
2009 (RO 05, RO 07, RO 10)[3]	4.60 (3.53–6.08)	13.55 (9.59–23.42)
Spain Central		
2009 (ES 07, ES 08)	3.09 (2.03–4.33)	11.98 (8.12–22.31)
2011 (ES.07, ES.08, ES15)[4]	**1.56 (1.27–1.91)**	**4.04 (3.12–5.91)**
Spain Northeast		
2008 (ES 04, ES 06)	7.03 (4.89–10.03)	23.91 (15.76–46.84)
2009 (ES 05, ES 06, ES 09)	6.40 (5.32–7.75)	13.68 (10.77–20.02)
2011 (ES.11, ES.12, ES.13, ES.14)[4]	**1.79 (1.54–2.07)**	**4.19 (3.45–5.48)**
Spain Southwest		
2008 (ES 01, ES 02, ES 03)	3.39 (2.94–3.89)	6.90 (5.79–8.89)
2010 (ES 02, ES 10)	5.76 (4.38–7.84)	11.85 (8.53–23.52)

[1] Pooled bioassay of three sites, [2] Replicated three times, [3] This data haven't been available when the 2010-report was submitted and are thus reported here, [4] second cry1Ab batch.

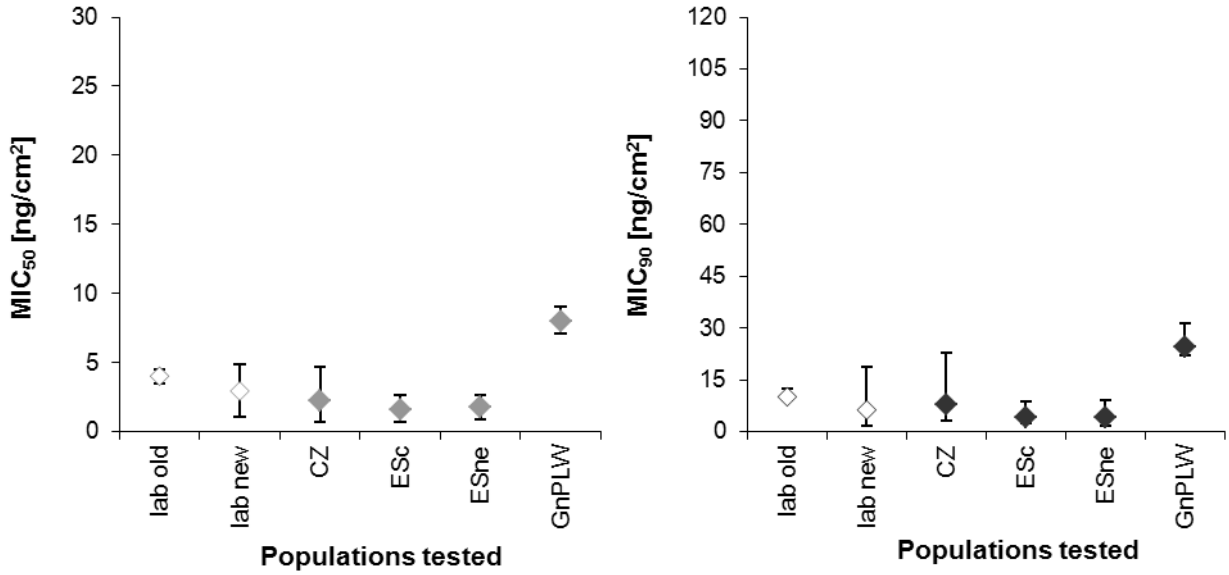

Figure 7: MIC_{50} (left hand site) and MIC_{90} (right hand site) ± 95 % CI of ECB neonates exposed to Cry1Ab in 2011 (Legend see Tab. 3; lab - reference strain; the strains lab new, Esc and ESne were tested using the second batch of cry1Ab).

4. Conclusions

During 2005–2012, 15 populations with 111 samples (including replicates and assays without concentration response relationship) of ECB were analysed. Thus far,

susceptibility to Cry1Ab have been assessed for one laboratory colony and populations collected in maize fields in Czech Republic, France, Germany, Italy, Hungary, Slovakia, Poland, Portugal, Romania, and Spain. ECB larvae were exposed to artificial diet treated with increasing Cry1Ab concentrations, and mortality and growth inhibition were evaluated after 7 days. Variation in Cry1Ab susceptibility (MIC_{50}) of field samples was up to 13.1-fold. A smaller variability was found for populations pooled according to geographic and climatic conditions (up to 6.6-fold). The results indicate that the observed population variation in susceptibility reflects natural variation in Bt susceptibility among ECB populations. Any evidence for a decrease of Cry1Ab susceptibility of populations during the monitoring duration from 2005–2012 could not be detected.

Further analyses have to be done to evaluate if the European populations of ECB are uniformly susceptible to Cry1Ab without any obvious genetic differentiation linked to geographical or other factors. In the future, other regional sources may be added to ensure that the monitoring program continues to represent the Cry1Ab maize market in Europe.

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7. Appendix

The graphs presenting the results of probit analyses of samples assayed from 2011–2012.

Reference-lab strain

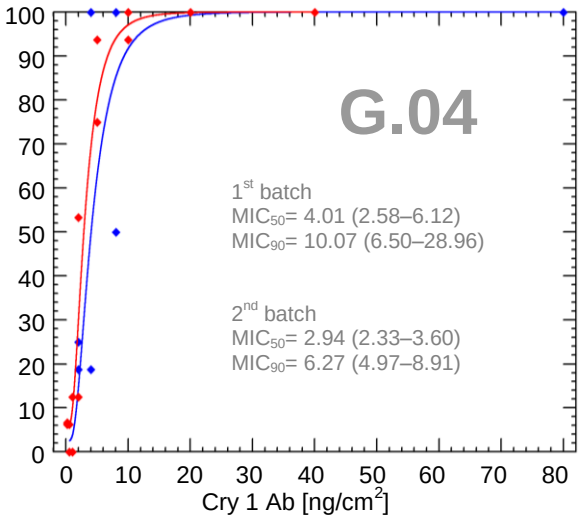

Fig. A1: Reference-lab strain (G.04);
Annual test 2012, 1st batch of cry1Ab (blue);
2nd batch of cry1Ab (red).

Czech Republic:

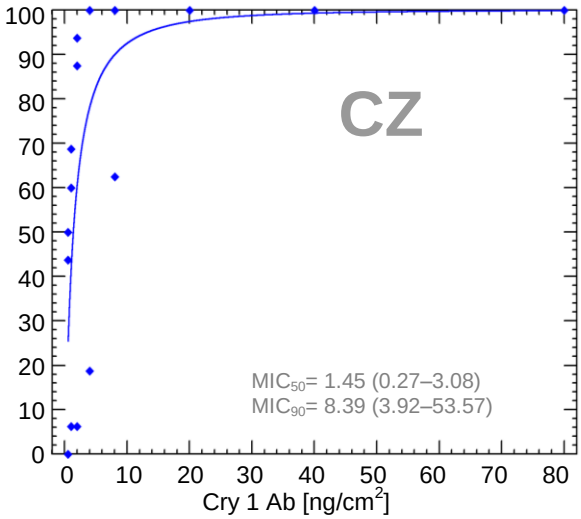

Fig. A2: Czech Republic (CZ.01, CZ.02, CZ.03)

Germany: North Germany (GnPL)

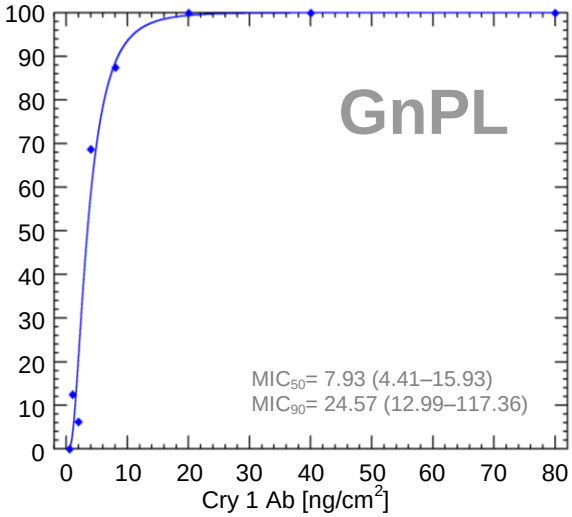

Fig. A3: Heinersdorf (G.03);Germany.

Spain: Central Spain (ESc)

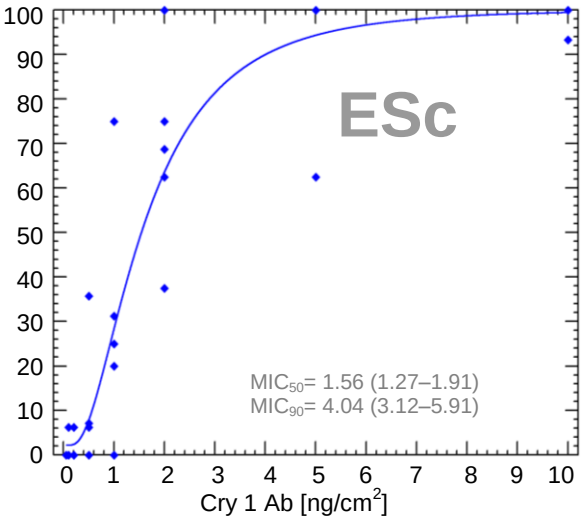

Fig. A4: Central Spain (ES.07, ES.08, ES.15)

Spain: Northeast Spain (ESne)

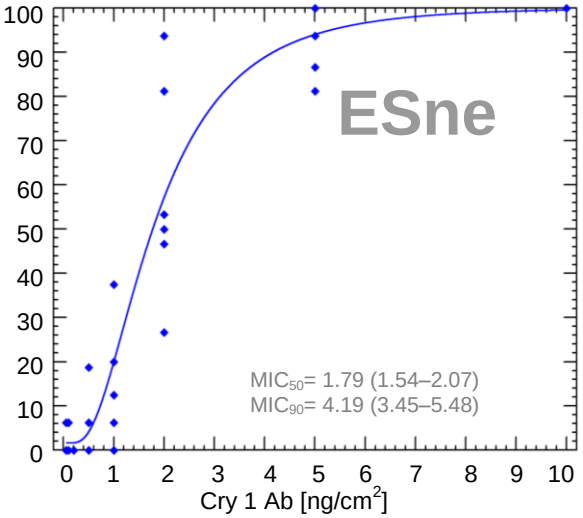

Fig. A5: Northeast Spain (ES.11, ES.12, ES.13, ES.14)

Bridging experiment

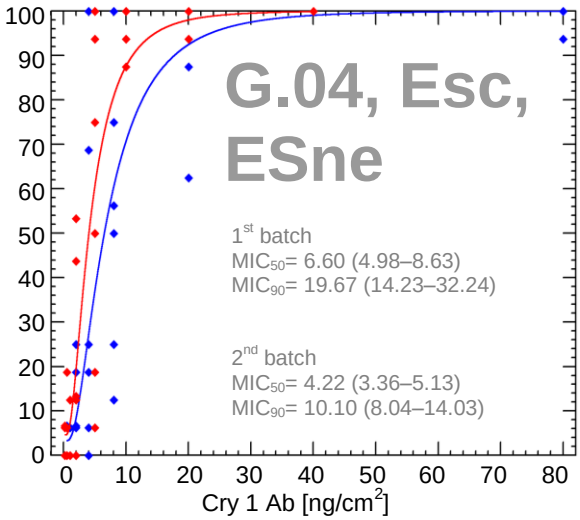

Fig. A6: Response of the reference-lab strain (G.04), ESc (ES.07, 08, 15) and ESne (ES.11-14) to 1st batch (blue) and 2nd batch of cry1Ab (red).