Appendix 8. Insect Resistance Monitoring in Iberian collections of *Ostrinia nubilalis* (ECB): 2012 Season

Report

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

- Results for 2012-2013 -

Date

23/07/2013

Testing Facility

BTL Bio-Test Labor GmbH Sagerheide
Birkenallee 19
D-18184 Sagerheide
Germany

Sponsor

Monsanto Europe SA
Avenue de Tervuren 270-272
B-1150 Brussels
Belgium

Statement of Confidentiality

This report contains confidential and proprietary information of Monsanto Europe SA, which must not be disclosed to anyone except the employees of this company or to persons authorized by law or judicial judgment without the expressed and written approval of Monsanto Europe SA.

Statement of Compliance with the Principles of Good Experimental Practice

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

- The Principles of Good Experimental Practice (GEP), (Plant Protection Products Ordinance, paragraph (5) of Article 1c, Germany).

Head of testing facility

Date / Signature: 23. July 2013 .....

Study director

Date / Signature: 23. July 2013 .....

Contents

Tables and Figures

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, Monsanto established an insect resistance monitoring program across Europe and in particular in areas where commercial activity of MON 810 is occurring or planned, *i.e.*, areas where the European target pests *O. nubilalis* and *S. nonagrioides* are prevalent. This monitoring program follows directions described in the plan of the industry working group on Insect Resistance Management (IRM) proposed to the Member State Competent Authorities and the European Commission (available since 2003 but published in 2007; ALCALDE et al., 2007 and subsequently updated as the EuropaBio harmonised IRM plan in September, 2012; see Appendix to the overall MON 810 monitoring report). This report focuses on the susceptibility monitoring of *O. nubilalis.*

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, *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). Now *O. nubilalis* is one of the most damaging pests of maize in North America and Europe and a major target pest for control with genetically modified maize expressing *Bacillus thuringiensis* (Bt) proteins.

In accordance with the EuropaBio Harmonised IRM plan (September, 2012; see Appendix to the overall MON 810 monitoring report) 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 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 grown 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, b and 2000). The 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, will be reported on a biennial basis in areas where MON 810 is grown. Two exceptions were described: (1) in case the adoption of MON 810 remains below 20% in the given area, no data will be reported in future, and (2) in case MON 810 adoption equals or exceeds the theoretical maximum of 80% (due to the required 20% refuge implementation), susceptibility monitoring will be performed on a yearly basis. Looking at all countries where MON 810 was grown during the 2012 growing season, reporting on the susceptibility of *O. nubilalis* to Cry1Ab in the areas covering the Czech Republic, Slovakia, Romania and the Central Iberia area was deemed not required since MON 810 adoption did not reach 20%. Also, samples were not taken in Northeast Iberia and Central Iberia since they were subject of last year's report. Therefore, the current report focuses on the

resistance monitoring of *O. nubilalis* in Iberia Southwest, the area where adoption of MON 810 was greater than 20%.

The objectives of the current report on the 2012 maize growing season are:

1) To determine the susceptibility of *O. nubilalis* in maize growing areas in Southwest Iberia to the Cry1Ab protein expressed in MON 810 maize varieties.
2) In addition, preliminary studies were conducted to explore the feasibility of monitoring resistance of *O. nubilalis* to Cry1Ab using the diagnostic dose method. This method was established to be the most efficient method and as effective as the dose-response method to detect changes in susceptibility to Cry proteins (SIMS et al., 1996).

2 Materials and Methods

2.1 Insect collection

The three areas identified in the entire EU where adoption of MON 810 in 2012 was expected to be greater than 20% are the Ebro valley (defined in earlier reports as Iberia Northeast), Central Iberia (particularly the province of Albacete) and the Southwest Iberia area. For these areas data on the susceptibility of *O. nubilalis* to Cry1Ab has been collected since 2007. In 2012, samples were collected from Iberia Southwest in two sites that were separated by at least 50 km. *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 (2012, see Appendix to the overall MON 810 monitoring report). 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). For each area, the aim was to collect 300 healthy larvae.

2.2 Insect culture

Field-collected *O. nubilalis* 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 (Figure 2 and 3, Table 1). 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) until May.

O. nubilalis larvae from different sampling sites separated by at least 50 km were analysed. Collected insects from different sites within the area tested were reared or kept under diapause separately to avoid cross contamination with *Beauveria* or *Nosema*.

Larvae surviving the diapause period were transferred to fresh containers and placed in incubators where the temperature was raised gradually from 15-25°C, humidity of 90% RH and a photoperiod of 20:4h (L:D) over a period of 10 days and kept at 25°C, humidity of 90% RH and a photoperiod of 20:4h (L:D) thereafter. Emerging adults were transferred to oviposition cages (Figure 4) and fed 15% honey water to increase fecundity (LEAHY & ANDOW, 1994). The inside of the cages was covered with filter paper serving as oviposition medium 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. Incubating egg masses were placed in an incubator for 20 h at 25 ± 2°C, 4 h at 20 ± 2°C, 90% RH and a photoperiod of 20:4h (L:D) (GUTHRIE et al., 1985).

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 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 analyze if the two batches differed 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 (see Table 1 for recipe). After the diet solidified, 100 µl of protein solution was applied to the surface and allowed to dry overnight at room temperature. To avoid contamination the trays were covered with a sheet of filter paper. Egg masses of each sampling location (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 (Bio-Cv-16, Color-Dec Italy) (Fig. 5). 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/cm² and those tested with the second batch to 0.2–40.0 ng Cry1Ab/cm². Field collected insects used in bioassays came from pooled samples of healthy insects collected in different fields within an area. A total of 3 (replicates) times 32 larvae were used for each concentration and each area.

All assays were conducted at 25°C, 70% RH and a photoperiod of 0:24h (L:D). After seven days, larval mortality and developmental stage were recorded. Larvae that had not grown beyond first instar would not survive under field conditions (e.g. SIEGFRIED et al., 2000). As a result, the criterion for mortality used in this study accounts for both death and complete moulting (or growth) inhibition.

Table 1. *O. nubilalis* diet recipe

Component	Amount	Provided
Distilled H_2O	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
Fumidil B	1 g	CEVA Salud Animal, S.A.
Ascorbic acid	3 g	BioServ
Vanderzant vitamin mix	2 g	BioServ

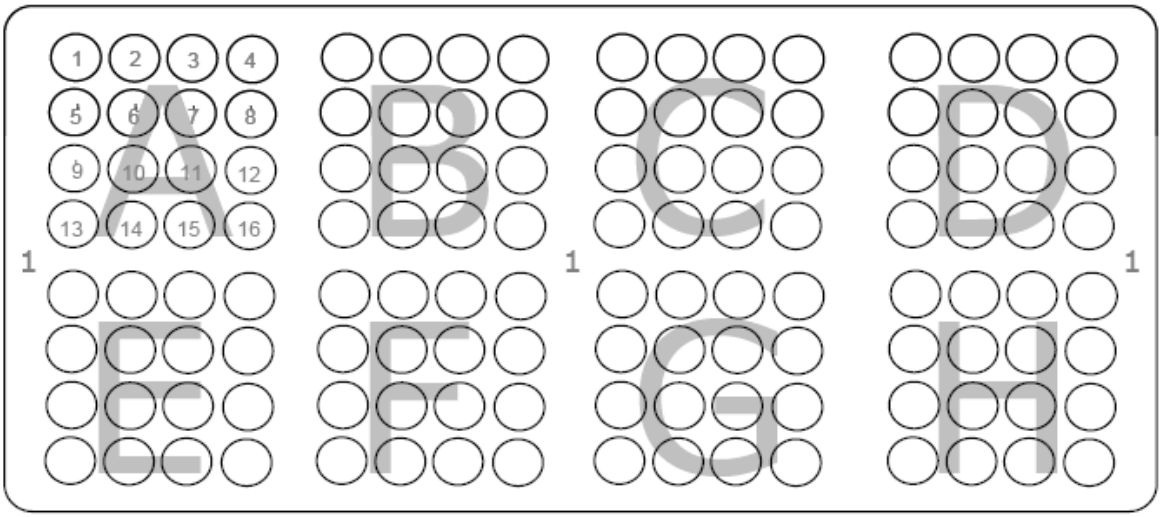


Figure 5. IDs of Bio-Ba-128 trays (tray number, field letter, well number; i.e.: 1.A.13)

2.3.2 Diagnostic dose

For the calculation of diagnostic dose the data for all experiments with ECB collected during the monitoring program from 2005-2012 in fields from Czech Republic, France, Germany, Italy, Panonia, Poland, Portugal, Romania and Spain were used with less than 20% response at the control after exposure to Cry1Ab. In total these calculations represent the responses of 11,502 larvae.

2.4 Statistical analysis

All statistical analyses were done using the computer program SYSTAT, Version 10.0, 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 be able to include the bioassay in the 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.

3 Results and Discussion

3.1 Collection of ECB

The area where ECB larvae were collected in 2012 is shown in Table 2, and the location is displayed on a map in Annex I.

Table 2. *O. nubilalis* collection details for the 2012-2013 season (lab reference strain).

Area	ID	Country	Collection site	Collected	Eggs	Larvae	Tested
lab	G 04	Germany		2005	x		2013
EsSW	ES.01	Spain	ES-06760 Villar de Rena	2012		x	2013
EsSW	ES.03		ES-06716 Navalvillar de Pela	2012		x	2013

3.2 Susceptibility to Cry1Ab in the 2012-2013 campaign

To determine the susceptibility to Cry1Ab, larval mortality and larval moult inhibition data at the different concentrations of Cry1Ab tested were analyzed by Probit analysis. Moulting inhibition concentrations at 50% (MIC_{50}) and 90% (MIC_{90}) for *O. nubilalis* collected in a particular area are provided in 0. The significance of differences in susceptibility between the laboratory strain (originally collected in Niedernberg, Germany, and kept in culture since 2005, causing poor performance according to MOAR et al., 2008) and the field collected insects was tested by determining the 95% confidence limits (CI) of MIC ratios (ROBERTSON et al., 2007). Moulting inhibition concentrations are significantly different ($P < 0.05$) if the MICR 95% confidence limits does not include 1. Fitted curves of susceptibility to the Cry1Ab protein of laboratory and field collections of *O. nubilalis* were generated taking into account the moulting inhibition concentration of neonate larvae after seven days feeding on treated diet (Figure 6).

Table 3. Results from probit analysis for the ECB origins collected in 2012.

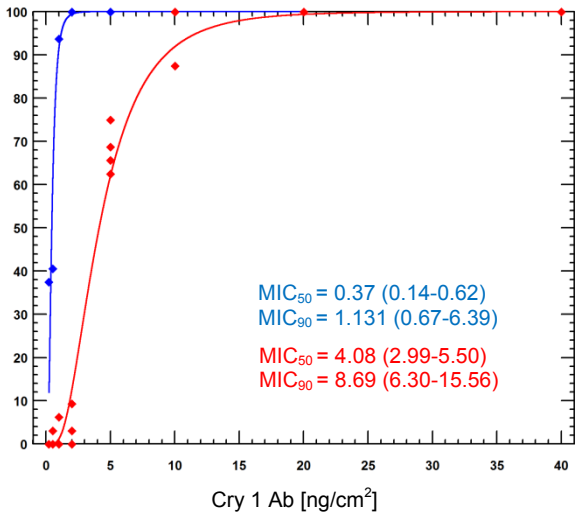
Area	n	Slope ± SE	χ^2	D.f.	MIC_{50} (95% CI) [a]	MIC_{90} (95% CI) [a]
lab	286	2.638 ± 0.429	12.24	5	0.37 (0.14-0.62)	1.131 (0.67-6.39)
EsSW	896	3.902 ± 0.285	189.77	28	4.08 (2.99-5.50)*	8.69 (6.30-15.56)*

[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 concentrations significantly different ($P < 0.05$) to the laboratory strain.

Figure 6. Fitted curve of susceptibility as moult inhibition after seven days feeding on treated diet of ECB (collected in 2012) to the batch 2 of protein Cry1Ab (PoloPlus, LeOra Software 2002-2009).

Reference laboratory strain (G.04, blue) vs. Iberia Southwest (EsSW, red)



3.3 Diagnostic Dose

For the calculation of diagnostic dose the data for all experiments using ECB collected from 2005-2012 in fields from Czech Republic, France, Germany, Italy, Panonia, Poland, Portugal, Romania and Spain representing the responses of 11,502 larvae. Using the average of the moulting inhibition concentrations (MIC) for 99% (MIC_{99}) the diagnostic dose for ECB larvae from Europe is 48.218 ng/cm^2 for batch 1 or 28.22 ng/cm^2 for batch 2.

3.4 Exposure to MON 810 tissue (confirmatory experiment)

It was planned that all *O. nubilalis* larvae from field collections that survived the bioassay at the highest dose should be assembled, transferred to plastic boxes in groups of approx. 50 larvae, provided with newly detached MON 810 maize leaves without the central nerve, and fed *ad libitum* to record any survivors. As for the season reported here, no surviving larvae were found after 10 days and confirmatory experiments were not necessary.

3.5 Historical susceptibility of corn borers to Cry1Ab

During 2008–2012, 23 samples of ECB from different areas were analyzed. Their susceptibility to Cry1Ab is shown in Table 4.

Table 4. Susceptibility of *O. nubilalis* neonates exposed to Cry1Ab as measured by the MIC over time for areas tested. ([a] ng Cry1Ab/cm^2; MIC moulting inhibition concentrations, CI confidence interval)

Area	Year	MIC_{50} (95% CI)	MIC_{90} (95% CI)
Iberia Central	2009	3.09 (2.03–4.33)	11.98 (8.12–22.31)
	2011[1]	1.56 (1.27–1.91)	4.04 (3.12–5.91)
Iberia Northeast	2008	7.03 (4.89–10.03)	23.91 (15.76–46.84)
	2009	6.40 (5.32–7.75)	13.68 (10.77–20.02)
	2011[1]	1.79 (1.54–2.07)	4.19 (3.45–5.48)
Iberia Southwest	2008	3.39 (2.94–3.89)	6.90 (5.79–8.89)
	2010	5.76 (4.38–7.84)	11.85 (8.53–23.52)
	2012[1]	4.08 (2.99-5.50)	8.69 (6.30-15.56)

[1] second batch of Cry1Ab

4 Conclusions

During 2005–2012, 14 areas 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 ECB 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 ECB pooled according to geographic and climatic conditions (up to 6.6-fold).

Variation in Cry1Ab susceptibility (MIC_{50} and MIC_{90}) of ECB collected in the field during the campaign 2012-2013 was 1.4-fold and 1.4-fold respectively. These results indicate that the observed variation in susceptibility reflects natural variation in Bt susceptibility among ECB origins. Any evidence for a decrease of Cry1Ab susceptibility of ECB during the monitoring duration could not be detected.

5 Acknowledgement

This report presents the results of research only and would not be possible without the kind help of all those who supplied insects: Michael Hoenig (Herbolzheim, G), Udo Hoffmann (BTL, Keindorf, G), Dr. Gustav Langenbruch (Darmstadt, G), Kai Lindner (Müncheberg, G), Andreas Schier (Nürtingen, G), Dieter Proff (Ansbach, G), Alain Weissenberger (Wiwersheim, F), Anne Mesas and Nathalie Eychenne (Castanet Tolosan, F), Isabell Rami and Nathalie Daste (Fredon Aquitaine, Villenave d'Ornon, F), Khalid Koubaïti (Biard, F), Prof. Frantisek Kocourek and Vladan Falta (Prague, CZ), Pawel Beres and Aleksandra Maslanka (Rzeszow and Warsaw, PL), Mihaly Czepo (Budapest, HU) and Prof. Ludovit Cagan (Nitra, SK), Prof. Ioan Rosca, Ioan Sabau (Bucharest, RO), Marco Gatti (Repros, Alonte, I) and the Spanish group around Félix Ortega Alonso and Gema Pérez Farinos (CSIC, Madrid, ES). All those persons are key public sector entomologists or local industry contacts.

6 References

BECK, S.D., 1987. Developmental and seasonal biology of *Ostrinia nubilalis*. Agric Zool Rev **2**: 59-96.

CAFFREY,D. J., & WORTHLEY, L.H., 1927. A progress report on the investigations of the European corn borer. USDA Bull. 1476.

FARINÓS, G.P., DE LA POZA, M., HERNANDEZ-CRESPO, P., ORTEGO, F. & CASTAÑERA, P., 2004. Resistance monitoring of field populations of the corn borers *Sesamia nonagrioides* and *Ostrinia nubilalis* after 5 years of Bt maize cultivation in Spain. Ent. Experimentalis et Applicata **110**: 23-30.

GUTHRIE WD, ROBBINS JC & JARVIS JL (1985) *Ostrinia nubilalis*. In: Handbook of insect rearing. Volume 1. SINGH, P. & R.F. MOORE (eds.) Elsevier, Amsterdam, Oxford, New York, Tokyo, pp. i-viii; 488.

HODGSON, 1928: The host plants of the European corn borer in New England. USDA Tech Bull **77**: 1-63.

HUDON, M., & LEROUX, E.J., 1986. Biology and population dynamics of the European corn borer (*Ostrinia nubilalis*) with special reference to sweet corn in Quebec, Canada. I. Systematics, morphology, geographical distribution, host range, economic importance. Phytoprotection **67**: 39-54.

LEAHY, T.C., & ANDOW, D.A., 1994. Egg weight, fecundity, and longevity are increased by adult feeding in *Ostrinia nubilalis* (Lepidoptera, Pyralidae). Annals of the Entomological Society of America **87**: 342-349.

LEORA SOFTWARE, 1987. POLO-PC. A user's guide to probit and logist analysis. LeOra Software, Berkley, CA.S

MARÇON, P.C.R. G., TAYLOR, D.B., MASON, C.E., HELLMICH, R.L., & SIEGFRIED, B.D., 1999a. Genetic similarity among pheromone and voltinism races of *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae): Insect Molecular Biology, **8** (2): 213-221.

MARÇON, P.C.R.G., YOUNG, L.J., STEFFEY, K.L. & SIEGFRIED, B.D., 1999b. Baseline susceptibility of European corn borer (Lepidoptera: Crambidae) to *Bacillus thuringiensis* toxins. Journal of Economic Entomology **92**: 279-285.

MARÇON, P.C.R.G., SIEGFRIED, B.D., SPENCER, T. & HUTCHISON, W.D. 2000. Development of Diagnostic Concentrations for Monitoring *Bacillus thuringiensis* Resistance in European Corn Borer. (Lepidoptera: Crambidae). Journal of Economic Entomology **93**: 925-930.

MOAR, W., ROUSH, R., SHELTON, A., FERRE´, J., MACINTOSH, S., LEONARD, B.R. & ABEL, C., 2008. Field-evolved resistance to *Bt* toxins. Nature Biotechnol. **26**: 1072-1074.

PONSARD, S., BETHENOD, M.-T., BONTEMPS, A., PELOZUELO, L., SOUQUAL, M.-C., & BOURGUET, D., 2004. Carbon stable isotopes: a tool for studying the mating, oviposition, and spatial distribution of races of European corn borer, *Ostrinia nubilalis*, among host plants in field. Can J Zool **82**: 1177-1185.

ROELOFS, W.L., DU, J.-W., TANG, X.-H., ROBBINS, P.S., & ECKENRODE, C.J., 1985. Three European corn borer populations in New York based on sex pheromones and voltinism. J. Chem. Ecol. **11**: 829-836.

SAEGLITZ, C., BARTSCH, D., EBER, S., GATHMANN, A., PRIESNITZ, K.U. & SCHUPHAN, I., 2006. Monitoring the Cry1Ab susceptibility of European corn borer in Germany. Journal of Economic Entomology **99**: 1768-1773.

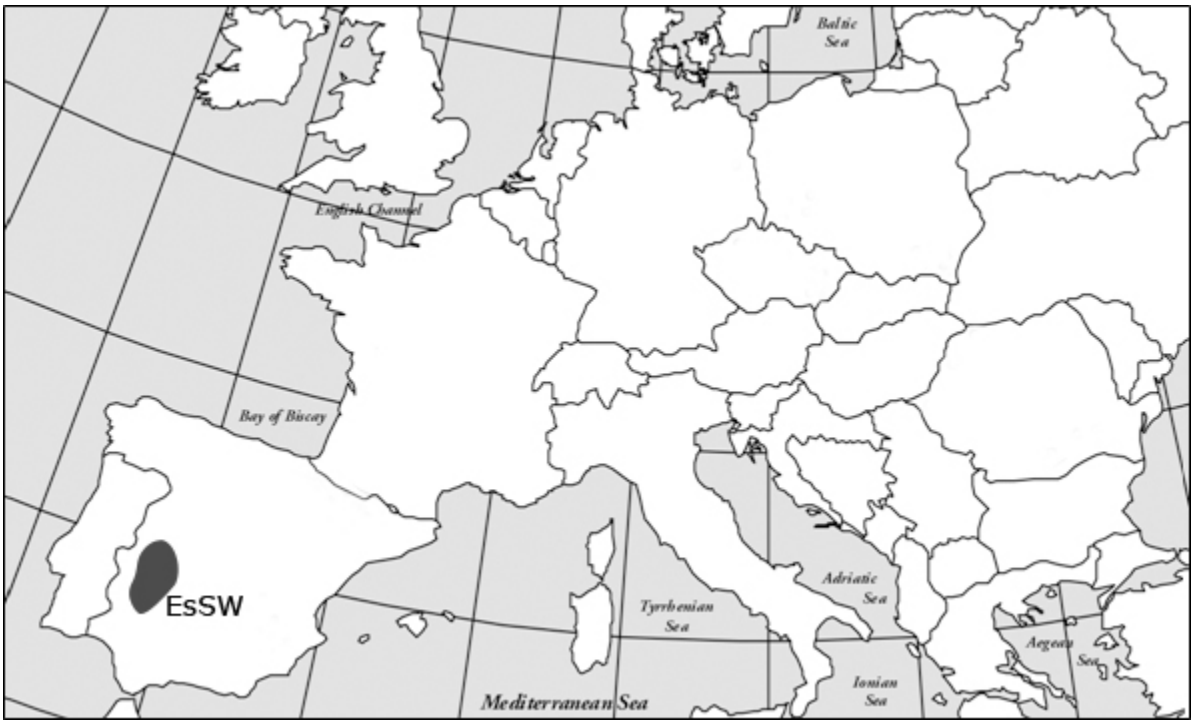
SHOWERS, W.B., 1993. Diversity and variation of European corn borer populations. In: Evolution of Insect Pests, KIM, K.C. & B.A. MCPHERON (eds.) (John Wiley and Sons) New York, pp. 287-309.

SIEGFRIED, B.D., SPENCER, T. & NEARMAN, J., 2000. Baseline Susceptibility of the Corn Earworm (Lepidoptera: Noctuidae) to the Cry1Ab Toxin from *Bacillus thuringiensis*. Journal of Economic Entomology **93**: 1265-1268.

SIMS, S.B., GREENPLATE, J.T., STONE, T.B., CAPRIO, M.A. & GOULD, F., 1996. Monitoring strategies for early detection of Lepidoptera resistance to *Bacillus thuringiensis* insecticidal proteins. In: Molecular Genetics and Evolution of Pesticide Resistance, BROWN, T.M. (ed.). ACS Symposium Series No. 645, Washington, DC, pp. 229-242.

VINAL, S.C., 1917. The European corn borer, *Pyrauta nubilalis* Hübner, a recent established pest in Massachusetts. Massachusetts Agric Exp Stn Bull **178**: 147-152.

Annex I
Areas of collection activities for ECB in 2012



Area where ECB was sampled in 2012 (EsSW-Iberia Southwest)