

Is there any change in susceptibility of European corn borer (*Ostrinia nubilalis*) to Cry1Ab protein?

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Abstract: In accordance with the EuropaBio Harmonised IRM plan (September, 2012) the baseline susceptibility of *Ostrinia nubilalis* (ECB) 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. During 2005-2014, 14 areas with 140 samples 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 (MIC50) 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). It was planned that all *O. nubilalis* larvae from field collections that survived the bioassay at the highest dose should be transferred to plastic boxes in groups of approximately 50 larvae, provided with newly detached MON 810 maize leaves, and fed *ad libitum* to record any survivors. As for the seasons reported here no surviving larvae were found after 10 days and thus confirmatory experiments were not conducted.

Key words: *Ostrinia nubilalis*, European corn borer, ECB, Cry1Ab, Maize, MON 810, *Bt* maize, resistance

Introduction

The Mon 810 maize has been engineered to produce a *Bt*-protein (Cry1Ab) that is toxic to butterfly pests like *Ostrinia nubilalis* (ECB). In accordance with the EuropaBio Harmonized IRM plan the baseline susceptibility of ECB to Cry1Ab protein was established and used in routine monitoring for changes in susceptibility. The objective is to detect quickly any changes in susceptibility that could indicate inadequate protection against the target species. This program will enable the early detection of any increase in the resistance of *O. nubilalis* to Cry1Ab protein and provide the necessary time for the proposal and implementation of additional control measures. During 2005-2014 15 areas and 140 samples of ECB were analyzed.

Material and methods

ECB collection

From 2005-2014 ECB were collected in the most important European maize growing regions where the use of MON 810 was expected to be greater than 20% (Figure 1). For each region,

different sampling sites separated by at least 50 km were chosen. In naturally infested fields ECB were mainly collected as larvae by dissecting maize stalks but also as adults by using light traps or collecting egg masses on leaves. This collection of insects and establishment of populations complies with the IRM (Insect Resistance Management) working group guidelines proposed by the EU Commission.

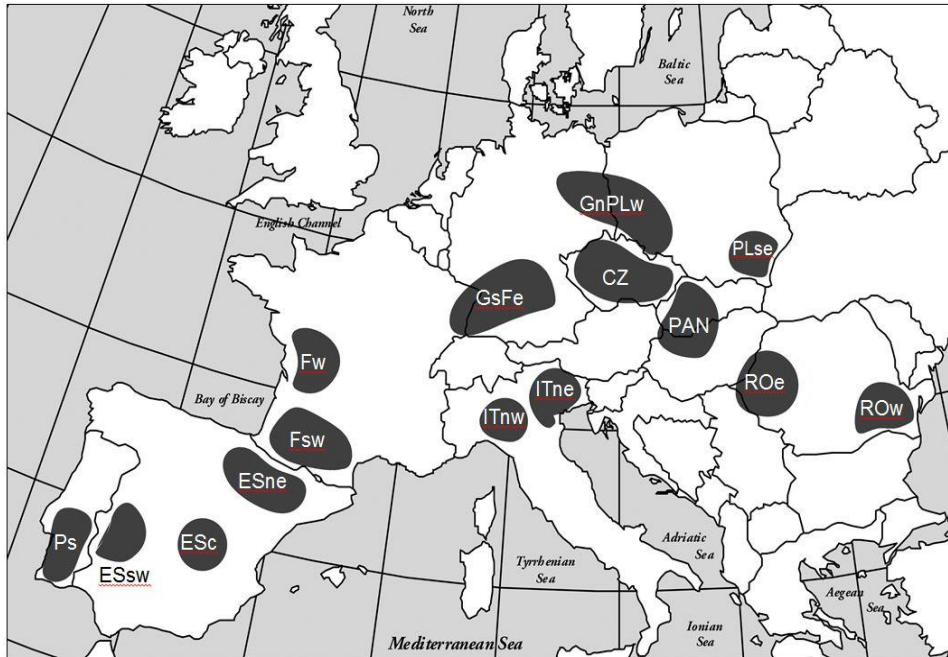


Figure 1. The major European maize growing regions from which ECB were collected from 2005-2014: Czech Republic/Moravia (CZ), Southwest and West France (Fsw, Fw), Northern Germany/Southwest Poland (GnPLw), Southern Germany and East France (GsFe), Northern Italy (ITne, ITnw), the Panonion region (PAN, Western Slovakia and North West Hungary), Southeast Poland (PLse), South Portugal (Ps), Romania (ROe, ROW) and Spain (ESne, ESc, ESsw)

ECB culture

Field-collected larvae of *O. nubilalis* were placed in plastic boxes containing corrugated cardboard and an agar-based maize diet, which were kept in a growth chamber at 28 °C, 85% RH and a photoperiod of 20:4 h (L:D). If the larvae had not pupated after two weeks, they were assumed to have entered diapause and transferred to another climatic chamber kept at 10 ± 2 °C, 70 ± 5% RH and a photoperiod of 0:24h (L:D) until the adults emerged in May. These adults were transferred to another growth chamber kept at 28 °C, 85% RH and a photoperiod of 20:4 h (L:D) prior to transfer to oviposition cages (Leahy & Andow, 1994), the insides of which were covered with filter paper (substrate for oviposition) that was changed daily. The eggs they laid were transferred to an incubator kept at 25 ± 2 °C, 85% RH and a photoperiod of 0:24 h (L:D) until they hatched. ECB sampled as adults were kept under the same conditions as described above. ECB sampled as egg masses were treated similarly to egg masses produced by adults reared in the laboratory. A laboratory reference non-diapausing strain (G04) from Niedernberg, Germany, which is highly susceptible to Cry1Ab, has been maintained under same conditions as described above at BTL since 2005.

Susceptibility to Cry1Ab

Two batches of Cry1Ab protein provided by Monsanto have been used since the start of the MON 810 monitoring plan, batch 1 (2 mg Cry1Ab/ml) from 2005 until its date of expiry in 2011 and batch 2 (1.64 mg Cry1Ab/ml) since 2012. To analyse if the two batches differed in efficacy a bridging experiment was done. The bioassays were performed in 128 well trays (BAW128, Bio Serv). In each cell 1 ml of artificial diet was dispensed to which after its solidification 100 µl of protein solution was applied to the surface. Egg masses from each location sampled were incubated and neonate larvae, within 12 h of hatching, were transferred to the cells (one per cell), which were covered with a lid (BACV16, Bio Serv). For batch 1 eight concentrations (0.5-80 ng Cry1Ab/cm²), batch 2 nine concentrations (0.2-28.22 ng Cry1Ab/cm²) and a control (bicarbonate buffer) for each batch were tested for each population. For each collection area, 16 larvae were tested using each concentration of Cry1Ab and 32 larvae for control batch 1 and 32 larvae for each Cry1Ab concentration and 64 larvae for control batch 2.

All assays were done at 25 °C, 70% RH and a photoperiod of 0:24 h (L:D). After seven 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 *et al.*, 2000). As a result, the criterion for mortality used in this study includes both death and inhibition of moulting (or growth).

Statistical analyses

All statistical analyses were done using the computer programme SYSTAT, Version 10.0, except for the concentration-response analysis for which PoloPlus 1.0 was used (LeOra Software Company).

Results and discussion

Diagnostic dosage

For calculating the diagnostic dosages the results of all the experiments using ECB collected from 2005-2012 in fields in the Czech Republic, France, Germany, Italy, Panonia, Poland, Portugal, Romania and Spain, which are the responses of 11,502 larvae, were used. Using the average of the moulting inhibition concentrations (MIC) for 99% (MIC₉₉), the diagnostic dose for ECB larvae from Europe is 48.218 ng/cm² for batch 1 and 28.22 ng/cm² for batch 2.

Performance of the laboratory strain G04

The laboratory strain G04 was kept as sub-strains since 2011 and checked regularly for performance (size of adults, size of egg masses and development of larvae). In 2011 applying a PCR based method (Saeglitz *et al.*, 2006) infection with *Nosema* was identified in some individuals in one sub-strain, which was terminated. One sub-strain has been used continuously until now. Individuals of this sub-strain produce good-quality egg masses and normal-sized adults, and according to the PCR analyses is not infected with microsporidia and especially not with *Nosema* (Figure 2).

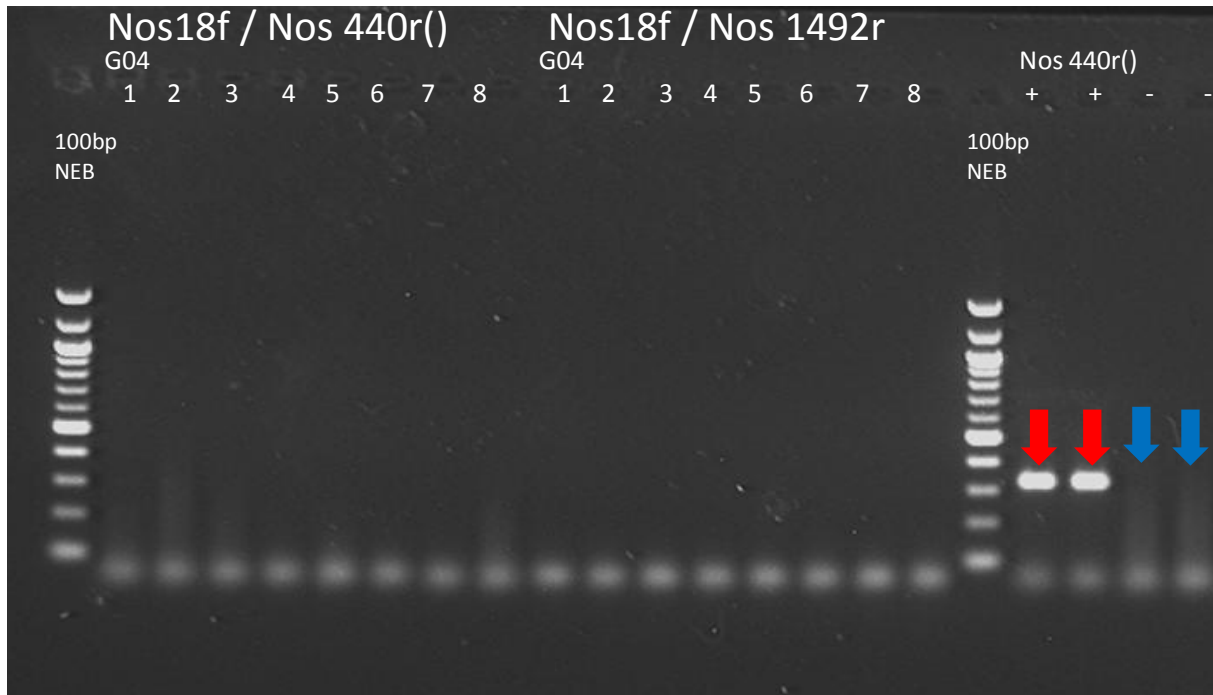


Figure 2. PCR analyses using different markers used to check if the reference strain of ECB (G04) was infected with *Nosema* (according to Saeglitz *et al.*, 2006). Red arrows indicate positive control; blue arrows indicate negative control.

Susceptibility to Cry1Ab in the years 2005-2014

During 2005-2014, 140 samples of ECB from 15 areas were analyzed. Thus far, susceptibility to Cry1Ab has been assessed for one laboratory colony (G04) and ECB collected from maize fields in the Czech Republic, France, Germany, Italy, Hungary, Slovakia, Poland, Portugal, Romania and Spain. ECB larvae were provided with an artificial diet treated with increasing concentrations of Cry1Ab and mortality and growth inhibition were evaluated after 7 days. Susceptibility of ECB collected in different geographic regions and exposed to purified Cry1Ab toxin differ only slightly. The variation in Cry1Ab susceptibility of field samples pooled according to geographic and climatic conditions was up to 6.6-fold (MIC₅₀) and up to 7-fold (MIC₉₀) for populations collected in 2005-2010 and tested with Cry1Ab batch 1; up to 3.6-fold (MIC₅₀) and up to 2.3-fold (MIC₉₀) for populations collected in 2011-2014 and tested using Cry1Ab batch 2. In all the years when bioassays were carried out no ECB survived diagnostic dosages of 48.218 ng/cm² of batch 1 Cry1Ab and 28.22 ng/cm² batch 2 Cry1Ab or reached the 2nd larval stage with exception of four specimens in a sample from Southern France in 2005.

In Iberia, the last European region to cultivate MON 810, ECB is highly susceptible to Cry1Ab protein. ECB sampled in South West Iberia in 2014 were even more susceptible than ECB in previous samples from this region in 2012 (Figure 3).

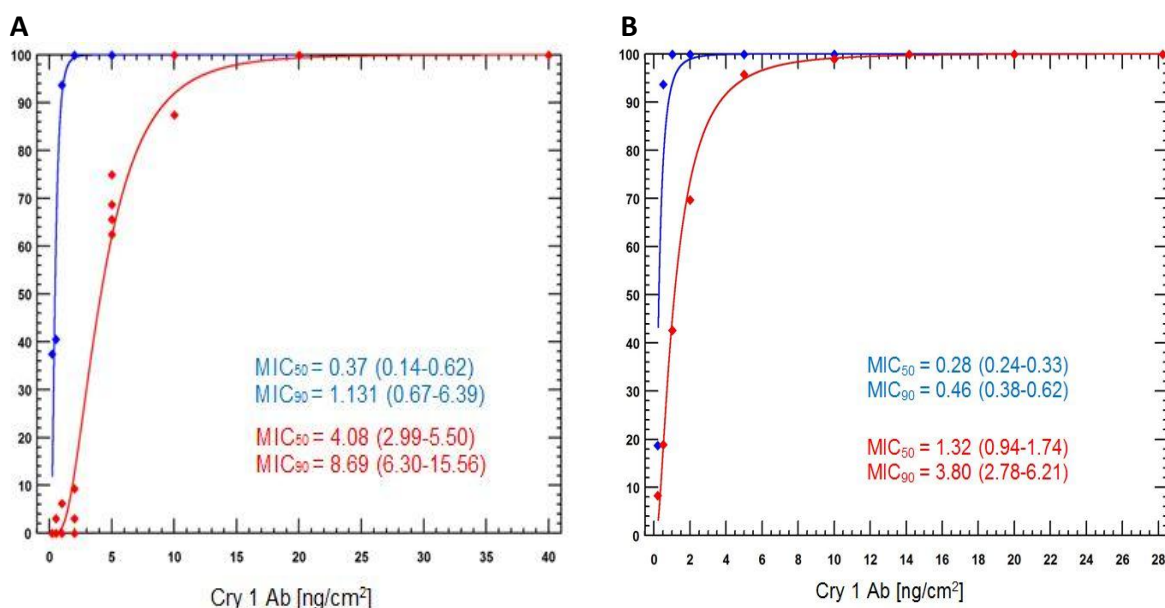


Figure 3. Fitted curves of the susceptibility of ECB in terms of the percentage inhibited from moulting seven days after feeding on a diet treated with the 2nd batch of protein Cry1Ab (A: collected in South West Iberia 2012, B: collected in South West Iberia 2014) (PoloPlus, LeOra Software 2002-2009). Reference laboratory strain (G04, blue) vs. Iberia Southwest (ESsw, red)

Confirmatory experiment

As some of the larvae reached the 2nd instar at the highest dosage applied in 2005 it was decided that in future bioassays all larvae of *O. nubilalis* collected in the field that survived the bioassay at the highest dosage should be placed in plastic boxes and provided *ad libitum* with newly detached leaves, without the central nerve, from MON 810 maize and any survivors recorded. For the seasons reported here, except for 2005, no larvae reached the 2nd instar after 10 days and therefore confirmatory experiments were not necessary.

Conclusion

The results indicate that the variation in ECB populations in their susceptibility to Cry1Ab reflects their susceptibility to this *Bt*-protein. There was no evidence of a decrease in the susceptibility to Cry1Ab in populations over the period 2005-2014.

Acknowledgements

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