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Managing resistance evolution to transgenic *Bt* maize in corn borers in Spain

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ABSTRACT

Since 1998, genetically engineered *Bt* maize varieties expressing the insecticidal Cry1Ab protein (i.e. event MON 810) have been grown in the European Union (EU), mainly in Spain. These varieties confer resistance against the European and Mediterranean corn borer (ECB and MCB), which are the major lepidopteran maize pests in the EU, particularly in Mediterranean areas. However, widespread, repeated and exclusive use of *Bt* maize is anticipated to increase the risk of Cry1Ab resistance to evolve in corn borer populations. To delay resistance evolution, typically, refuges of non-*Bt* maize are planted near or adjacent to, or within *Bt* maize fields. Moreover, changes in Cry1Ab susceptibility in field populations of corn borers and unexpected damage to maize MON 810, due to corn borers, are monitored on an annual basis. After two decades of *Bt* maize cultivation in Spain, neither resistant corn borer populations nor farmer complaints on unexpected field damage have been reported. However, whether the resistance monitoring strategy followed in Spain, currently based on discriminating concentration bioassays, is sufficiently sensitive to timely detect early warning signs of resistance in the field remains a point of contention. Moreover, the Cry1Ab resistance allele frequency to *Bt* maize, which has recently been estimated in MCB populations from north-eastern Spain, might exceed that recommended for successful resistance management. To ensure *Bt* maize durability in Spain, it is key that adequate resistance management approaches, including monitoring of resistance and farmer compliance with refuge requirements, continue to be implemented and are incorporated in integrated pest management schemes.

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Introduction


Genetically engineered (GE) crops expressing insecticidal proteins from the biocontrol agent *Bacillus thuringiensis* (*Bt*) have been cultivated globally for 25 years over a continuously growing area. In 2019, the worldwide surface of insect-resistant *Bt* crops, alone or in combination with other traits such as herbicide tolerance, has exceeded 107 million ha [1]. Commercialized *Bt* crops mostly include maize, soybean, cotton, and rice, and, to a lesser extent, the eggplant (brinjal). Cultivation of other *Bt* crops such as cowpea in Nigeria might soon follow [2].

Bt proteins expressed in GE crops confer protection against either certain lepidopteran or coleopteran pests or both, offering a complementary means of control

against crop insect pests. The presumed mode of action of *Bt* proteins is to bind selectively to specific receptors on the epithelial surface of the midgut of susceptible insect species, leading to death through pore formation, cell burst and subsequent septicemia [3–7]. Owing to the targeted specificity of insecticidal *Bt* proteins expressed in *Bt* crops to insects from the order of Lepidoptera and Coleoptera, such crops are considered less harmful to other (valued or beneficial) insects (i.e. non-target organisms) and the ecosystem services they contribute to (such as biological control, pollination or decomposition) than broad-spectrum insecticides [8–16]. Deployment of *Bt* crops has led to some agronomic, environmental and economic benefits compared to conventional crops and their associated farm

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management practices [17–28]. For example, their adoption has been associated with a reduction in the use of insecticides that are more harmful to the environment, because less or no treatments with soil or broad-spectrum foliar insecticides may be needed [29]. However, there is concern that *Bt* crops could adversely affect some susceptible non-target insect species when they are exposed to harmful amounts of *Bt* protein through ingestion/feeding [e.g. 30,31]. Moreover, the widespread and repeated use of *Bt* crops expressing the same *Bt* protein by individual farmers as the sole pest management option against target insect pests will create significant selection pressure, increasing the potential for pests to evolve resistance to the *Bt* protein (i.e. heritable reduction in the susceptibility of the target insect pest population to the *Bt* protein) [32,33]. Resistance evolution in target insect pests is not a direct environmental harm, but resistant populations may require altered pest management practices due to their reduced susceptibility to the *Bt* protein expressed in a GE crop. Thus, farmers may need to revert to the previously used pest management tools that are more harmful to the environment, and ultimately alter cultivation/farming systems, which may decrease farm income [34] and affect the sustainability of the cropping system. Therefore, in some jurisdictions, the susceptibility of target insect pests to *Bt* proteins, including those expressed in GE crops, is viewed as a common good to preserve [35,36].

To delay the potential resistance evolution of target insect pests to *Bt* crops, globally, risk managers typically require that their cultivation is accompanied by the implementation of insect resistance management (IRM) plans, including monitoring strategies. In general, these strategies consist of planting “structured” refuges (i.e. areas with the crop that does not express *Bt* proteins that are active against the target insect pest) near or adjacent to, or within the *Bt* crop fields (e.g. in large blocks or as row strips) or mixing non-*Bt* with *Bt* seeds in each sowing seed bag. In addition, changes in *Bt* protein susceptibility in field populations of the targeted insect pests and unexpected damage to GE crops due to the pest are monitored regularly (see below for further details). Despite the adoption of IRM strategies, there are at least 20 instances of field-evolved resistance documented worldwide at present (April 2021). These cases involve eight major agricultural insect pests, nine *Bt* proteins and in six countries, with reduced pesticide efficacy and practical consequences for pest control [37,38]. The main reasons for the cases of field-evolved reported resistance are the deployment of *Bt* crop varieties that do not express the *Bt* protein(s)

at a concentration high enough to kill most of the individuals of the targeted pest population and the insufficient planting of non-*Bt* crop refuges. While the instances of field-evolved resistance have steadily increased over the last decade [39], there are examples of successful implementation of IRM programs accompanying the long-lasting cultivation of *Bt* crops. In the European Union (EU), this is the case for Spain, where after more than two decades of continuous cultivation of *Bt* maize (mainly maize event MON 810 that expresses the Cry1Ab protein against the European corn borer (ECB), *Ostrinia nubilalis* (Lepidoptera: Crambidae), and the Mediterranean corn borer (MCB), *Sesamia nonagrioides* (Lepidoptera: Noctuidae)), there have been no reports of resistant corn borer populations [40–42]. However, the resistance allele frequency recently estimated in MCB populations from north-eastern Spain might exceed that recommended for a successful implementation of the IRM strategy [43]. Moreover, an unresolved point of contention is whether the current monitoring activities performed in Spain are sufficiently sensitive to timely detect possible field-evolved resistance before becoming irreversible and irremediable [44]. The belated detection of early signs of resistance will significantly hamper the timely implementation of remedial measures to appropriately respond to confirmed resistance, and hence prevent the spread of resistance to other areas or eradicate resistance. Therefore, after briefly reporting on the main lepidopteran pests of maize and the adoption of *Bt* maize for cultivation in the EU, we: (i) review all relevant ECB and MCB resistance monitoring data gathered for the cultivation of *Bt* maize in the EU since 1998, focusing on north-eastern Spain where adoption rates of *Bt* maize are the highest, (ii) discuss whether IRM measures implemented for *Bt* maize in Spain are adequate to delay resistance evolution, (iii) present the main limitations encountered in the monitoring and management of resistance evolution to *Bt* maize in Spain so far, and (iv) present recommendations on how to overcome shortcomings to ensure the durability of *Bt* maize.

Lepidopteran pests of maize in the European Union

ECB and MCB are the most damaging lepidopteran pests to maize in the EU. ECB is widespread throughout Europe, while MCB has a narrower geographical distribution being solely present in the Mediterranean region [45,46]. However, climate changes may favor the further expansion of MCB to other EU territories where it could establish as a new agricultural pest [47].

Both species complete a variable number of generations per year depending on the latitude, ranging from one generation in northern countries (for ECB) up to three generations in Spain and Portugal (for MCB) [48,49].

ECB and MCB larvae cause direct damage to maize plants (i.e. reduced physical strength of the stalk that ultimately may lead to plant lodging, significant metabolic and physiological alterations due to disrupted transport and use of nutrients and water) by boring into the plant stem (tunneling) and feeding on it for most of their larval stage. Occasionally, second-generation larvae feed on maize ears causing wounds through which fungal pathogens can enter the maize plant. Some of these pathogens produce mycotoxins that are toxic to vertebrates [50–52]. Thus, damage to maize plants caused by corn borers may reduce yield, and lead to broken stalks, dropped ears and low grain quality. Insecticidal sprays are frequently ineffective against corn borers because once a larva has entered the maize stem, it is no longer exposed to the chemical treatment and thus protected from the insecticide sprays. In contrast, lepidopteran-active *Bt* maize plants directly target ECB/MCB larvae feeding on them through the *Bt* proteins' expression in plant tissues, so they can be a highly efficient pest control measure against corn borers [53].

ECB and MCB have been defined by *Bt* crop developers as the main target insect pests of *Bt* maize in the EU (i.e. target organisms). Other noctuids such as *Sesamia cretica*, *Agrotis* spp., *Helicoverpa armigera* and *Mythimna unipuncta* that occasionally cause damage to maize in certain EU regions [45,46] are not considered the target pests of *Bt* maize: they are not prevalent over the entire European maize growing area, do not regularly occur at high population densities, or cause damage to maize only in certain seasons [54]. The potential for *Bt* maize resistance evolution in non-target lepidopteran pest species is not addressed in this review.

Cultivation of *Bt* maize in the European Union

Since 1998, the European Commission has approved the cultivation of two single *Bt* maize events, i.e. *Bt*176 and MON 810. Both events express the Cry1Ab protein conferring protection against ECB and MCB larvae. Maize hybrids derived from the event *Bt*176 were grown in France in 1998, Portugal in 1999, and Spain between 1998 and 2005. Cultivation of maize *Bt*176 in the EU was discontinued because it contained an antibiotic-resistant marker gene [55], and failed to provide season-long protection against corn borers in some

cases [42]. Maize MON 810 cultivation in the EU started in 2003 in Spain when the *de facto* moratorium on new approvals of genetically modified organisms introduced by the Member States was lifted following the adoption of new legislation. Since then, maize MON 810 varieties have been grown at different commercial scales in nine Member States (Figure 1(A)). However, the cultivation of *Bt* maize remains a controversial issue in the EU [56]. Following the adoption of new legislation in 2015 allowing individual Member States to ban or restrict the cultivation of GE crops in their territory, 17 EU Member States and one region have prohibited *Bt* maize cultivation on their territory (https://ec.europa.eu/food/plant/gmo/authorisation/cultivation/geographical_scope_en). In 2019, only two Member States have cultivated MON 810, with significant acreage in Spain (107,127 ha) and less in Portugal (4,718 ha). *Bt* maize represents approximately 35% of Spain's total maize area and less than 10% in Portugal [27]. However, in regions with a high incidence of corn borer infestation such as the Ebro basin in north-eastern Spain, adoption rates of *Bt* maize can exceed 60% (<https://www.mapa.gob.es/>).

Insect resistance management for *Bt* maize

In most jurisdictions, the cultivation of GE crops is subject to risk assessment and regulatory approval. In the risk analysis process, the role of risk assessors such as the European Food Safety Authority (EFSA) is to assess any plausible risk that the cultivation of a GE crop may pose to human and animal health and the environment. Decisions to approve cultivation, given potential risk management, are taken by risk managers (i.e. European Commission, EU Member States). As part of the regulatory approval process of *Bt* crops in the EU and other jurisdictions, applicants (also termed registrants) submitting an application/dossier for cultivation proactively provide an IRM plan. IRM plans are designed to prevent or at least delay resistance evolution in the target insect pests and extend *Bt* crop durability [34,57–59].

In 2003, the European Association for Bioindustries (EuropaBio) designed a long-term, EU-wide, IRM plan for various lepidopteran-active *Bt* maize events, including MON 810 (and derived varieties). The IRM plan proposed a standard methodology to manage and monitor potential resistance evolution to Cry1Ab and Cry1F proteins in ECB and MCB populations in the EU [58]. Four main pillars underpin the IRM plan: (i) compliance with refuge obligations, (ii) establishment of baseline susceptibility of corn borers to the *Bt* protein and monitoring of resistance evolution, (iii) communication and

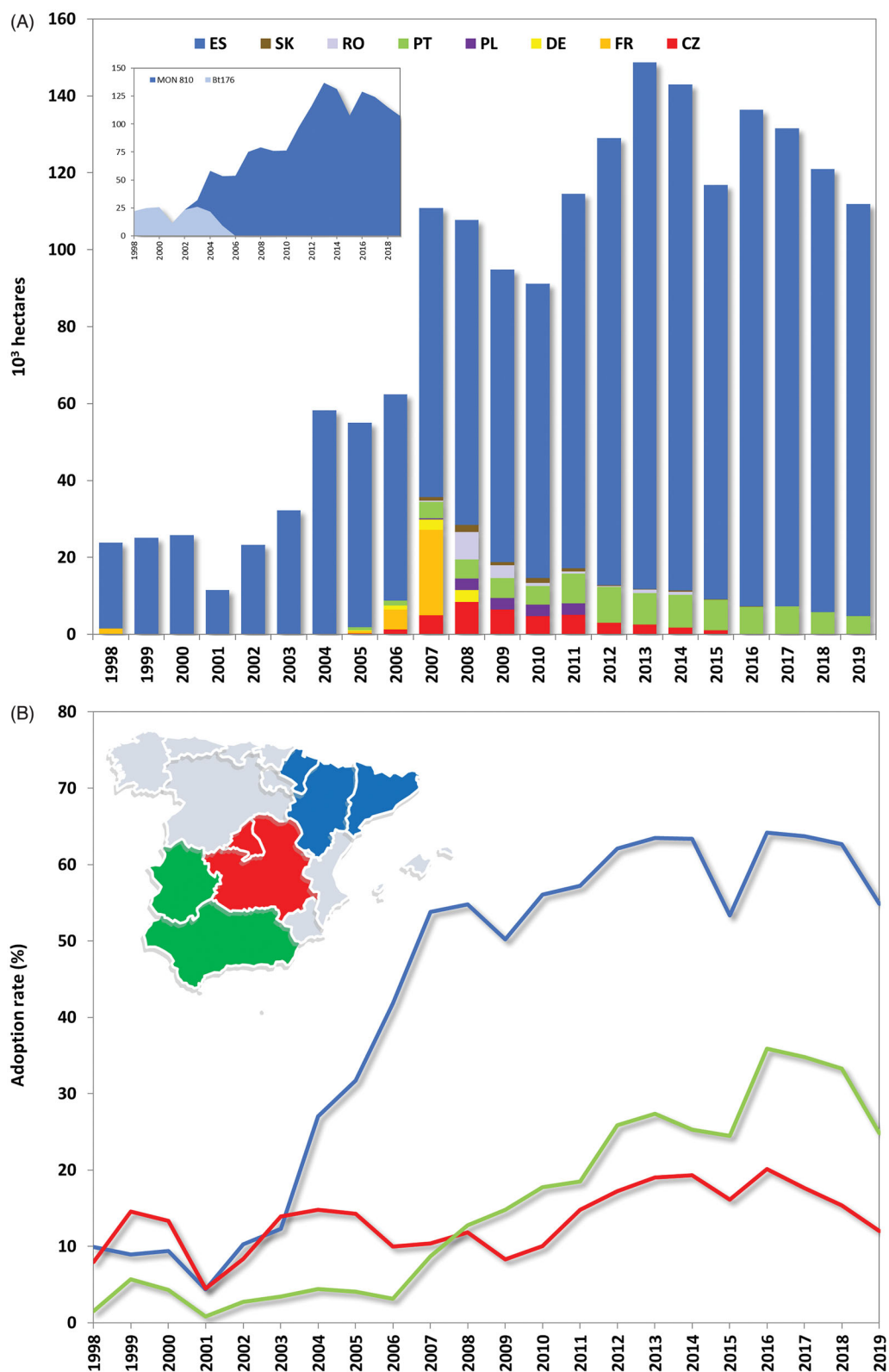


Figure 1. (A) Total area (10³ ha) devoted to Bt maize varieties in the European Union since 1998. The small graph on the upper left represents the area (10³ ha) planted with varieties Bt maize events Bt176 and MON 810 in Spain. (B) Adoption rate of Bt maize in the main maize growing areas in Spain since 1998. CZ: Czech Republic; DE: Germany; ES: Spain; FR: France; PL: Poland; PT: Portugal; RO: Romania; SK: Slovakia.

education of farmers, and (iv) remedial actions in case of field-evolved resistance.

High-dose refuge strategy to delay resistance evolution

Currently implemented IRM measures for *Bt* crops typically are based on the 'high-dose refuge' (HDR) strategy, which also forms the cornerstone of the harmonized IRM plan for *Bt* maize in the EU. This strategy requires that: (i) *Bt* plants produce sufficiently high concentrations of the *Bt* protein (i.e. 25 times the amount needed to kill 99% of susceptible individuals and 95% of heterozygous individuals for resistance alleles [59]) so that heterozygotes do not survive exposure in the *Bt* crop, thus making resistance functionally recessive, and (ii) refuge areas of non-*Bt* host plants are planted near or adjacent to, or within, the *Bt* crop fields [60–63]. Refuge areas provide a reservoir of susceptible target insects that will mate with those rare resistant individuals emerging from *Bt* crop fields. If resistance is a recessive trait, then the heterozygous offspring produced by such crosses will not survive on *Bt* plants. The optimal size and configuration of refuge areas and their distance to the *Bt* crop fields depends on: population size, feeding habits and the dispersal abilities of the target insect pest [64,65].

The underlying assumptions of the HDR strategy are that: (i) the *Bt* protein is expressed at appropriate levels in relevant plant parts, (ii) alleles conferring resistance to *Bt* proteins are rare or absent in pest populations (ideally their frequency should be below 10^{-3}), and (iii) random mating occurs between resistant insects emerging in *Bt* crops and susceptible insects preserved on refuges at sufficient levels [34]. Whether the underlying assumptions of the HDR strategy are met for ECB and MCB and *Bt* maize MON 810 in Spain is described below.

***Bt* protein is expressed at appropriate levels in relevant parts to the plant:**

Cry1Ab protein expression levels in *Bt* maize may fluctuate during the growing season, resulting in variable selection pressure on different annual ECB/MCB generations. In maize *Bt*176 varieties, levels of expression of the Cry1Ab protein decreased after anthesis [66]. This decline allowed heterozygous ECB and MCB larvae from the second and third generations to complete their development on those *Bt* plants, thus accelerating selection for resistant populations [67]. In maize MON 810 plants, however, high concentrations of the Cry1Ab protein are maintained throughout

vegetative and reproductive development stages of maize [68,69], conferring season-long protection against all generations of ECB and MCB [67,70,71]. While the genetic background of a maize MON 810 variety, environmental conditions and/or agricultural practices can cause variation in Cry1Ab protein concentration levels, such levels remain sufficiently high to maintain any resistance alleles in corn borers functionally recessive [72].

Initial resistance alleles are rare in the targeted insect pest populations

Allele frequencies for Cry1Ab resistance as expressed in *Bt* maize were first estimated in MCB populations from north-eastern Spain in the 2004 and 2005 growing seasons [73], following seven and eight successive years of cultivation (Figure 1(B)). No resistance alleles were detected in any of the 85 isofemale lines tested by F_2 screening [74] and the frequency was estimated to be 2.9×10^{-3} , with 95% credibility intervals (CI) between 0 and 8.6×10^{-3} (Supplementary material, Table S1). The number of lines tested by Andreadis et al. [73] was insufficient to confirm whether the initial frequency of resistance was below the recommended value of 10^{-3} . However, considering the relatively low adoption rate of *Bt* maize in that area at that time ($\sim 35\%$) and the initial allele frequencies estimated in other southern MCB populations [73], the actual frequency was presumed to be lower than the estimated 2.9×10^{-3} . More than a decade later, Camargo et al. [43] re-calculated the allele frequency in MCB populations collected in 2016 from the same area. One of the 137 lines screened was shown to carry a resistance allele. The updated frequency was estimated to be 3.6×10^{-3} (95% CI between 4×10^{-4} and 10^{-2}) (Supplementary material, Table S1). These most recent estimates indicate that the frequency of resistance alleles in MCB populations from north-eastern Spain might exceed the value recommended for effective implementation of the HDR strategy. The authors attributed the increased frequency of Cry1Ab resistance alleles to maize MON 810 to the strong selective pressure caused by the widespread, repeated and exclusive use of *Bt* maize in the Ebro basin.

Unlike MCB, there are no recent estimates of the frequency of Cry1Ab resistance alleles to maize MON 810 in Spanish populations of ECB. This parameter has previously been estimated in populations of ECB collected in: France, Germany, Italy and Slovakia between 1999 and 2004 before *Bt* maize was grown in these countries. There was no evidence of resistance to *Bt* maize in any of the 2156 lines tested from those populations, and

the expected frequency of resistance alleles was estimated to be 0.0001, with a 95% CI between 0 and 0.0003 [75,76]. Considering the low genetic differentiation of ECB populations in Europe [77], a similarly low initial rate of Cry1Ab resistance alleles was assumed for Spanish populations.

Random mating occurs between resistant insects emerging in Bt crops and susceptible insects preserved on refuges at sufficient levels

For refuges to be effective, their placement, configuration and size should ensure that resistant and susceptible insects mate randomly and that refuges harbor a sufficiently large population of susceptible insects to outnumber resistant ones. How much mingling and mating would occur between individuals emerging from refuges and *Bt* crop fields is determined by the scale of adult movement.

A series of release and recapture studies, carried out in the United States using light and pheromone traps, showed that adult dispersal for ECB was influenced by agronomic practices and environmental conditions [78]. Adults could move over 40 km in short periods [79], though most males were often recaptured at shorter distances, in the range of 200–800 m from the release site [78,79]. Allozyme analyses of several ECB populations collected from maize fields across different European countries revealed an extensive level of gene flow and thus dispersal between regions [77,80]. In the case of MCB, data from studies carried out in north-eastern Spain using pheromone traps showed that most MCB adults disperse at least 400 m away from emergence points [81,82].

A concern may be the non-synchronous emergence of insects from refuges and *Bt* crop fields, as this could result in nonrandom (assortative) mating and accelerate resistance development. For MCB, random mating between individuals emerging from refuges and *Bt* maize fields is assumed to be driven by the dispersal of adult males, because adult females rarely disperse before mating [81–85]. By contrast, adult females of ECB might move out of their fields of emergence before mating [86,87], though virgin females disperse less often than males and mated females [88]. Since field-evolved resistance to *Bt* maize has not yet occurred in corn borers in the EU, the level of potential local assortative mating remains uncertain. Consequently, this is an important assumption to test for reducing uncertainty about the potential of the HDR strategy to delay resistance evolution should resistance occur.

Refuge requirements

In the EU, farmers growing more than 5 ha of *Bt* maize must plant structured refuges equivalent to at least 20% of the total *Bt* maize area. Refuge areas should be planted near (within a 750 m distance) or adjacent to, or within the *Bt* maize fields either as blocks or strips of non-*Bt* maize. For clusters of *Bt* maize fields with an aggregated area greater than 5 ha, EFSA advocated planting refuges, irrespective of individual field and farm size [89]. Whenever possible, refuge maize should be selected based on equivalent maturity to *Bt* maize and be planted with the same planting window as *Bt* maize. Moreover, farmers should manage refuges using comparable agronomic practices (fertilization, weed and pest management and irrigation) to the *Bt* maize areas, and avoid the spraying of microbial *Bt* formulations [90].

Considering the host range of ECB and MCB, the use of wild or cultivated plants other than maize is not considered a suitable alternative to a structured refuge for both corn borers [91,92]. While ECB may lend itself to the seed blend concept (also termed seed mixtures or “refuge-in-the-bag”) [93], the use of seed blends is not recommended in the EU [40].

Resistance and compliance monitoring

IRM programs for *Bt* crops require routine monitoring for resistance evolution, so that early warning signs, indicating a decrease in susceptibility to the *Bt* protein expressed in *Bt* crops in field populations of the target insect pests, are detected [94]. Timely detection of such signs enables taking action to limit the survival of resistant insects, and slow or prevent their spread should resistance have already evolved [95]. Data generated through resistance monitoring also allows researchers, risk assessors, risk managers and regulators to assess whether the HDR strategy delays resistance evolution in the target insect pest adequately and efficiently. Moreover, since ensuring high levels of compliance with refuge requirements is a critical factor contributing to the success of IRM plans, monitoring activities are also tailored to collect information on the implementation of refuges, *Bt* crop adoption levels and farmer use patterns (such as applied pest management practices). This information gives relevant indications on whether farmers follow and adhere to the refuge requirements, and on compliance levels. An overview of the two programs that have been implemented in the EU to monitor the resistance evolution of target insect pest populations to *Bt* maize, as well as the

Table 1. Historical data on Cry1Ab susceptibility from concentration-response assays with Mediterranean and European corn borer populations collected in maize fields from north-eastern Spain between 1998 and 2015.

Growing season ^a	Spanish monitoring program (1998–2011)						Seed company monitoring program (2004–2015)					
	Field type ^b	Cry1Ab protein source ^c	North-east Spain	Central Spain	South-west Spain	Reference population	Field type ^b	Cry1Ab protein source ^e	North-east Spain	Central Spain	South-west Spain	Reference population
			LC ₅₀ (95% CI) ^d						MIC ₅₀ (95% CI) ^d			
Mediterranean corn borer												
1998	C	Syn-1	70 (56–87)	23 (16–30)	27 (16–39)	–	R/C	Mon-B1	63 (34–99)	12 (5–22)	–	18 (11–25)
1999	Bt176	Syn-2	23 (14–31)	10 (8–13)	3 (2–5)	–	R/C	Mon-B1	–	7 (1–17)	–	–
2000	Bt176	Syn-2	20 (15–25)	10 (6–14)	18 (6–28)	8 (5–11)	R/C	Mon-B1	14 (8–20)	–	17 (10–25)	16 (12–22)
2001	Bt176	Syn-2	–	–	19 (11–28)	–	R/C	Mon-B1	–	–	–	19 (10–30)
2002	Bt176	Syn-2	34 (27–42)	19 (13–26)	–	5 (4–6)	R/C	Mon-B1	–	–	–	–
2003	Bt176	Syn-2	–	18 (15–21)	–	–	R/C	Mon-B1	–	–	–	–
2004	Bt176	Syn-2	11 (7–16)	15 (9–23)	24 (16–32)	10 (7–12)	R/C	Mon-B1	–	–	–	–
2005	Bt176	Syn-2	22 (15–29)	25 (18–31)	–	18 (13–24)	R/C	Mon-B1	22 (16–28)	–	–	–
2006	Bt176	Syn-2	16 (7–27)	23 (18–28)	21 (18–26)	26 (18–34)	R/C	Mon-B1	20 (14–27)	12 (5–22)	–	18 (11–25)
2007	R	Syn-2	–	–	28 (21–36)	26 (18–30)	R/C	Mon-B1	9 (3–15)	–	11 (5–18)	–
2008	R	Syn-2	22 (17–26)	14 (11–16)	24 (18–31)	23 (16–30)	R/C	Mon-B1	–	–	–	–
2009	R	Syn-2	30 (24–37)	–	17 (12–21)	–	R/C	Mon-B1	–	–	–	–
2010	R	Syn-2	–	–	12 (6–19)	–	R/C	Mon-B1	–	–	–	–
2011	R	Syn-2	29 (22–35)	10 (8–13)	18 (14–23)	15 (12–20)	R/C	Mon-B1	–	–	–	–
2012	R	Syn-2	30 (21–40)	21 (11–35)	–	–	R/C	Mon-B1	14 (8–20)	–	–	–
2013	R	Syn-2	23 (18–30)	–	–	26 (22–30)	R/C	Mon-B1	–	–	–	–
2014	R	Syn-2	40 (33–47)	30 (23–39)	–	18 (12–24)	R/C	Mon-B1	22 (16–28)	10 (6–14)	–	8 (5–11)
2015	R	Syn-2	–	–	29 (22–38)	23 (18–29)	R/C	Mon-B2	–	–	–	9 (6–13)
European corn borer^{e,f}												
1998	C	Syn-1	109 (77–162)	104 (82–140)	–	–	R/C	Mon-B1	7.0 (4.9–10.0)	–	3.5 (2.9–4.2)	2.2 (1.9–2.5)
1999	Bt176	Syn-1	81 (51–122)	–	–	–	R/C	Mon-B1	6.4 (5.3–7.8)	3.1 (2.0–4.3)	–	2.3 (1.5–3.0)
2000	Bt176	Syn-2	–	–	4 (2–6)	–	R/C	Mon-B1	–	–	–	3.6 (2.8–4.9)
2001	Bt176	Syn-2	–	–	5 (2–9)	–	R/C	Mon-B2	1.8 (1.5–2.1)	1.6 (1.3–1.9)	–	2.8 (2.2–3.3)
2002	Bt176	Syn-2	34 (28–41)	–	3 (2–5)	3 (2–4)	R/C	Mon-B2	–	–	–	2.9 (2.3–3.6)
2008	Bt176	Syn-2	9 (6–12)	–	6 (5–7)	4 (3–6)	R/C	Mon-B2	2.4 (1.9–3.0)	2.4 (2.0–2.8)	–	0.4 (0.1–0.6)
2009						3 (2–4)	R/C	Mon-B2	–	–	–	2.0 (0.8–4.6)
2010						4 (3–6)	R/C	Mon-B2	–	–	–	0.3 (0.2–0.3)
2011						3 (2–4)	R/C	Mon-B2	2.1 (1.7–2.6)	1.9 (1.7–2.1)	–	4.0 (2.8–4.9)

^aMaize varieties derived from event Bt176 were cultivated between 1998 and 2005; maize varieties derived from event MON 810 have been cultivated since 2003.

^bC: conventional (not genetically engineered) maize field; R: refuge area.

^cCry1Ab protein provided by Syngenta: Syn-1: 25% purity; Syn-2: 81% purity. Syn-1 and Syn-2 differ in their biological activity¹⁰ and, therefore, the results are not comparable.

^d50% and 90% lethal/molting inhibition concentration (LC/MIC₅₀ and LC/MIC₉₀) and their 95% confidence intervals (CI 95%) are expressed in ng Cry1Ab/cm² of diet surface area.

^eData provided by Monsanto Europe S.A. confirmed that the Cry1Ab protein batches B1 and B2 have similar insecticidal activity.

^fResults of the concentration-response assays performed with the European corn borer populations collected after 2002 have not been published.

Table based on data provided in Farinós *et al.* [40,127–130], Thieme *et al.* [41], Castañera *et al.* [71], González-Núñez *et al.* [98].

available data on farmer's compliance with refuge requirements, are presented below.

Resistance monitoring

Resistance monitoring programs for *Bt* crops usually follows a two-pronged approach, consisting of (i) regular monitoring for changes in susceptibility to the *Bt* protein in field populations of the target insect pest, and (ii) monitoring of unexpected field damage caused by the insect pest [57,58,96,97].

Baseline and monitoring target insect pest susceptibility to *Bt* maize

Two programs for monitoring the evolution of Cry1Ab resistance in corn borer populations have been put in place in the EU: (i) one run by the Spanish authorities between 1998 and 2011, and (ii) an ongoing one conducted by the applicants marketing maize MON 810 since 2004.

Spanish resistance monitoring program (1998–2011).

In 1998, following the commercial cultivation of maize *Bt*176, the Spanish authorities initiated resistance monitoring activities for corn borers. Before the widespread cultivation of *Bt* maize varieties, baseline susceptibility to Cry1Ab and natural variability in ECB and MCB field populations was established for many maize-growing regions based on the highest anticipated levels of adoption [98]. For both pest species, geographical differences in the susceptibility to *Bt* protein among populations were low. The observed differences were attributed to natural variations due to a lack of usage of previous *Bt* spray formulations in the studied areas.

Changes in baseline susceptibility were measured periodically by collecting between 300 and 500 corn borer larvae of the last generation from maize fields (either from *Bt*176, non-*Bt* maize fields or refuge areas in the three regions where *Bt* maize was grown, i.e. north-eastern, central and south-eastern Spain). The concentration causing 50% mortality (LC_{50}) was then estimated in diet-overlay concentration/response bioassays with microbially-produced Cry1Ab protein using the progeny of the field-collected individuals. Reference susceptible populations, initiated in 1998 and 2000 from MCB and ECB larvae collected from non-*Bt* maize fields, and maintained in the laboratory ever since without any exposure to the Cry1Ab protein, were used in the bioassays as an additional comparator. To preserve its vigor and avoid inbreeding depression, populations were refreshed periodically with new individuals.

The results of the Spanish monitoring programs indicated that susceptibilities of field populations of ECB and MCB to *Bt* maize remained within a narrow range for thirteen years (1999–2011) and were comparable to those of the relevant laboratory reference populations (Table 1).

Seed-companies program (2004–present).

As required by the EU decision approving the cultivation of maize MON 810, the applicant marketing maize MON 810 commenced a resistance monitoring program in 2004. Baselines of susceptibility to the Cry1Ab protein were established for ECB and MCB populations from several EU maize growing areas in accordance with the geographic distribution of corn borers. Small variations in susceptibility of populations (no higher than 6.6-fold for ECB and 9-fold for MCB) were observed across the continent. Given that the cultivation of maize MON 810 varieties has only continued in Portugal and Spain, yet on a small scale (i.e. less than 10% of maize cultivation) in Portugal, routine monitoring efforts for insect resistance have focused primarily on Spain.

Between 2004 and 2015, ECB and MCB populations were sampled from the same three geographical areas included in the Spanish monitoring program and their susceptibility was assessed using the same concentration/response bioassay. For each area and target insect pests, between 300 and 500 corn borer larvae were collected at least every second year from a minimum of three refuge areas or non-*Bt* maize fields. Then, the Cry1Ab concentration causing 50% and 90% of molt inhibition ($MIC_{50,90}$), denoted as larvae that had either died or failed to molt to the second instar, was estimated after seven days of exposure using the progeny of the field-collected individuals. $MIC_{50,90}$ values were also estimated from laboratory reference populations and served as additional comparators to account for any potential issues derived from the unavailability of a stable purified protein over time [40].

In 2016, EuropaBio revised the sampling strategy and monitoring protocol of the IRM plan accounting for the experience gained with its practical implementation, several recommendations made by EFSA [99] and new available scientific evidence. From then onwards, monitoring has focused on the Ebro basin (north-eastern Spain), as this is the area with the highest adoption rate of maize MON 810 in the EU (>60% between 2015 and 2019) and where both ECB and MCB complete two generations per year. Adhering to the revised plan, corn borer populations were collected every year from at least three 10 km × 10 km areas within that region. To increase the sensitivity of the monitoring strategy, the

sampling size has been increased with an annual goal of at least 1000 larvae of each target insect pest, and bioassays using a single concentration, which is expected to discriminate between susceptible and (homozygous) resistant individuals, have superseded concentration/response tests.

In addition to the bioassays with microbially-produced Cry1Ab protein, supplementary tests using maize MON 810 leaves have been performed since 2011 with ECB and MCB larvae that survive the diagnostic concentration as well as with spare larvae not used in the bioassays. These tests aim to verify whether resistant individuals are already present in the field-collected populations.

Results of insect bioassays. Table S2 of the Supplementary material provides an overview of the different types of bioassays that have been performed within the seed companies monitoring program, covering: (i) concentration/response, (ii) diagnostic concentration, and (iii) plant-tissue assays.

- **Concentration/response assays:** The outcome of the concentration/response assays does not show any trends in Cry1Ab susceptibility of corn borers that were sampled and monitored in different geographical areas in Spain for more than 10 years (Table 1). Estimated MIC₅₀ values did not fluctuate considerably as the difference between the most and least susceptible samples in a given area ranged between 2.0- and 3.9-fold in ECB, and 2.8- and 7.0-fold in MCB. Oscillations in Cry1Ab susceptibility were attributed to natural variability, as they are comparable to the changes observed in the respective susceptible reference strains (reared in the laboratory without any exposure to *Bt* proteins) and to historical values of field populations collected in Europe [40,41]. Similar conclusions can be drawn from the results of the concentration/response studies performed in both programs, regardless of the endpoint measured and the source of the microbially-produced Cry1Ab protein used in each program. While fluctuations occurred over time (Table 1), LC/MIC values followed no trend. Moreover, resistance ratios, which indicate the number of times that the susceptibility of the field population is higher than the laboratory susceptible population, never exceeded 10 (Figure 2). They were much lower compared to those reported for instances of field-evolved and laboratory-selected resistance of lepidopteran pests to *Bt* proteins [e.g. 37,39].

- **Diagnostic concentration assays:** Diagnostic concentration assays revealed no symptoms of Cry1Ab resistance in corn borer populations collected in north-eastern Spain between 2016 and 2019. Molt inhibition values of ECB larvae exposed to the discriminating concentration for seven days were always higher than the expected >99% (Table 4). For MCB populations, molt inhibition rates averaged for the three sampling zones were lower than the expected >99% in all four years (Table 2). However, statistically significant differences between the field-collected population and the susceptible reference strain were only observed in 2017. Follow-up studies using plant material indicated that none of the second instars that survived the diagnostic concentration in that each year reached the third instar after feeding on maize MON 810 leaves [100].
- **Plant tissue assays:** Larvae that survived the concentration/response or the diagnostic concentration bioassays with Cry1Ab and spare individuals from those assays were fed maize MON 810 leaves in plant tissue assays. None of the tested larvae were able to molt to the following instar and all died within a few days (Table 3).

Unexpected field damage caused by corn borers.

Applicants marketing maize MON 810 seeds have established a reporting system allowing farmers to report complaints about product performance and related issues to seed suppliers *via* the local sales representatives or customer service routes. Complaints can include unexpected crop damage to *Bt* maize possibly caused by corn borers due to product failure. So far, none of the complaints received by seed industries (5417 over the 2016–2019 growing seasons) have been attributed to the loss of efficacy of *Bt* maize varieties in Portugal and Spain [44,100,101].

Evaluating the results of the resistance monitoring program.

As required by EU legislation on GE plants, IRM activities accompanying the cultivation of *Bt* maize in the EU and their outcomes are reported to the European Commission and the EU Member States on a yearly basis in annual post-market environmental monitoring (PMEM) reports. Since 2010, EFSA has assessed the methodology and results of the IRM activities associated with the cultivation of maize MON 810, issued scientific outputs on each of the annual PMEM reports, and made concrete recommendations on how to further improve the implementation and reporting of the resistance monitoring strategy [44,100–109].

Based on the analysis of the resistance monitoring data provided in the 2009–2018 PMEM reports, EFSA concluded that there are no indications of decreased Cry1Ab susceptibility in Spanish ECB and MCB populations. However, due to the number of field-collected larvae represented in the diagnostic concentration bioassays and limitations of those bioassays to detect recessive resistance alleles at low frequencies [95], EFSA is of the opinion that the current monitoring strategy for maize MON 810 is not sufficiently sensitive to enable timely detection of a surge of field-evolved resistance. Therefore, the applicant marketing maize MON 810 has been recommended to (i) increase the number of larvae sampled in the field across relevant geographical areas and reduce their mortality during rearing under laboratory conditions (which reached approximately 50% for ECB and between 40 and 60% for MCB in 2017–2019) prior to diagnostic concentration assays, or (ii) replace the diagnostic bioassay by a more sensitive testing method. Yet, finding sampling sites with sufficient corn borer larvae and reducing larval mortality before laboratory testing is a challenge. So, the alternative is the replacement of current resistance monitoring activities with the use of the F_2 screen that would provide an estimation of resistance alleles in corn borer populations [74,94,95]. Such data could be gathered on a periodic basis, as resistance allele frequencies are not expected to increase rapidly from one year to another, provided that enough isolines are screened.

Monitoring of compliance with refuge requirements

The evolution of resistance to *Bt* crops can rapidly accelerate if farmers fail to plant refuges. Thus, ensuring and monitoring compliance with refuge requirements, especially in hotspot areas where target insect pest populations are subject to significant selection pressure and where resistance is most likely to arise, is key to the success of IRM [39,110–114]. Seed companies, national competent authorities and farmers' associations play a pivotal role in reinforcing grower's awareness of the importance to follow refuge obligations. Besides education (i.e. training and stewardship activities), compliance can be maximized via farmer contracts, certification tests, audits, rewards for compliance, crop insurance for refuges, databases of non-compliant farmers, sales restrictions, and fines for noncompliance. Some stakeholders have also proposed refuge planting to be a prerequisite for direct payments under the EU's common agricultural policy (<https://ec.europa.eu/info/food-farming-fisheries/key-policies/common-agricultural-policy/>) or other national rules.

If a structured refuge is to be planted, then monitoring the farmer's compliance with refuge obligations is an important element of the IRM plan. The reporting of noncompliance with refuge requirements, especially in areas where the uptake of *Bt* crops is high, may serve as a trigger to strengthen education (training) programs that aid farmers understanding the importance of adhering to refuge requirements and to impose penalties for noncompliance (i.e. sales restrictions and fines).

Data on compliance with refuge requirements by growers planting *Bt* maize in Spain are reported by applicants in annual PMEM reports and typically gathered through farmer questionnaires. During the first eight years of cultivation of *Bt* maize, only around 60% of Spanish farmers required to plant refuge areas followed this requirement. However, since 2009, annual surveys have reported consistently greater levels (~90%) of compliance by Spanish growers (Supplementary material, Table S3), perhaps associated with the implementation of training programs directed at personnel, distributors, cooperatives and individual farmers to raise awareness on the importance of refuge compliance. Overall, Head and Greenplate [61] indicated that monitoring farmer compliance remains challenging due to the resources required to visit an appropriate number of farmers, and the potential bias present in telephone- and computer-based surveys.

Currently, farmers that grow less than 5 ha of *Bt* maize are not required to plant refuges. However, exempting "small" farmers from planting refuges may be an important bottleneck for IRM. Such farmers represent a significant portion of *Bt* maize growers [44], so the effective percentage of refuges planted would be less than the expected 20% of *Bt* maize growing area. Therefore, EFSA considers that refuge requirements should also apply to clusters of small *Bt* maize fields in which the aggregated area is greater than 5 ha, irrespective of individual field and farm size [89], but acknowledges that the implementation of this recommendation may be challenging. It would require adequate information systems on GE crop cultivation [44] and incentives for the farmers who plant refuges, e.g. rebate on seed costs [115].

Simulations with resistance evolution models

Mathematical models have been widely used to predict the possible adaptation of target insects to *Bt* crops [e.g. 62,114,116,117], and design IRM strategies to delay resistance evolution [e.g. 118–120].

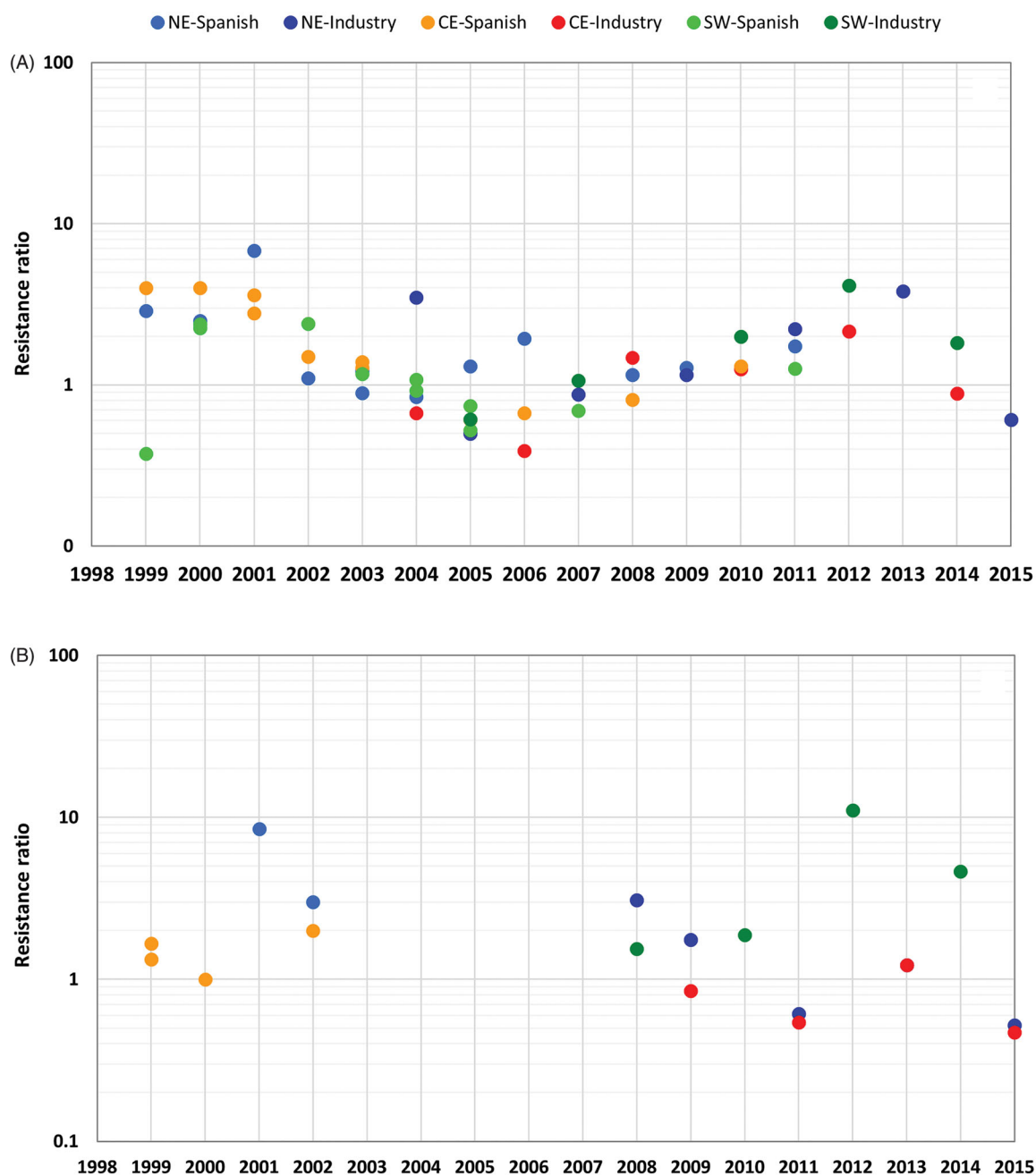


Figure 2. Changes in susceptibility to the Cry1Ab protein for Mediterranean corn borer (A) and European corn borer (B) in populations from north-eastern (NE), central (CE) and south-western (SW) Spain. Resistance ratios were calculated based on Cry1Ab concentration causing 50% mortality (LC_{50}) (Spanish monitoring plan) and inhibition (MIC_{50}) (seed-companies monitoring plan).

Simulations to predict resistance occurrence

A resistance evolution model was developed by Castañera et al. [71] for MCB and maize cropping systems in north-eastern Spain, the EU area with the highest adoption rates of *Bt* maize. This model has considered a wide range of parameters related to the biology, ecology and genetics of the pest and agricultural practices, among others. By modifying some of the parameters of the model (refuge compliance,

female dispersal, assortative mating, local colonization, adoption rate of *Bt* maize events *Bt*176 and MON 810), the authors established which factors have contributed to the success of IRM, and those expected to accelerate the resistance evolution. Model simulations revealed that local assortative mating (nonrandom mating between susceptible and resistant individuals) has more influence in accelerating the development of resistance than any other factor. The low initial adoption rates of

Table 2. Molting inhibition (%) of European and Mediterranean corn borer populations from north-eastern Spain tested with a diagnostic concentration of the Cry1Ab protein.

Growing season	Sampling zone	No. larvae collected	No. adults used in bioassay	No. F ₁ larvae tested	% Molting inhibition	
					Field population	Reference population
European corn borer^a						
2016	North-eastern Spain ^c	1111	554	1562	99.23	nt
2017	North-eastern Spain ^c	1111	628	1488	99.19	nt
2018	Huesca-I	480	206	744	99.97	nt
	Huesca-II	367	185	544	99.63	
	Navarra	297	143	480	100	
	Total/mean ± SE	1144	534	1768	99.87 ± 0.11	
2019	Huesca-I	368	169	496	99.40	nt
	Huesca-II	547	414	704	99.86	
	Navarra	195	151	288	99.65	
	Total/mean ± SE	1110	734	1488	99.63 ± 0.13	
Mediterranean corn borer^b						
2016	Huesca-I	428	282	1024	98.86	99.20
	Huesca-II	524	350	1004	98.47	
	Zaragoza	412	279	1202	96.56	
	Total mean ± SE	1364	911	3230	97.96 ± 0.71	
2017	Huesca-I	504	285	1048	91.65	97.69
	Huesca-II	493	231	1111	96.50	
	Navarra	455	233	1174	94.28	
	Total/mean ± SE	1452	749	3333	94.14 ± 1.4 ^{*,**}	
2018	Huesca-I	516	153	1120	97.85	97.75
	Huesca-II	553	160	1148	99.06	
	Navarra	421	241	1181	99.05	
	Total/mean ± SE	1490	554	3449	98.65 ± 0.40	
2019	Huesca-I	655	352	1162	98.20	97.02
	Huesca-II	560	309	1195	97.26	
	Navarra	429	207	1194	98.44	
	Total/mean ± SE	1644	868	3551	97.97 ± 0.36 ^{**}	

nt: not tested; SE: standard error.

^aA diagnostic concentration of 28.22 ng Cry1Ab/cm² of diet surface area was used.

^bA diagnostic concentration of 1091 ng Cry1Ab/cm² of diet surface area was used.

^cField-collected individual from the three sampling zones were pooled and one diagnostic concentration bioassay was performed.

*Statistically significant differences were observed between the pooled north-eastern populations and the reference susceptible strain.

**Statistically significant differences were observed between the pooled north-eastern populations and the expected value of 99%.

Table based on data provided in EFSA [41,97–99].

Bt maize varieties and the full replacement of maize *Bt*176 by MON 810, in which season-long protection is ensured through the continued Cry1Ab expression throughout the entire plant life cycle, have been pivotal to delay resistance evolution in MCB populations.

Camargo et al. [43] ran simulations with the same model as Castañera et al. [71], but using the latest estimates of resistance allele frequency in MCB populations collected in northeast Spain in 2016. The new simulations indicated that resistance is not evolving faster than the initial projections and that field-evolved resistance to maize MON 810 varieties in MCB in the Ebro basin still might take 25 years or more to appear.

Simulations to design appropriate insect resistance management strategies

Predictive models for resistance evolution have been used to determine and design proper IRM strategies. In 2015, using a resistance evolution model [99], EFSA tested several scenarios of density-dependent mortality for ECB under different adoption rates of

Bt maize. The outcomes of the scenario analysis enabled the estimation of the time needed for resistance to evolve and evaluate whether the IRM approach implemented in the EU allows for the early detection of resistance. For instance, for a maize MON 810 adoption rate of 60%, the estimated number of generations of ECB populations required the detection of a resistance allele frequency of 3% and 5% to reach field-evolved resistance (corresponding to the frequency of 50%) is 7–10 and 5–7, respectively (Figure 1S). Based on these figures and acknowledging that there should be a correct balance between sampling efforts and timely detection of a surge of field resistance, EFSA advocated setting a maximum detection limit for resistance allele frequency at 3% in areas with 60% maize MON 810 adoption rates. EFSA also recommended annual sampling of populations of both target insect pests in areas where the maize MON 810 adoption rate is at least 60% of the total area cropped to maize. Since 2016, this latter recommendation has been followed by the applicant marketing maize MON 810 [101].

Table 3. Number of European and Mediterranean corn borer neonates fed maize MON 810 leaf tissue from the offspring of field-collected populations collected between 2011 and 2019.

Growing season	European corn borer			Mediterranean corn borer		
	Sampling area ^a	Bioassay survivors ^b	Spare larvae ^c	Sampling area ^a	Bioassay survivors ^d	Spare larvae ^c
2011	CE/NE	nt	nt	NE	721	nt
2012	SW	nt	nt	CE/SW	491/432	nr
2013	CE/NE	nt	nt	NE	1003	nr
2014	SW	nt	nt	CE/SW	803/847	≈3000
2015	NE	2	nt	NE	574	≈1400
2016	NE	12	nt	NE	66	>10,000
2017	NE	12	nt	NE	nt	10,650
2018	NE	3	10,267	NE	40	10,294
2019	NE	5	12,415	NE	67	17,300

nr: not reported; nt: not tested.

^aCE: central Spain; NE: north-eastern Spain; SW: south-western Spain.

^bAll surviving larvae from the highest or half the highest (2015) concentration of the concentration-response (2012–2015) or the diagnostic concentration (2014–2019) bioassays.

^cSpare neonates from field-collected individuals not used in the susceptibility assays.

^dAll surviving larvae from the concentration-response (2011–2015) or the diagnostic concentration (2016–2019) bioassays.

None of the larvae survived exposure to maize MON 810.

Table based on data provided in the annual post-market environmental monitoring reports of maize MON 810. Available at: https://ec.europa.eu/food/plant/gmo/post_authorisation/plans_reports_opinions_en

Concluding remarks

The number of instances of field-evolved resistance to *Bt* crops documented in agricultural pests globally has risen in the last decade [39]. In most cases, resistance evolution has been attributed to the deployment of non-high dose *Bt* crop events and the lack of or only partial compliance with refuge requirements. In Spain, however, there have been no reports of resistant corn borer populations after more than 20 years of cultivation of *Bt* maize varieties expressing the Cry1Ab protein. Resistance monitoring data and the lack of farmer complaints on unexpected damage to *Bt* maize caused by corn borers confirm maintenance of ECB and MCB susceptibility to Cry1Ab [40–42,44]. While resistance is not evolving faster than predicted by initial model simulations, the most recent estimation of Cry1Ab resistance allele frequency in MCB populations from north-eastern Spain might exceed the value recommended for the successful implementation of the HDR approach [43]. Relevant factors contributing to the current durability of *Bt* maize in Spain include gradual adoption rates of *Bt* maize, replacement of *Bt* maize event *Bt*176 by the high-dose event MON 810, the low initial frequency of resistance alleles in corn borer populations, and increased levels of growers' compliance with refuge requirements [71].

The IRM strategy for *Bt* maize has been implemented successfully in Spain for more than 20 years, with consistently elevated levels (~90%) of compliance with refuge requirements since 2009. Moreover, the IRM strategy has been regularly updated in the light of the resistance monitoring data gathered annually, the periodic assessment of IRM appropriateness and cost-effectiveness, EFSA's recommendations for methodological

improvements and new relevant evidence published in the scientific literature. Despite the diligent implementation of the IRM strategy, and some efforts to further fine-tune insect resistance monitoring, an unresolved point of contention between EFSA and the applicant is whether the resistance monitoring strategy followed in Spain, based on discriminating concentration bioassays, is sufficiently sensitive to timely detect early warning signs of resistance in the field [44]. EFSA considers this a concern, as the belated detection of early signs of resistance will significantly hamper the timely implementation of remedial measures to appropriately respond to confirmed resistance, and hence prevent the spread of resistance to other areas or eradicate resistance [99]. However, it is also acknowledged that tailoring monitoring activities to the early detection of resistance would demand additional sampling and monitoring efforts, which may raise practical challenges.

To achieve the recommended 3% resistance allele frequency, EFSA previously recommended using a more sensitive testing strategy that combines periodic estimations of allele frequencies for Cry1Ab resistance to *Bt* maize through F₂ screening, with a robust farmer alert system for reports of unexpected damage [44]. Alternatively, resistance allele frequencies could be estimated by F₁ screening of a colony set up with a proper number of field-collected larvae in case that resistant corn borer populations are available (e.g. through laboratory selection) [121]. Matched F₂ and F₁ screens are regularly conducted to manage resistance in *Helicoverpa* sp. to *Bt* cotton in Australia [122]. Such data enable adjusting predictive models for resistance evolution to account for the new resistance allele frequencies and other changes in model parameters. The outcome

of model simulations would help to evaluate whether the frequency of the Cry1Ab resistance alleles is evolving as predicted and decide when to conduct the next F_2 screening. Finally, obtaining more precise estimates on some of the factors influencing the evolution of resistance, such as the level of local assortative mating, would be crucial to reduce uncertainties associated with model projections [71].

The recently estimated increase of Cry1Ab resistance allele frequency to *Bt* maize in MCB populations from north-eastern Spain confirms the continued need to implement adequate IRM approaches (including resistance and compliance monitoring), and incorporate those in IPM schemes to ensure *Bt* maize durability in Spain. This is particularly pertinent in the EU context as current chemical and biological control and cultural methods against ECB and MCB are subject to limitations in Spain [46], while no new GE lepidopteran-active maize events (except for the *Bt* maize event 1507 that expresses the Cry1F protein, see below) for cultivation are in the regulatory approval pipelines to offer additional means for managing corn borers and resistance to *Bt* maize (Supplementary material, Table S4). Such new products are at various development stages or approved for use in non-EU jurisdictions (<https://www.isaaa.org/gmapprovaldatabase/default.asp>). These GE maize events either rely on new *Bt* proteins, combine existing ones, or are based on alternative strategies involving a different mode of action than *Bt* proteins (e.g. chimeric proteins). For instance, *Bt* maize pyramids (such as *Bt* maize 1507 × MON 810 expressing the Cry1F and Cry1Ab proteins and *Bt* maize MON 89034 expressing the Cry1A.105 and Cry2Ab2 proteins), which expresses at least two existing *Bt* proteins with different modes of action targeting the same insect pests [123], can effectively delay the evolution of resistance to each *Bt* protein if most individuals that are resistant to one *Bt* protein are killed by the other, and when selection for resistance to one of the *Bt* proteins does not cause cross-resistance to the other. Under such conditions, IRM could be greatly simplified with the possibilities of reduced refuge amounts. An exception to this is the *Bt* maize 1507 for which regulatory approval is pending in the EU. If approved for cultivation, this *Bt* maize could be grown in rotation with maize MON 810 enabling the alternation of *Bt* proteins with a different mode of action, thus avoiding repeated selection pressure encountered in continuous maize MON 810 cultivation, as is often practiced in Spain [71]. Additional approaches, such as combining the HDR strategy with mass releases of sterile insects [124] or male GE insects with a female-specific self-limiting gene [125,126] have

been proposed or applied for simultaneously managing agricultural insect pest populations and resistance to *Bt* crops. In the southwestern United States and northern Mexico, for instance, the combined use of *Bt* cotton and mass release of sterile pink bollworm (*Pectinophora gossypiella*) moths has enabled the successful suppression of this invasive pest [124] and the replacement of refuges with mass releases of sterile insects.

Education (training) programs aiding farmers to understand the importance of adhering to IRM requirements are essential to the success of the HDR strategy and thus should continue to form an integral part of IRM plans for *Bt* maize, in order to maximize compliance with refuge requirements, especially in hotspot areas (i.e. north-eastern Spain) where corn borer populations are subject to significant selection pressure and resistance is most likely to arise.

Author contributions

Fernando Álvarez-Alfageme: Conceptualization, Formal analysis, Writing – Original Draft, Writing – Review and Editing; Yann Devos: Conceptualization, Writing – Sections of Original Draft, Writing – Review and Revising; Ana M. Camargo: Review and Editing; Salvatore Arpaia: Review and Editing; Antoine Messéan: Review and Editing.

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