Appendix 1. EuropaBio Harmonised insect resistance management (IRM) plan for cultivation of *Bt* maize (single insecticidal traits) in the EU, September 2012

Harmonised insect resistance management (IRM) plan for cultivation of Bt maize (single insecticidal traits) in the EU

- September 2012 -

EuropaBio Monitoring Working Group

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Summary

With the introduction of Bt crops, concerns have been raised about the possible development of insect resistance that could deprive growers of the benefits of Bt crops and Bt microbial preparations. This concern has been addressed pro-actively in a number of countries by the implementation of insect resistance management (IRM) plans to delay the potential development of pest resistance and to enable the timely detection of changes in pest susceptibility allowing remedial actions to be put in place. In the EU, with the introduction of Bt maize cultivation in 1998 in Spain, research programmes were established to monitor the potential development of insect resistance. In 2001, with the introduction of Directive 2001/18/EC, IRM plans became mandatory. Given that different Bt maize varieties targeting the same insect pests were commercialized in the EU at that time (Bt176 and MON 810) and other varieties were under review for approval (TC1507, Bt11), developers of the technology united efforts and proposed a common IRM plan. The purpose of this harmonized plan was to develop and use common methodology to establish the baseline susceptibility of European corn borer (ECB; *Ostrinia nubilalis*) and Mediterranean corn stalk borer (MCB; *Sesamia nonagrioides*) to Cry1Ab and Cry1F endotoxins and to monitor the potential development of resistance following the cultivation of these Bt maize varieties. The plan was implemented in 2003 and has been in place since then. Despite fourteen years of use of Bt maize in the EU and high adoption rates of the technology in some areas, no decreases in the susceptibility of ECB or MCB to Cry1Ab have been detected. This suggests that the implemented harmonized IRM plan was effective. In the EU as well as worldwide, no field resistance to any Cry1Ab and Cry1F-containing event or formulation has been observed in any species of *Ostrinia* or *Sesamia.* However, one of the elements described in the plan was to maintain it updated in view of the findings and in view of new scientific information. Since the first implementation of the harmonized IRM plan there have been some updates in the regulatory framework. Additionally, there has been a large amount of data generated by the previous plan and in the scientific literature, and experience has been gained from IRM plans established in other regions. Taking together all this information, the EuropaBio Monitoring working group has now updated the IRM plan.

This document describes the updated IRM plan including the key elements to follow and the rationale behind the recommendations. The current IRM plan is in line with the recommendations and guidance provided by the current regulatory framework.

Abbreviation/definition of technical terms

Area	Area is defined as a geographical zone where a given crop is typically grown following similar agronomic practices and is isolated from other areas by barriers that might impair an easy exchange of target pests between those areas
Bt	*Bacillus thuringiensis*
Bt maize	Maize plants expressing Bt Cry proteins
Cry protein	Crystal protein derived from Bt
EC_{50}	Effective concentration: the concentration, which affects 50% of a test population after a specified exposure time
EC_{99}	Effective concentration: the concentration, which affects 99% of a test population after a specified exposure time
ECB	European corn borer
Endotoxin	Toxic molecule associated with the outer membrane and cell wall of bacteria
GM	Genetic modification
Grower	Individual responsible for seed purchasing and planting
IPM	Integrated pest management
IRM	Insect resistance management
MCB	Mediterranean corn stalk borer
MIC_{50}	Moulting Inhibition Concentration (*i.e.*, the concentration/dose of substance that is estimated to inhibit molting of 50% of the test organisms)
Field resistance	Field resistance is defined as a genetically-mediated ability of a target pest to survive on one or more commercial line(s) of Bt corn under field (or near fieldsuch as greenhouse) conditions. This ability may be conferred to heterozygotes, but must be conferred to homozygotes. It is demonstrated by an ability of the insect to feed and complete development on Bt corn. Fitness costs (e.g. delayed development, reduced competitiveness, or fecundity) may be associated with the resistance.
Lab resistance	Lab resistance is defined as a genetically-mediated reduction in sensitivity of a target pest to Bt toxins, either in artificial diet or leaf-disc bioassays. Such resistance may be observed as an increase in population MIC_{50}, or as enhanced growth or survival at a discriminating concentration, compared to a known susceptible line. Such resistance does not necessarily confer the ability to develop on Bt corn plants in the field.

Population resistance Population resistance occurs when a large portion of a pest population is field-resistant and causes the Bt maize to fail to confer economic control of the population.

1 Introduction

Maize is an important crop in the European Union (EU) and infestations of European corn borer (ECB; *Ostrinia nubilalis*) and Mediterranean corn borer (MCB; *Sesamia nonagrioides*) can result in considerable crop damage and yield loss. In Spain for example, the losses in maize production due to these insect pests can be as high as 15% in areas of high corn borer pressure. Given the biology of corn borers, the use of conventional insecticides is not very effective as chemical sprays cannot reach the boring pest larvae. Genetically modified (GM) maize plants have been developed to control these pests. These GM plants express *Bacillus thuringiensis* (Bt) proteins, such as Cry1Ab and Cry1F, that provide specific control of lepidopteran pests, by consumption of the proteins when feeding on the maize, and are very effective against ECB and MCB. The plants are commonly known as Bt maize.

Bt is a Gram-positive bacterium capable of producing large crystal protein inclusions that have insecticidal properties. The efficacy and specificity of Bt strains and individual toxins produced by Bt isolates are such that a large number of insecticidal products based on this bacterium and/or its toxins have been developed and sold commercially since the late 1950's. Historically, Bt has been considered a safe option for pest control and it has often been the preferred pest control method in Integrated Pest Management (IPM) programmes.

Using modern biotechnology, the genes coding for specific Bt toxins were isolated in the 1980's and introduced into various crop plants to provide insect protection. Such insect-protected crops now represent an important new management tool to control crop damage and losses due to insect pests. In addition, the use of insect protected crops provides important benefits to growers, society, and the environment (Gómez-Barbero, 2008; Brookes and Barfoot, 2009; MacIntosh, 2009; Park *et al.*, 2011; Brookes and Barfoot, 2012).

Maize plants expressing Bt proteins for pest control were first registered for commercial use (deregulated) in the USA in 1996. Currently, genetically modified (GM) maize containing an insect protection trait (as such or in combination with herbicide tolerance) is the second most widely planted GM crop, with approximately 43.3 million hectares commercially grown worldwide in 2011, mainly in the USA, Canada, Argentina, Brazil and South Africa (James, 2012).

With the introduction of Bt crops, concerns were raised about the possible development of insect resistance that could deprive growers of the benefits of Bt crops and Bt microbial preparations. The development of resistance in insect pests to pesticides is a well known phenomena and the development of insect resistance to Bt crops is accepted in the scientific community as a possibility. Anticipating this concern, biotechnology companies have been working with academic experts, regulators and growers to design and implement proactive insect resistance management plans for Bt crops. As a result of the implementation of these IRM plans no field evolved resistance in ECB or MCB have been reported, neither in the EU nor on a global scale. Only isolated reports of field evolved resistance to other pests have been cited globally (Kruger *et al.*, 2012). However, these reports have been linked to non-compliance by farmers with IRM recommendations, and in particular non-compliance with refugia requirements (Kruger *et al.*, 2012). This shows that the implementation of good IRM practices and customer education and resistance monitoring have slowed the spread of field evolved resistance (Van Rensburg, 2007; Kruger *et al.*, 2009).

In the EU, Bt crops were introduced in 1998 and the adoption has steadily increased every year. In 2011, six EU countries (Spain, Portugal, Czech Republic, Poland, Slovakia and Romania) planted a total of 114,508 hectares of Bt maize, 26% more than in 2010, with Spain growing 85% of the total in the EU with an adoption rate of 28% (James, 2012).

With the introduction of Bt maize cultivation in 1998 in Spain, research programmes were established to monitor the potential development of insect resistance. In 2001, with the introduction of Directive 2001/18/EC (EC, 2001), IRM plans became mandatory. Given that different Bt maize varieties targeting the same insect pests were commercialized in the EU at that time (Bt176 and MON 810) and other varieties were under review for approval (TC1507, Bt11), developers of the technology united efforts and proposed a common IRM plan. The purpose of this harmonized plan was to develop and use common methodology to establish the baseline susceptibility of European corn borer (ECB; *Ostrinia nubilalis*) and Mediterranean corn stalk borer (MCB; *Sesamia nonagrioides*) to Cry1Ab and Cry1F endotoxins and to monitor the potential development of resistance following the cultivation of these Bt maize varieties. The plan was implemented in 2003 and has been in place until now. Despite fourteen years of use of Bt maize in the EU and high adoption rates of the technology in some areas, no decreases in the susceptibility of neither ECB nor MCB to Cry1Ab have been detected. This suggests that the harmonized IRM plan was effective for Cry1Ab. However, one of the elements described in the plan was to maintain it updated in view of the findings and in view of new scientific information. Since the first implementation of the harmonized IRM plan there have been some updates in the regulatory framework. Additionally, there has been a large amount of data generated by the previous plan and in the scientific literature, and experience has been gained from IRM plans established in other regions. Taking together all this information, the EuropaBio Monitoring working group has now updated the IRM plan.

This document describes the updated IRM plan including the key elements to follow and the rationale behind the recommendations. The current IRM plan is in line with the recommendations and guidance provided by the current regulatory framework. The goal of the IRM plan is to detect resistance far enough in advance of population resistance to allow adequate time for the confirmation and characterization of resistance, and to take steps to reduce the likelihood or extent of product failure. The plan has been designed so it is effective, balanced and practical for the growers of Bt maize.

2 Scope of the plan

The goal of the IRM plan described in this document is to detect resistance far enough in advance of population resistance to allow adequate time for the confirmation and characterization of resistance, and to take steps to reduce the likelihood or extent of product failure. The IRM plan includes timely detection of changes in pest susceptibility to Cry proteins and remedial actions in case of any confirmed development of resistance. A timeframe of two to two and a half years between initial resistance detection and implementation of an appropriate remediation plan is considered adequate. The transformation events currently included in the proposal are presented in Table 1.

Table 1. Proteins and transformation events currently included in the harmonised IRM plan

Transformation event	OECD unique identifier	Protein	Notifier
Bt11	SYN-BT∅11-1	Cry1Ab	Syngenta
MON 810	MON-∅∅81∅-6	Cry1Ab	Monsanto
1507	DAS-∅15∅7-1	Cry1F	Pioneer; Mycogen/DAS

The main insects targeted by the plan are the European corn borer and the Mediterranean corn stalk borer, as shown in Table 2.

Table 2. Insects targeted by the harmonised IRM plan

Common name	Abbrev.	Scientific name	Family
European corn borer	ECB	*Ostrinia nubilalis* (Hubner)	Crambidae
Mediterranean corn stalk borer	MCB	*Sesamia nonagrioides* (Lefebvre)	Noctuidae

3 Approach and rationale of the plan

3.1 Regulatory framework in the European Union

The updated IRM plan proposed by the EuropaBio Monitoring working group has taken into account the recommendations and guidance provided by the current regulatory framework.

Directive 2001/18/EC (EC, 2001) was the first to establish that notifiers should develop and submit a monitoring plan together with the notification for placing on the market of a genetically modified (GM) crop. The design of the post-market environmental monitoring plan (PMEM) was outlined in Annex VII of this Directive. The objectives of the monitoring plan were described as: (1) to confirm that any assumptions made regarding the occurrence and impact of potential adverse effects of the GMO or its use in the environmental risk assessment (ERA) are correct, and (2) to identify the occurrence of adverse effects of the GMO or its use on human health or the environment which were not anticipated in the ERA. In line with the regulatory framework, Annex VII to Directive 2001/18/EC was later supplemented by the Council Decision 2002/811/EC providing further guidance on the objectives, general principles and design of monitoring plans. More recently, the European Food Safety Authority (EFSA) GMO Panel updated its scientific opinion on the PMEM of GM plants (EFSA, 2011a), following the opinion on the ERA of GM plants (EFSA, 2010).

3.2 Practical experience from IRM plans implemented around the world

The first country to introduce Bt maize for commercial cultivation was the USA in 1996. The rapid success of this technology and the high rates of adoption led to the US Environmental Protection Agency (EPA) to view Bt crops as a "public good" and to adopt measures to protect the technology. The US EPA now requires the monitoring of insect resistance development to Bt crops as a condition of registration (US EPA, 2001; MacIntosh, 2009). Developers of the technology in collaboration with experts from academia, USDA, EPA and the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) developed a harmonized industry IRM plan for Bt maize (ABSTC, 2003). This plan is based on the high-dose/refuge strategy (also further addressed in this document below) and comprises all the elements required by US EPA[1], such as the use of structured refuge requirements, grower agreements and resistance monitoring programs for Cry1Ab and Cry1F Bt maize (Siegfried *et al.*, 2007). To date, despite the high level of adoption of Bt maize in the USA during almost two decades, there are no reports of field-evolved resistance in populations of ECB to Cry1Ab or Cry1F (Tabashnik *et al.,* 2003; Siegfried *et al.*, 2007; Tabashnik *et al.,* 2009; Head and Greenplate, 2012).

In Argentina the first Bt maize product was approved in 1997. Bt maize was initially introduced with a variety of voluntary IRM practices, but in 1999, building upon the experiences in the USA, a joint industry IRM plan was developed in collaboration with experts from academia and the Instituto Nacional de Tecnología Agropecuaria (INTA). The harmonised IRM plan was also based on the high-dose/refuge strategy and proposed the use of a 10% refuge requirement. The proposal of this refuge size was based on knowledge of the biology of the local target pest and on grower behaviour. In particular, it was noted that the presence of abundant alternative hosts for the target pests justified refuge sizes smaller than in the USA for target pests that were otherwise similar in their biology. The IRM plan also included the development of baseline susceptibility measurements for the target pests, the creation of standardised educational literature for growers and the use of regular surveys to assess grower compliance with the requirements. The joint industry IRM plan was accepted by the regulatory agency Comisión Nacional Asesora de

[1] http://www.epa.gov/oppbppd1/biopesticides/pips/bt_corn_refuge_2006.htm#websites (Accessed July, 2012).

Biotecnología Agropecuaria (CONABIA) and implemented. To date, there are no reports of field resistance of target pests to Cry1Ab or Cry1F.

Similar approaches have been followed in other countries such as Canada, South Africa and Brazil, where harmonized industry plans have been developed in collaboration with experts from academia and regulatory authorities, to protect the technology. All these IRM plans for Bt maize crops are based on the high-dose/refuge strategy, although the size of the refuge may vary depending on the biology and ecology of the target pest in that country. In South Africa, there have been some cases of insects other than ECB or MCB developing resistance to Bt maize in the field, however, these cases have been linked to poor implementation of IRM practices among customers; further customer education efforts and monitoring efforts have slowed the spread of resistance (Van Rensburg, 2007; Tabashnik *et al.,* 2009; Kruger *et al.*, 2012).

3.3 Practical experience from the previous IRM plan implemented in the EU

In the EU, Bt maize has been cultivated since 1998 in Spain. Research programmes supported by the Spanish authorities and the industry harmonized IRM plan that has been in place since 2003 have provided a large body of information on baseline susceptibility of the target pests populations in different EU countries, on susceptibility levels after continuous exposure to Bt maize and on the ecology of the pests (See Section 3.4 below for a summary). In addition, since the only Bt maize currently cultivated in the EU is MON 810, Monsanto, in compliance with current regulatory requirements, has submitted annual monitoring reports to the European Commission since 2005 (the two most recent reports: Monsanto, 2010 and Monsanto, 2011 are accessible through the European Commission's website[2]). These reports provided information on the findings of the IRM plan in place for Cry1Ab, including baseline susceptibility data for ECB and MCB and susceptibility data following exposure to MON 810 (See Section 3.4 below for a summary of the findings). The two most recent reports have now been reviewed by EFSA and scientific opinions have been adopted (EFSA, 2011; EFSA, 2012). These scientific opinions have been taken into consideration while updating this IRM plan.

One of the key conclusions of all this research is that no susceptibility shifts have been established for field populations of MCB or ECB after fourteen years of Bt maize cultivation, showing that the IRM plan in place has been effective.

3.4 Current scientific knowledge

This IRM plan is based on the high-dose/refuge strategy. The strategy consists in planting Bt maize that produces sufficiently high concentrations of the insecticidal Cry protein so that even partially resistant target pest individuals do not survive. A non-Bt refuge is planted nearby providing a safe and large enough habitat for susceptible target pest individuals, so resistant insects emerging from the Bt maize field are likely to mate with susceptible insects from the refuge producing a heterozygous progeny that is phenotypically susceptible to Bt-maize (Head and Greenplate, 2012). The value of this approach has been demonstrated through mathematical modelling and field experiments (Ives and Andow, 2002; Shelton *et al.*, 2000) and is considered an effective tool in delaying the development of resistance in Bt crops (MacIntosh, 2009; Huang *et al.*, 2011; Head and Greenplate, 2012).

Three key assumptions underlie the high-dose/refuge strategy: the plant must express the toxin at sufficient levels so that resistance is functionally recessive, resistant insects must mate randomly with susceptible individuals surviving in the refuge, and resistance alleles must be rare (Andow, 2008).

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[2] http://ec.europa.eu/food/plant/gmo/reports_studies/index_en.htm (Accessed July, 2012)

The Bt maize crops included in this IRM plan, MON 810, Bt11 and TC1507 express the Bt protein at high dose. Current scientific knowledge suggests that the frequency of resistance alleles in populations of ECB and MCB in Europe is low and that these alleles are recessive (Bourguet *et al.*, 2003; Gaspers, 2009). Knowledge on ECB and MCB biology and previous experience of cultivation of Bt maize in Spain and implementation of IRM measures following the high-dose/refuge strategy suggest that the high-dose refuge strategy is a suitable tool for delaying the development of resistance in ECB and MCB in Europe.

For ECB, many studies have been conducted to determine the genetic diversity and baseline susceptibility of ECB populations to Cry1Ab and Cry1F. The results showed that there is a low genetic differentiation of ECB populations in Europe and no geographic clusters of populations have been detected (Gonzalez-Nuñez *et al.*, 2000; Chaufaux, 2001; Farinós *et al.*, 2004; Gaspers, 2009; Gaspers *et al.*, 2011). This was also confirmed by analysis conducted with ECB in Europe by Saeglitz (2006). Baseline susceptibility of ECB in populations collected from different EU countries showed some variability, but no consistent pattern emerging, suggesting that there is an intra-species variability in susceptibility to Cry1Ab and Cry1F (Gaspers, 2009 and Gaspers *et al.*, 2011).

For MCB, studies have also been conducted to determine the genetic diversity and baseline susceptibility to Cry1Ab and Cry1F (Gonzalez-Nuñez *et al.*, 2000; De La Poza et al., 2008; Farinós *et al.*, 2012). The results showed that population genetics of MCB collected in populations in Spain and southwest France were closer than populations from Italy, Greece, and Turkey (De la Poza *et al.,* 2008), suggesting a small genetic differentiation between West Mediterranean and East Mediterranean populations. However, no significant differences in the susceptibility to Cry1F and Cry1Ab were found when comparing MCB populations from these two areas (Farinós *et al.*, 2011 and 2012).

As discussed in Head and Greenplate (2012) there are a number of factors that can influence the development of resistance in insect pests. Apart from the characteristics of the product and the genetics of resistance, the pest ecology (such as movement and mating and the number of generations per year) can influence the development of resistance. In Europe, ECB completes one or two generations per year depending on latitude, generally with one generation in the North of Europe and two in the South (Farinós *et al.*, 2004). Whereas MCB completes a variable number of generations per year depending on latitude, ranging from two in southern France to up to four in Morocco (Farinós *et al.*, 2012). The mating behaviour and movement of these species has also been studied (Showers *et al*. 2001, Hunt et al., 2001; Tate *et al.,* 2006; Eizaguirre *et al.*, 2004 and 2006; Reardon and Sappington, 2007).

In summary, there is a lot of information on the baseline susceptibility of ECB and MCB populations to Cry1Ab and Cry1F in Europe, the genetic diversity within populations of these species and their ecology. The scientific findings suggest that the implementation of a high-dose/refuge strategy is a suitable tool to delay the onset of resistance to Bt maize in ECB and MCB in Europe.

4 Characteristics of the IRM plan

The goal of the IRM plan is to detect resistance in advance of population resistance to allow adequate time for the confirmation and characterization of resistance, and to take steps to reduce the likelihood or extent of product failure. The plan has been designed to be effective, balanced and practical for the growers of Bt maize.

4.1 Effective

Based on current knowledge of pest biology and insect resistance, combined with information from simulation models incorporating highly generous safeguard margins, a science based IRM plan has been developed.

Recognising that available data may not be representative of all pest populations and that a degree of uncertainty exists, the present IRM plan incorporates generous safeguard margins to ensure that the IRM plan is precautionary. In particular, the added safeguard margins are manifested by a larger refuge than would be necessary in the EU on strictly technical grounds. A comparable refuge strategy has been used in the USA where Bt maize has been grown widely on a commercial scale since 1996. Despite extensive monitoring efforts over the past 16 years, there has been no report of development of ECB resistance to Bt maize in the USA (Siegfried *et al.*, 2007; Crespo *et al.*, 2009; Head and Greeplate, 2012). The effectiveness of the IRM plan will be reviewed regularly, taking into account the results of resistance monitoring to incorporate any new scientific developments relevant to the IRM plan.

4.2 Balanced and practical

It is important that all stakeholders of Bt maize technology adopt and implement the elements of the IRM plan. Seed companies have experience in cooperating with regulatory agencies, providing grower education, implementing product stewardship and working with experts on resistance management initiatives. However, farming practices are also critical to the success of the IRM plan. This highlights the importance of the decision-making of individual growers in the implementation of the IRM plan, in particular the refuge strategy. These important factors have been taken into consideration whilst developing the IRM plan, in particular the recommendations for implementation of a refuge, which have been carefully designed to be pragmatic, clear and consistent across relevant regions as well as provide a degree of flexibility where necessary according to variable cropping systems.

The refuge requirement is part of the IRM plan and is designed to delay the potential development of resistance by target pests to Bt maize. This is a precautionary measure to reduce the selective pressure on local populations of target pests. Details on refuge size, location, configuration and a tested process for investigating unexpected damage are provided in the IRM plan. The practices described in this plan balance a grower's opportunity to benefit from Bt maize in the short term with the longer-term objective of preserving the efficacy of Bt maize. All developers subscribing to the present IRM plan are committed to provide farmers with the necessary guidance, technical support and advice on best practices for growing Bt maize.

5 Elements of the IRM plan

The IRM plan is comprised of the following elements:

- Maintaining an adequate level of non-Bt maize refuge in the vicinity of Bt maize to support a sufficient local population of susceptible target pests.
- Baseline susceptibility data for ECB and MCB have been established for Cry1Ab and Cry1F.
- Monitoring for any potential development of resistance.
- Remedial action plan in case of any confirmed development of resistance.
- Programme of grower education for greater awareness of Bt maize cultivation and proper stewardship

The first four elements are elaborated below. Details about grower education can be found in Section 6.

5.1 Refuge

Currently, it is widely accepted that resistance to single insecticidal trait Bt crops is rare and genetically recessive (Head and Greenplate, 2012). This has led to the development of IRM plans using a high-dose/refuge strategy based on the following assumptions:

- Bt maize that produces sufficiently high concentrations of the insecticidal Cry protein so that even partially resistant target pest individuals do not survive
- Resistance alleles typically are partially or fully recessive and rare so there will be few homozygous survivors
- Refuges are set up so that resistant homozygotes will mate randomly with susceptible individuals.

In summary, the purpose of the refuge is to maintain high numbers of susceptible homozygotes that will breed with the few surviving heterozygotes as well as with the rare resistant homozygotes, thereby delaying the evolution of resistance.

The effectiveness of a refuge is dependent on biological, genetic, behavioural and social or cultural factors. Therefore, the refuge strategy described below takes into account EU target pests, agronomic conditions and cultural practices. Moreover, it draws from experience gained through several years of implementing refuge strategies in countries where Bt maize is routinely cultivated. The result is a refuge strategy that incorporates generous safeguard margins and will delay or avoid resistance of target pests to Bt maize without compromising grower accessibility to Bt maize or grower ability to implement refuge requirements.

5.1.1 Refuge size

An appropriate level of refuge should be determined based on a comparative analysis of refuge strategies and maize-growing conditions in countries where Bt maize is regularly cultivated. The minimum proportion of non-Bt refuge implemented in other countries ranges from 10% to 20%. Such refuge sizes are considered to contain generous safeguard margins taking into consideration the local growing conditions.

For the purpose of the present IRM plan for the EU, a grower is defined as the individual responsible for managing and taking planting decisions on one farm or a group of farms. Growers planting more than 5 hectares (ha) of Bt maize would be required to plant a non-Bt maize refuge whereas growers planting less than 5 ha of Bt maize would not. This 5 ha threshold relates to the total amount of Bt maize, within or among fields, planted by one grower and is independent of the size of the individual fields or the total land area managed by this grower.

The EFSA scientific opinion on the annual monitoring reports by Monsanto on the cultivation of GM maize MON 810 in 2009 and 2010 concluded that the 5 ha threshold proposed is reasonable and conservative, given current scientific knowledge on the mating and movement behaviour of ECB and MCB in maize (EFSA, 2011 and 2012). However, the EFSA opinion highlighted that there could be areas of high Bt maize adoption where most of the maize fields could be 5 ha or less. Therefore, this IRM plan takes into account that in those areas the recommendations for refuge plantings will be reassessed to achieve the requested refuge levels.

5.1.2 Refuge configuration and placement

Refuge maize can be located near, adjacent to or within Bt maize fields. Refuges within a Bt maize field can be planted as a block, perimeter border or strips (see example in Appendix 1). Growers should also ensure that the refuge maize and the Bt maize share similar growth and development characteristics.

Growers should plant the refuge within 750 meters of their Bt maize field(s) although lesser distances are preferred. The objective of this distance requirement is to maintain a high probability of pest immigration into Bt maize, and consequently, a high probability that any rare individuals surviving on Bt maize will mate with susceptible individuals from the refuge. The scientific basis for this distance requirement is outlined in the work of Showers *et al.* 2001; Hunt *et al.* 2001 and Eizaguirre *et al.*, 2004 and 2009. This distance is also consistent with refuge strategies practiced in other countries.

Guidelines for planting a refuge will be clearly communicated in the product use guide that accompanies Bt maize.

5.1.3 Refuge management

Refuge zones should be managed in the same way as the Bt crop areas, where possible. Growers are encouraged to monitor their maize crop. Control of pest populations in non-Bt refuge maize should only be applied when the level of pest damage reaches economic importance. Where necessary, insecticides should be used according to their label recommendations. Microbial Bt sprays are the only class of insecticide that must not be used in refuge maize.

5.2 Resistance monitoring

5.2.1 Objectives and underlying principles

The goal of the IRM plan is to detect resistance far enough in advance of population resistance to allow adequate time for the confirmation and characterization of resistance, and to take steps to reduce the likelihood or extent of product failure. A timeframe of two to two and a half years between initial resistance detection and implementation of an appropriate remediation plan is considered adequate (See Section 5.2.3).

Insect resistance monitoring encompasses two basic approaches (MacIntosh, 2009):

1. Monitoring the insects for changes in susceptibility to the expressed protein. The establishment of baseline susceptibility measurements for Bt maize provides the opportunity of early detection of shifts in susceptibility well before widespread resistance occurs at field level.
2. Monitoring fields for signs of unexpected damage allows the detection of resistant insect populations and the application of remedial action to avoid the spread of resistance._This approach may also allow the detection of resistance in secondary lepidopteran pests.

This IRM plan focuses on the first approach, where the baseline data collected since the previous harmonized IRM plan was implemented in 2003 will be used to monitor potential shifts in susceptibility in ECB and MCB once Bt is cultivated. This approach is considered more appropriate as it allows the observation of potential changes in susceptibility before resistance in the field takes place. The second approach is already covered by the stewardship programmes that individual companies establish for their products, where customers can contact companies in case of product failure and the reasons for that failure are investigated. Performance and unexpected damage are also elements included in farmer questionnaires as part of General Surveillance programmes.

5.2.2 Monitoring focus

Resistance monitoring will focus in areas where the development of resistance of target pests to Bt maize is more likely due to the ecology of the pest and relatively high adoption of Bt maize cultivation.

As discussed in Head and Greenplate (2012) there are a number of factors that can influence the development of resistance in insect pests. Apart from the characteristics of the product and the genetics of resistance, the pest ecology (such as movement and mating and the number of generations per year) can influence the development of resistance. The data collected for ECB and MCB regarding baseline susceptibility, genetic diversity and ecology (See Section 3.4 for summary), support the differentiation of two major regions in the EU (See Table 3):

- Southern Europe: comprising Mediterranean countries including the South of France
- Northern Europe: the rest of EU countries cultivating maize.

Table 3. Characteristics of ECB and MCB in the EU relevant for development of resistance to Cry1Ab and Cry1F

Geography	ECB			MCB		
	Genetics	**Susceptibility**	**Ecology**	**Genetics**	**Susceptibility**	**Ecology**
Northern Europe	No difference (1)	No difference (1)	Univoltine (2)	NA(3)	NA(3)	NA(3)
Southern Europe	No difference(1)	No difference (1)	Bivoltine (2)	Small difference(5)	No difference (6)	Multivoltine(5)

(1) Farinós *et al*., 2004; Gaspers, 2009; Gaspers *et al*., 2011; Gonzalez Nuňez *et al*., 2000; Saeglitz *et al*., 2006
(2) Farinós *et al*., 2004.
(3) NA: Not applicable. The distribution of *Sesamia nonagrioides* is currently confined to the mediterranean areas.
(5) de La Poza *et al*., 2008.
(6) Farinós *et al.,* 2012.

From this table, it is clear that in terms of genetic variability and susceptibility there is little or no difference within a specific geography for each individual pest. The ecology of the pest (particularly number of generations per year), however, appears to be a geography-specific determinant for resistance development, *i.e.*, northern or southern Europe. Therefore, it is reasonable to differentiate the monitoring strategy on this characteristic. In addition, the level of adoption of Bt maize in a given area (causing the selective pressure) is an important factor contributing to potential resistance development. Therefore, the sampling strategy for this IRM plan will be made dependent on the ecology of the pests (based on current knowledge) and the published adoption levels of Bt maize, comprising Cry1Ab and Cry1F expressing maize varieties, which are independent from companies or brand names. Considering that the recommended size of the non-Bt maize refuge in the EU is 20%, the approach that will be followed for sampling is outlined in Table 4.

Table 4. Sampling approach for insect resistance monitoring of ECB and MCB based on their ecology and levels of Bt maize adoption

Bt maize adoption rate per area[(1)]	Generations of ECB and MCB	
	Univoltine	Multivoltine
< 20%	No sampling	No sampling
20-80%	Monitoring every two years	Monitoring every two years
80%	Monitoring every two years	Monitoring every year[(2)]

(1) A maize area is defined as a geographical zone where maize is typically grown following similar agronomic practices isolated from other maize areas by barriers that might impair an easy exchange of target pests between those areas, *e.g.*, Ebro Valley. The Bt maize adoption rate is expressed as a fraction of total maize cultivation in the same area, which is based on official numbers published for this area.

(2) This type of monitoring would be conducted in an area that EFSA would call a 'hotspot', *i.e.*, an area with a high adoption rate of Bt maize where multivoltine types of the target pest are present.

If, in accordance with Table 4, an area has to be sampled for susceptibility testing, three independent samples of the relevant target pests will be collected.

Sample site selection within an area will be driven by ecological factors. The target pest population must be large enough to provide sufficient numbers of healthy individuals for collection. Bt maize adoption levels could also vary from year to year. The sampling methodology will therefore be adapted to these variations and it may not be possible to always collect the targeted number of insects per area. More details on sampling can be found in Appendix 2. These protocols apply to both ECB and MCB collections.

In addition, target pest collections should be made in non-Bt fields within the dispersal range of the insects coming from the nearest Bt maize field. Samples taken close (or within a Bt maize field) may have been subjected to local Bt selection and may demonstrate reduced sensitivity due to selection pressure by exposure to sub-lethal doses or elevated resistance gene frequencies misrepresentative of the population average of the area. Since this typically overrepresents resistance gene frequencies, this sampling method is a conservative approach for detecting reduced sensitivity. The precise collection locations will be varied from year to year to provide thorough coverage across years.

The implementation of all the above described practices ensures that the sampling will be intensified in areas where high levels of Bt maize adoption occur and where target pests pressure is higher.

5.2.3 Monitoring protocol

As discussed in Section 3.4, since the first harmonized industry IRM plan was implemented in 2003 a number of studies have already been performed to measure baseline susceptibility of ECB and MCB to Cry1Ab and Cry1F (Marçon *et al.*, 1999 and 2000; Gonzalez-Nuñez *et al.,* 2000; Chaufaux *et al.*, 2001; Farinós *et al.*, 2004; Saeglitz *et al.*, 2006; Gaspers *et al.*, 2011; Farinós *et al.*, 2011 and 2012). In addition, data collected by developers was submitted in the annual monitoring reports prepared by Monsanto in 2010 and 2011 (Monsanto, 2010 and 2011). The baseline susceptibility data for ECB and MCB to Cry1Ab and Cry1F in the EU is therefore already established (See Section 3.4).

Insect collections will be coordinated by participating companies. Susceptibility tests will be performed using the same methodology used for the measurement of baseline susceptibility, which was based on a discriminating dose (Marçon *et al.,* 2000) with F1 progeny larvae obtained by field collected individuals. To provide sufficient detection sensitivity, about 200 larvae, 200 adults, 100 mated females or 100 egg masses will be collected per population. A minimum population size of 50 larvae, or 50 adults, or 25 mated females or 25 egg masses will be considered a valid sample for testing. The detailed procedures for sampling are provided in Appendix 2.

Conventionally, a population is considered resistant to a certain toxin when the resistance allele reaches a relative frequency of 0.5 (or 50%). To understand the sensitivity needed to detect recessive alleles two and a half years before population resistance occurs, a simple model was used to determine average population fitness on Bt maize five generations (*i.e.*, 2 ½ years) after resistance detection. Model assumptions included a 20% refuge (*i.e.*, 80% of maize is Bt), complete random mating, and no fitness cost associated with resistance. Using this simple model and assuming a 20% refuge, a functionally recessive allele (heterozygote survival = 0%) would need to be detected at a frequency of 0.075 (or 7.5%) to allow 2½ years (5 generations if the pest is bivoltine) before a population becomes resistant. The monitoring approach outlined in this document is designed to detect resistance when the frequency of the resistant allele reaches about 0.01 – 0.05 (or 1-5%). This level of sensitivity should allow for early detection of potential pest resistance before field failures occur and therefore would enable additional management measures to be effectively implemented in the two to two and a half year timeframe.

5.3 Remedial plan in case of Bt maize failure to protect against target pests

5.3.1 Procedures for unexpected damage

The following procedures are proposed in case of reports of unexpected damage:

a. The seed company will require distributors to instruct purchasers of Bt maize seed to report unexpected levels of damage caused by target pests.

b. The seed company will provide distributors specified information, including details of the report.

c. If the company is a licensee for the Bt trait, it will transmit this information to the notifier.

d. Companies will investigate the cause of these reports using available methods to confirm that the damaged plants express Cry protein, the damage resulted from a target pest and the damage is unexpected.

e. Insects will be collected for the purpose of further evaluation and confirmation subject to subsequent investigations to confirm that damage is unexpected.

5.3.2 Steps to confirm resistance

a. If damage is unexpected, the collected insects will be tested in a laboratory following specific guidelines used to confirm resistance.

   - confirm field resistance;
   - confirm resistance is heritable;
   - use crosses to determine the nature of resistance (i.e. recessive or dominant, and level of functional dominance);
   - estimate r-allele frequency in the original population;

- determine whether the r-allele frequency is increasing by analyzing field collections in subsequent years sampled from the same site where the resistant allele(s) was originally collected;
- determine the geographic distribution of the r-allele by analyzing field collections in subsequent years from sites surrounding the site where the resistant allele(s) was originally collected;

b. Both of the following conditions must be met to confirm resistance: the collected insects or their progeny must have an MIC_{50} that exceeds the upper 95% confidence interval of the historical (susceptible) mean MIC_{50} for the appropriate Bt protein and the collected insects or their progeny must achieve > 30% survival and > 25% leaf area damage in a 5-day bioassay under laboratory conditions using the appropriate protein-positive leaf tissue.

If resistance is confirmed**,** the notifier will inform the European Commission and other relevant Authrities according to the relevant legislation and take appropriate measures as described below.

5.3.3 Remedial actions if insect resistance is confirmed

a. A remedial action plan will be developed, involving the relevant notifier and others concerned with the cultivation of affected Bt maize in collaboration with the relevant Authorities.

b. Components of an appropriate remedial action plan may include:

   - Informing customers and extension agent in the affected areas of confirmed resistance.
   - Increasing monitoring in affected areas.
   - Implementing alternative means to reduce or control target pest populations in affected areas.
   - Modifying and amending the IRM strategy accordingly.
   - If the above measures are not efficient, then cessation of sales in the affected and bordering areas may be necessary until an effective local management plan approved by the pertinent Member State has been put in place.

If interrupted, sale of Bt maize in the affected area will restart when an effective management plan has been implemented.

6 Implementation (Grower education)

An extensive grower education programme is essential for the successful implementation of the IRM plan. Growers should have a clear understanding of the importance of IRM to preserve the long-term efficacy of the Bt technology and realise that their participation in this IRM stewardship programme is vital to prolonging the success and benefits of Bt maize. Each of the seed companies participating in this IRM plan is committed to continuing with their ongoing comprehensive education programmes.

A technical user guide will provide each purchaser of Bt maize with latest information on the recommendations for the IRM plan, Bt technology, the approval status of various Bt maize hybrids in the relevant country and contact details of the responsible seed provider (technology provider, licensee). The user guide will request growers to implement the required IRM measures such as recording where Bt maize is planted, planting a non-Bt maize refuge and monitoring product performance.

In addition, the IRM plan will be communicated using a combination of the following means:

- Slide and video presentations to growers and distributors, co-ops, seed dealers and distributors.
- Information via company and relevant country specific associations as well as agricultural extension services web sites.
- Newsletters.
- Country specific hotlines.
- Relevant competent authorities.

An example of the IRM guidance given to costumers in Spain is provided in Appendix 1 and will be adapted to the conditions of the local market.

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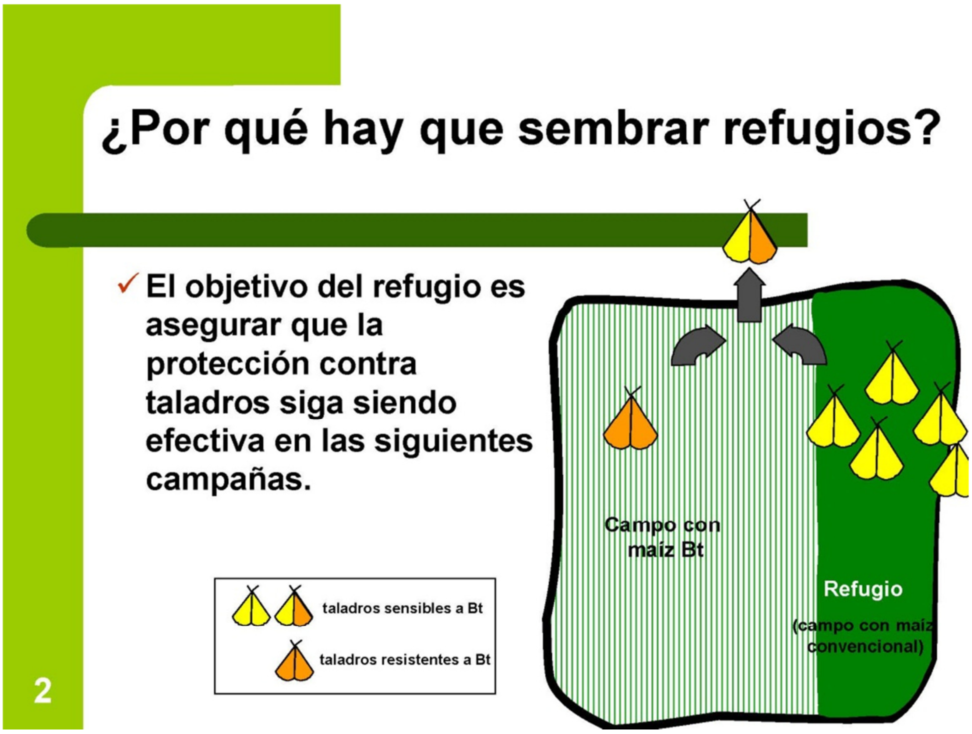
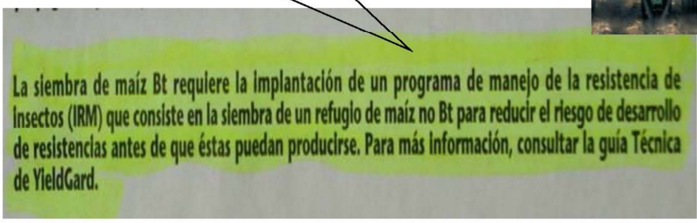
Appendix 1: Example of grower information material



- ✓ Es obligatorio, de acuerdo con la autorización europea y se especifica en las condiciones de empleo que el agricultor asume al adquirir la semilla (Etiquetas y Guías en sacos)

Ejemplo de la información recogida en las Guías Técnicas

Ejemplo de la información recogida en los sacos



La siembra de maíz Bt requiere la implantación de un programa de manejo de la resistencia de insectos (IRM) que consiste en la siembra de un refugio de maíz no Bt para reducir el riesgo de desarrollo de resistencias antes de que éstas puedan producirse. Para más información, consultar la guía Técnica de YieldGard.

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3

¿Qué consecuencias tendría el desarrollo de poblaciones de taladro resistentes?

- ✓ **El maíz Bt dejaría de ser efectivo** contra los taladros.
- ✓ **Todos los agricultores** de esa zona **perderían** el acceso a la tecnología.
- ✓ Se crearía una **imagen negativa** de los agricultores españoles por descuidar el respeto a las Buenas Prácticas.

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4

¿Cómo debe sembrarse el refugio ?

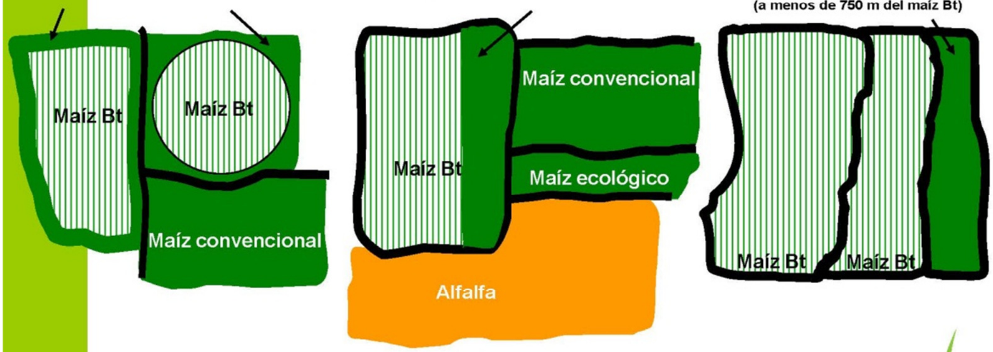
- ✓ **Obligatorio cuando se siembren más de 5 ha de maíz Bt, aunque éstas estén distribuidas en varias parcelas.**
- ✓ **Un tamaño de al menos un 20% de la superficie dedicada a maíz.**
- ✓ **Sembrado lo más cerca posible al campo con maíz Bt (distancia inferior a 750 m).**
- ✓ **Empleando una variedad convencional de ciclo y fecha de siembra similar.**
- ✓ **No sirve la parcela del vecino, ya que una parcela con maíz convencional de otra finca puede ser el refugio del propietario de dicha finca.**
- ✓ **Se puede tratar contra taladro, siempre que no se utilicen preparados de *Bacillus thuringiensis* (Bt)**

Diferentes opciones son posibles

Refugio sembrando las cabeceras o las esquinas del pivot con maíz convencional

Refugio sembrando maíz convencional en un bloque, que sirva de aislamiento a la parcela de maíz Bt

Refugio para agricultores con parcelas pequeñas, sembrando maíz convencional en una parcela completa. (a menos de 750 m del maíz Bt)



6

Las empresas que comercializan maíz Bt se toman muy en serio el empleo correcto de la tecnología

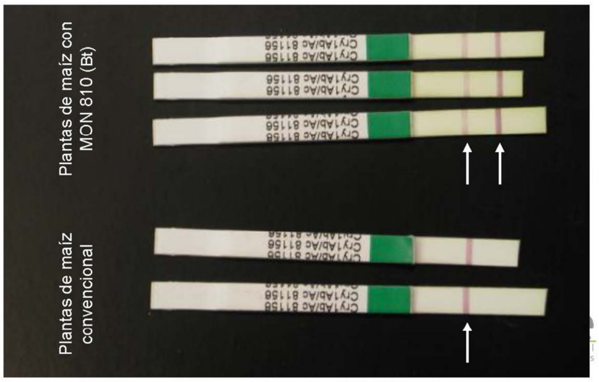
- ✓ Asegurando que los usuarios reciban información completa sobre Refugios y Buenas Prácticas, para cumplir con la legislación vigente.
- ✓ Recogiendo larvas de taladro cada año, que son analizadas en laboratorio para comprobar que siguen siendo sensibles a Bt.
- ✓ Evaluando a través de encuestas a agricultores el comportamiento de Bt en el campo y el grado de cumplimiento en la siembra de refugios.

7

ANOVE y las empresas comercializadoras ofrecen sus servicios para información, o comprobación en campo

✓ Comprobación de refugios en campo mediante análisis de hoja

→



8

Recuerde que:

Si el taladro se vuelve resistente, perdemos todos.

Muchas Gracias por su Colaboración

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9

Appendix 2: Standard Operating procedures for eggs and larvae sampling

Standard Operating Procedure
How to collect European corn borer egg-masses?

This document describes how to collect egg- masses of the European co rn borer (ECB, *Ostrinia nubilalis* [Hübner 1796]). The samples will be used to assess and monitor the Bt-susceptibility of different European *Ostrinia*-populations.

Content Page

1. Definitions and basic requirements

To avoid misunderstandings or confusion please use the following terms only:

Egg masses:	Batch of at least 10 single eggs (see fig.1).
Field:	Collection site: consider point 2 below.
Population:	ECB of three different fields/sites within a geographic homogeneous area.

To cover the genetic variability it is con sidered to collect **150 egg masses per field**. If you have less than 150 egg m asses, we can accept that field, if we end up with a total sum of 450 egg masses per population. **But we can not accept a field with less then 75 egg masses!**

Fields need to be free from any insecticide or other pest control methods (i.e. *Trichogramma*). For (first) baseline collections avoid any Bt m aize in the v icinity. For subsequent monitoring collections, a refuge zone/field can be sampled. In any case insects should **not** be taken from a Bt GM field!

Note: If once, it may occur that some insects may survive in Bt-fields, such a collection and case has to be considered in another procedure and will be a part of another investigation process. In such a case, please contact local Monsanto representatives.

2. Field selection and timing

Please identify fields with high ECB infestation. The distances between the three fields forming a population should be at least 50 km but not more than 250 km. Ask the farm owner for access prior to the collection! With respect to plant protection measurements consider point 1.

Insure collection of following data

- Field address, postal code, name of the area or bigger town the field is close to
- If available coordinates (GoogleEarth)
- Name of farmer
- Date of collection
- Field description: intensity of the attack, % of plants attacked
- Variety name/names in the field

Timing: for the collection of egg masses it is crucial to be out in the field to the right time.
If you are early, the number of egg masses will be low; if you're late, the eggs will be developed and first larvae are already hatched, or short before hatch. – To match the right time it is important to track the development of the **adult flight!** The important dates are the first flight and flight peak.
Female ECB begin laying eggs only a few days after eclosion. The flight-peak usually occurs 10–14 days after first flight. **That's the time to collect egg masses.**
To complicate the issue, both first flight and flight peak do not only differ between regions, but are weather-dependent and differ between the years. But in most European maize growing areas, there are experts monitoring the flight to coordinate insecticide treatments.
As rough estimation consider the first flight in the mid of June and egg masse in early July (German dates!). But as mentioned this varies between regions and years (up to two weeks earlier or later).

In regions with more than one generation per year, please collect the eggs of the first generation. The adult flight (and oviposition) is more synchronized than in the subsequent generation(s).

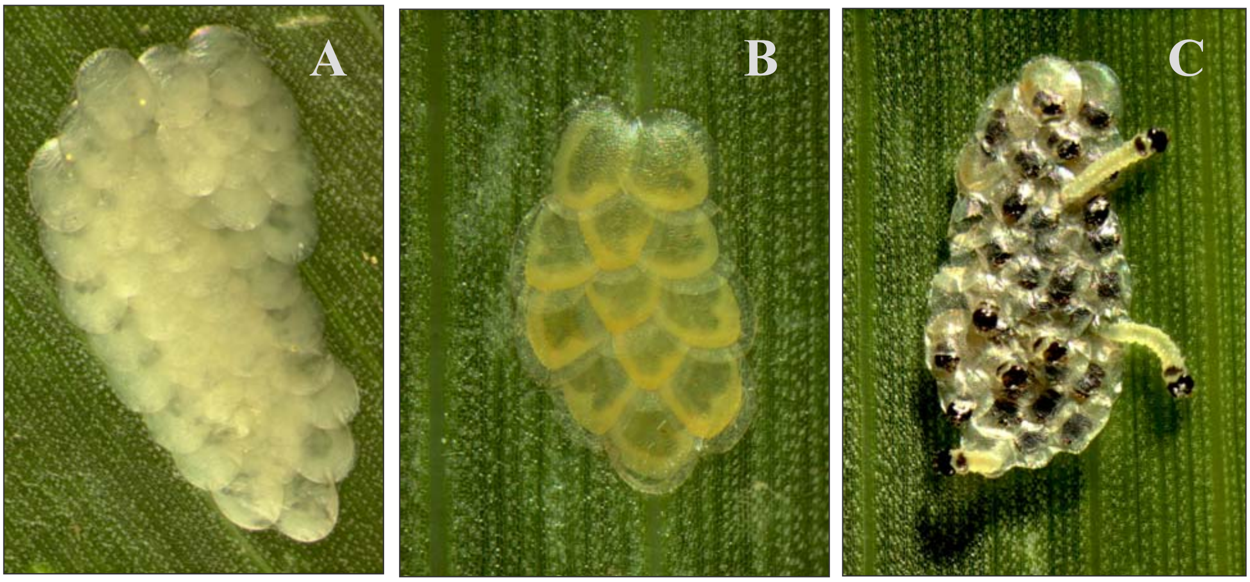

Figure 1: Fresh egg masses (A) are pale white and will be laid on upper maize leafs, mostly bottom side. Older eggs turn first yellow–orange (B) and will be black (visible head capsules) if the larvae are ready to hatch (C).

3. Collection procedure

3.1. Material per person

- 1 scissors
- 1 small box or bag for storing leaf peaces
  (you can use any plastic box, but pay attention, that it will fit in an insulation box)
- A moistened peace of filter paper or paper towel, placed in the box.
- Insulation box for transportation/storage, cooled with thermal packs: **not below 5°C!**

  Please let us know if you are in need of collection- and/or insulation boxes and we'll send it!

3.2 Collecting egg masses

- Please schedule collections for **Monday – Wednesday** to avoid shipments later than Wednesday! If you have to collect egg masses Thursday or Friday please store them cooled (again: not below 5° C!) over the weekend and we'll arrange a shipment for Monday of the subsequent week.
- If available, use a light trap, or most advanced a light trap-cage (Fig. 2). Both will ease the collection by "concentrating" ECB/egg masses around the trap/within the cage.
- Look for **white/young** eggs and cut them (with a leaf-margin of at least 1 cm) off
- Please don't collect black egg masses; because the larvae will be hatched before arrival and can not be used for bio-assays!
- Important is an appropriate labelling, especially if more than one field is collected and send at once (consider point 2 above)!
- If you have to store egg masses (i.e. over a weekend) 8–10°C are optimal. In any case avoid temperatures below 5 and above 20 °C . Don't store egg masses longer than five days cooled!

Figure 2: Light-trap cage (A). The trap is joined to the cage with a funnel. Large number of ECB will be attracted, fall down and lay eggs on the maize plants within the cage.

4. Transportation procedure

- BTL will arrange a pickup service by **World Courier,** so you don't have to pay for the shipment.
- To be able to arrange the shipment we do need the following information:
  - **When** will the collection boxes be ready for the shipment?
  - **Where** can the courier pick up the larvae: –> complete address of your office.
  - **The number and size** of boxes you want to ship.
  - **Name** of a contact person who hand out the boxes.
  - **Phone and Email** of the contact person (to enable World Courier to clarify details).
  - **Availability** of the contact person (daily working hours, lunch time …)
- A World Courier colleague will contact you to clarify any details and will come along to pick up the collection boxes.
- **Please don't forget** to send the required field-information (postal code …) as stated at the end of page one!
- If (for any reason) you arrange the shipment by yourself:
  –> select an air plane/over-night or express service
  –> inform us about the shipment and let us know the courier and tracking number

If there remain any questions or if you want to improve this SOP, please don't hesitate to contact us via email or phone!

Standard Operating Procedure
How to collect European corn borer larvae?

This document describes how to collect larvae of the European corn borer (ECB, *Ostrinia nubilalis* [Hübner 1796]). The samples will be used to assess and monitor the Bt-susceptibility of different European *Ostrinia*-populations.

Content **Page**

1. Definitions and basic requirements

To avoid misunderstandings or confusion please use the following terms only:

Field: Collection site: consider point 2 below.
Population: ECB of three different fields/sites within a geographic homogeneous area.

To cover the genetic variability it is considered to collect **300 larvae per field**. If you have less than 300 larvae, we can accept that field, if we end up with a total sum of 900 larvae per population. **But we can not accept a field with less then 100 larvae!**

Fields need to be free from any insecticide or other pest control methods (i.e. *Trichogramma*). For (first) baseline collections avoid any Bt maize in the vicinity. For subsequent monitoring collections, a refuge zone/field can be sampled. In any case insects should **not** be taken from a Bt GM field!

Note: If once, it may occur that some insects survive in a Bt-field, such a collection and case has to be considered in another procedure and will be a part of another investigation process. In such a case, please contact local Monsanto representatives.

2. Field selection process

In August, please identify fields with high ECB infestation. The distances between the three fields forming a population should be at least 50 km, but not more than 250 km. Best timing for collecting larvae is prior to the harvest. Ask the farm owner for access prior to the collection ! With respect to plant protection measurements consider point 1.

Insure collection of the following data

- Field address, postal code, name of the area or bigger town the field is close to
- if available coordinates (GoogleEarth)
- Name of farmer
- Date of collection
- field description: intensity of the attack, % of plants attacked
- variety name/names in the field

3. Collection procedure

3.1. Material per person

- 1 pruning shears
- 1 solid and sharp knife
- 1 soft forceps
- Boxes for storing larvae, filled with crumpled filter paper and/or bee boards

  You can use any plastic box usually used to freeze food, but please pay attention for a **tight closing** lid and provide small **ventilation holes**.
  We use boxes as shown below (volume 1.5 L, about 23 x 15.5 x 7.5 cm LxWxH)

  If necessary we will provide such boxes!

- 1 water bottle for moistening the filter paper
- Insulation box for transportation/storage (depending on weather condition)

3.2 Method of extracting larvae

- Please schedule collections for **Monday – Wednesday** to avoid shipments later than Wednesday! If you have to collect larvae Thursday or Friday please store them over the weekend and we'll arrange a shipment next Monday.
- Look for plants with boreholes and fresh faeces, cut them off as deep as possible (some times larvae are already below the ground!).
- Cut the maize stalk in pieces (length: approximately 15 cm)
- Cut the corn cob in pieces if infested (length: approximately 5 cm)
- Split each piece by using the knife/shear blade as lever (save your fingers!)
- Take one larvae per plant solely to avoid collecting siblings and to maximize the genetic variation of the collection.
- Transfer larvae into the box **(not more than 150 larvae per box!)** Ensure that the box is not exposed to direct sunlight during the collection process and moisten the filter paper/bee board as necessary.
- Some folded leaves can be added as larval food into the collection boxes. Avoid adding stalk peaces, which hurt free larvae in most cases.
  Important: please fix the content of collection boxes with crumpled filter paper to avoid damaging larvae during transportation.
- Assure that the lid of each box is closed carefully, the larvae will find any gap!
- Important is an appropriate labelling, especially if more than one field is collected and send at once!
- If you have to store larvae (i.e. over a weekend) 10–15°C are optimal. Avoid temperatures below 5 and above 20 °C.
- Potential bags to collect grain heads cut from the destroyed plants could be considered to show to the farmer our commitment to limit yield destruction.




4. Transportation procedure

- BTL will arrange a pickup service by **World Courier,** so you don't have to pay for the shipment.
- To be able to arrange the shipment we do need the following information:
  - **When** will the collection boxes be ready for the shipment?
  - **Where** can the courier pick up the larvae: –> complete address of your office.
  - **The number and size** of boxes you want to ship.
  - **Name** of a contact person who hand out the larvae.
  - **Phone and Email** of the contact person (to enable World Courier to clarify details).
  - **Availability** of the contact person (daily working hours, lunch time …)
- A World Courier colleague will contact you to clarify any details and will come along to pick up the collection boxes.
- World Courier provides insulation boxes, (**inner dimensions 23.5 x 23.5 x 18 cm WxLxH**) and cooling/gel packs. So you don't have to care about that.
- Two of the shown collection boxes fit perfectly in such a box. So if you don't use our ones please pay attention that your boxes will fit in the insulation box.
- **Please don't forget** to send the required field (postal code …) as stated at the end of page one!
- If (for any reason) you arrange the shipment by yourself:
  –> select an air plane/over-night or express service
  –> inform us about the shipment and let us know the courier and tracking number

If there remain any questions or if you want to improve this SOP, please don't hesitate to contact us via email or phone!

Standard Operating Procedure for the collection of the Mediterranean corn borer (*Sesamia nonagrioides*)

Definition of a population and basic requirements:

Each region is made up by at least 3 different fields. Field distances should be 50 Km as a minimum and not more than 250–300 km. Fields need to be free from any insecticide or other pest control methods. For the baseline first collection, Bt maize in the vicinity should be avoided. For the routine monitoring collection once the baseline has been established, larvae will be collected from refuge zone/fields, near Bt crops

A region can be considered complete with a minimum of 400 larvae in the 3 fields. Similarly, we should not take a field with less than 100 larvae. We take into account a mortality of 10%, so it would be necessary to collect a total of 450 larvae to ensure that we can use 400 larvae for the bioassay.

Field selection process:

Before September month (about 15th August for collections in the South of Spain), ask your local crew to help in the identification of fields having an optimal to maximum symptoms of MCB attack.
Ask the farm owner for access prior to the collection!
Best timing for collection of larvae is prior to the harvest.

Field collection procedure for MCB larvae:

Insure collection of:

- Field address, postal code, name of the area or bigger town the field is close to
- Name of farmer
- Date of collection
- field description: intensity of the attack, % of plants attacked
- variety name/names in the field
- each field insect has to be treated separately

Material per person:

- a solid and sharp knife
- soft and thin forceps
- boxes for storing larvae (we use 21x16x5cm), filled with rumpled moistened filter paper

Method for MCB:

- Look for plants with boreholes and fresh faeces, remove the leaves.

- Cut the stalk about 5 cm over the hole

- Split carefully the stalk with the knife following the line of the tunnel, using the knife as a lever, until you find the larva.

- Take one larvae per plant solely to avoid collecting siblings and to maximize the genetic variation of the collection. If both the stalk and cob show boreholes, choose better the stalk, it is easier.
- Transfer larvae into the storing box (not more than 50 larvae per box 21x16x5 cm; but the number will depend on the size of the box) containing stalk parts. Ensure that the box is not exposed to direct sunlight during the collection process and moisten the filter paper as necessary.
- During the collection larvae should be kept in ventilated boxes containing thin stalk parts. In the case of MCB, the plastic should be hard enough to avoid that the larvae eat the plastic and escape (for instance, polyethylene plastic of Tupperware® can be bored by MCB, do not use it!). A wire mesh for ventilation should be used for the same reason.
- If a box is filled up, add again some 10 ml water to the media (paper) and then place this box in the insulation box for transport. Assure to label the box appropriate if more than one population is collected and sent in one box!

Plastic box with MCB larvae and pupae, including leaves and small stalks for the shipment

Transportation procedure:

- Don’t store the boxes for longer than 2 days before they were sent to labs.
- For MCB shipments the same ventilated boxes of hard plastic described before should be used. They should be closed tightly by means of a rubber band and/or adhesive tape. Thin stalk parts and green leaves (which keep the suitable humidity inside the box) should be added for feeding and protection. Most of the larvae will bore the stalks and will be protected during the delivery. If necessary, add soft crumpled paper to fill out the box, avoiding that the movement crush the larvae.
- If you schedule a collection assure that the boxes will not be shipped later than **Wednesday!**
- A Friday collection should be avoided to do not put insects in difficult storage conditions. As well as a Friday parcel service delivery is not appropriate to avoid an over the weekend insect storage in transportation process.
- Do not expose the insect to hot condition or direct sun light! Cool temperatures are optimal.
- If you arrange the shipment by yourself select an air plane rapid shipment service, and please use the company recommended by Monsanto. Please inform about the arranged shipment and the tracking number.