**UNIVERSIDAD COMPLUTENSE DE MADRID**
FACULTAD DE CIENCIAS BIOLÓGICAS


# TESIS DOCTORAL

## Uso sostenible del maíz BT: optimización del manejo de la resistencia en noctuidos plaga

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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**Madrid 2019**

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# USO SOSTENIBLE DEL MAÍZ BT: OPTIMIZACIÓN DEL MANEJO DE LA RESISTENCIA EN NOCTUIDOS PLAGA

Tesis doctoral presentada por Ana Martín Camargo
para optar al grado de Doctora en Biología
por la Universidad Complutense de Madrid

Madrid, 2018

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*There is one quality that characterizes all of us who deal with the sciences of the earth and its life - we are never bored.*
***Rachel Carson***

*What you do makes a difference, and you have to decide what kind of difference you want to make.*
***Jane Goodall***

# *Agradecimientos*

Esta Tesis Doctoral se ha realizado en el laboratorio del Grupo de Interacción Planta-Insecto del Centro de Investigaciones Biológicas (CSIC), y no habría sido posible sin la financiación del Ministerio de Economía y Competitividad a través del Proyecto del Plan Nacional AGL2012-34289, la Ayuda para contratos predoctorales para la formación de doctores BES-2013-064987, y las ayudas a la movilidad predoctoral EEBB-I-2015-10396 y EEBB-I-17-11975.

En primer lugar, mi más sincero agradecimiento a mis directores de tesis, Pedro y Gema, por confiar en mí desde el principio pese a venir de un campo muy distinto, por guiarme en el mundo de la entomología aplicada y por estar siempre ahí para ayudarme con cada una de mis miles de dudas.

Gracias a toda la gente del grupo de interacción planta-insecto. A Félix y Pedro por resolver mis dudas y prestarme su ayuda en momentos críticos de picos de trabajo. A todos mis compañeros de poyata, desayunos y comidas, por ayudarme, enseñarme y hacerme reír tanto como para disfrutar hasta los días más duros de trabajo en el laboratorio. A Nuria por ser como es, una de las mejores personas que conozco, por ayudarme a no perder el norte y hacer que las tardes en el 201 fuesen casi psicodélicas. Gracias a Carol por ser la eficiencia personificada, por ayudarme con todos los ensayos y pasarme tu sabiduría de muestreos. A Miguelín por estar siempre de ese buen humor contagioso que levanta el ánimo del laboratorio, y por invitarme a planes a los que nunca puedo apuntarme. A Clara, por ser una gran amiga con la que sé que siempre puedo contar para hablar de tonterías o de cosas importantes. A Guille, por introducir los paint en nuestra vida y meter aire fresco en el labo. Gracias a Bea por ser mi apoyo en el amor a lo salado y entender lo que es bajar desde las montañas cada día. A Elena por estar siempre pendiente de mí, especialmente estos últimos meses tan duros, y estar dispuesta siempre a un café con charlas de viajes, conciertos o lo que se tercie. Gracias a Ana por ser una gran compañera que hace grupo, por esas galletas de chocolate y por ser la voz del horóscopo cada tarde. A Cristian por entender y compartir mi humor negro y por resolverme dudas que tendría que saber desde

primero de carrera sin perder (del todo) la paciencia. A Miguelón, gracias por discutir cada detalle de mis experimentos que te comentaba y realmente preocuparte por ayudarme, y por esos "¿¿perdona??" diarios que me encantaban. Muchas gracias a todos los que habéis continuado fuera del laboratorio: María, Gabriela, Matías y Enric, me habéis ayudado mucho tanto en la parte científica como en la personal, y espero que podamos trabajar de nuevo juntos en algún momento. A los patitos Marcos y Alberto gracias por haber metido un poco de juventud en nuestro grupo de viejales y haberos adaptado tan bien a nuestras locuras. También quiero agradecer a Matoya, parte indispensable de nuestro laboratorio, por tantos cafés, risas, celebraciones de cumpleaños por todo lo alto y por supuesto por tu gran trabajo en la organización de los viajes, que a menudo no dejaban mucho margen de maniobra.

Gracias también a los servicios del CIB, especialmente a Pablo en fotografía y Guillermo de estadística, por su paciencia y su disponibilidad siempre que les necesitaba, así como a Abel y Luis en el invernadero y a todo el equipo de Servicio Técnico.

Mi agradecimiento también a toda la gente que conocí en mis estancias en Estados Unidos. Gracias al Dr. Huang por facilitarme todo durante mi tiempo en Louisiana, por su disponibilidad que dura hasta hoy en día y por sus palabras de reconocimiento. A Ying, Binod, Fei, Emily y la gente del departamento de entomología que hicieron mi estancia allí muy interesante, científica y culturalmente. A todos mis amigos músicos y a mis profesores españoles, que me ayudaron a sacar una parte muy positiva de una experiencia complicada. Al Dr. Andow por ayudarme en el difícil análisis de datos de la $F_2$, a Becky por estar ahí para absolutamente todo, a Mercy, Karl, Hannah y toda la gente que me enseñó un pedacito de Minnesota y me integró en sus vidas durante dos meses.

Gracias también a mis amigos de El Escorial que han hecho más llevaderos los últimos meses de escritura (y todos los años de carrera, máster y doctorado). Gracias especialmente por esos juernes en los que descargamos tensión y nos ponemos al día cada semana, y por los paseos campestres que ayudan a ver que hay vida más allá de las plagas del maíz.

Por último, gracias a mi familia, a mis padres y a mi hermana, por aguantarme tantos largos años de ser una empollona, por animarme y apoyarme en cada paso que he dado desde que decidí estudiar biología y por creer siempre en mí y en que puedo lograr todo lo que me propongo. Gracias también a Stefano por ser un verdadero artista y ayudar a que esta tesis tenga color y gracia. A mi familia política y amigos catalanes por interesarse siempre por mis “gusanitos” y animarme a seguir en el difícil mundo de la investigación.

Y, como no, gracias a Marc, por quererme como soy, por cuidarme en mis múltiples dolencias, alimentarme con manjares, soportarme cuando estoy insoportable y saber siempre cuando necesito un paseo y una copa de vino para volver a poner los pies en la tierra y no perder la perspectiva de lo que realmente importa en la vida.

Muchas gracias a todos vosotros y a los que me haya dejado en el tintero. Esta tesis sería imposible sin todos vosotros.

# *Index*

# *Resumen*

El maíz Bt MON 810 es el único maíz modificado genéticamente cultivado en la Unión Europea (UE). Este maíz, que expresa la toxina Cry1Ab, controla eficazmente a una de las plagas clave en el área Mediterránea, *Sesamia nonagrioides*, y también es efectiva frente a la plaga secundaria *Mythimna unipuncta*, ambos noctuidos. La mayor parte del maíz Bt cultivado en la UE está en España (alrededor del 91%) y más concretamente en el Valle del Ebro, con una adopción superior al 50% desde 2007. La evolución de resistencia a la toxina es considerada la mayor amenaza para la sostenibilidad a largo plazo del maíz Bt, siendo mayor el riesgo de resistencia en zonas con alta adopción de maíz Bt, con la consiguiente presión de selección sobre las plagas. En este contexto, se considera el Valle del Ebro como el único "hotspot" para el desarrollo de resistencia en la UE. La estrategia dosis-alta/refugio (HDR en inglés), aplicada en España, es el sistema de manejo de la resistencia en insectos (IRM en inglés) más usado para retrasar el desarrollo de resistencia. Esta tesis ha estudiado varios factores asociados con el desarrollo de resistencia al maíz Bt, con el objetivo de optimizar las estrategias de IRM para diferentes noctuidos plaga.

El seguimiento de la resistencia al maíz Bt en *S. nonagrioides* se realiza anualmente evaluando poblaciones de campo procedentes de distintas zonas del Valle del Ebro. El primer objetivo de esta tesis fue estudiar en el Valle del Ebro la variabilidad inter- e intrapoblacional de la susceptibilidad a la toxina Cry1Ab en poblaciones de *S. nonagrioides* de zonas espacialmente próximas, para determinar si el seguimiento en dichas zonas podría optimizar los muestreos. No se han detectado poblaciones con una menor susceptibilidad a la toxina Cry1Ab, por lo que el seguimiento de la resistencia en *S. nonagrioides* no debería restringirse a zonas concretas de esta área. Por otro lado, se han estudiado las diferencias en la susceptibilidad a la toxina Cry1Ab entre poblaciones de *M. unipuncta* de áreas con alta adopción de maíz Bt (Valle del Ebro) y áreas donde el maíz Bt nunca se ha cultivado (Galicia). La susceptibilidad a la toxina Bt obtenida fue baja y similar en ambas poblaciones, sugiriendo que esta plaga ha estado poco expuesta al maíz Bt

en el Valle del Ebro, dado el alto potencial de desarrollo de resistencia al maíz Bt que ha mostrado en el laboratorio.

Recientemente se ha modelizado la evolución de resistencia en poblaciones de *S. nonagrioides* del Valle del Ebro, basándose en parámetros biológicos de esta plaga que son relevantes para el desarrollo de resistencia. El segundo objetivo de la tesis aborda dos de estos parámetros. Primero, se estudió la oviposición y el desarrollo larvario de *S. nonagrioides*, en relación al maíz, en dos plantas huésped cultivadas y cuatro silvestres. Los resultados indican que el maíz es la planta más adecuada para el desarrollo de este noctuido. Además, las larvas completaron su ciclo biológico en arroz, sorgo cultivado y sorgo silvestre, pero en ellos se encontró mayor mortalidad, retraso en el desarrollo y menor peso. Consecuentemente, estas plantas no servirían como refugios no estructurados de maíz Bt. Segundo, se estimó la fecundidad de las hembras en plantas de maíz en antesis (VT) y maduración de grano lechoso (R4) por ser los estados fenológicos que coinciden temporalmente con adultos de la segunda y tercera generación, respectivamente. La menor fecundidad obtenida en plantas R4 frente a las plantas VT sugiere que la fenología de las plantas es determinante en la mayor o menor fecundidad de *S. nonagrioides*, lo cual tiene repercusiones importantes para el modelo.

El tercer objetivo de la tesis ha sido determinar la evolución de la frecuencia de alelos de resistencia en poblaciones de *S. nonagrioides* del Valle del Ebro, utilizando la técnica de la "$F_2$ screen". El modelo de evolución de resistencia incluía una estima de 2004-2005. Los resultados indican que esta frecuencia ha aumentado ligeramente, pero al ritmo predicho por el modelo. Además, es la primera vez que se detecta un alelo de resistencia al maíz Bt en la UE. La versión actualizada del modelo predice fallos de control del maíz Bt en esta área en 2047, lo cual sugiere que sería necesario el total cumplimiento de los refugios para asegurar la sostenibilidad a largo plazo del maíz Bt, dado que la frecuencia de alelos de resistencia (0.0036) triplica el valor recomendado (<0.001) para que la estrategia HDR funcione.

El conocimiento de la herencia de la resistencia es fundamental para estudiar el desarrollo de resistencia. Además, determinar la base genética de la resistencia en

poblaciones de distintas áreas es clave para evaluar si la resistencia comparte un origen común en las poblaciones. El cuarto objetivo fue estudiar la herencia de la resistencia al maíz Cry1F en dos poblaciones resistentes de *Spodoptera frugiperda* procedentes de Puerto Rico (PR) y Florida (FL). Los resultados indican que en ambas poblaciones la resistencia se hereda como un gen recesivo y autosómico, y que las dos colonias comparten un locus de resistencia. Sin embargo, estudios recientes han revelado que la mutación responsable de la resistencia en PR no está en FL, sugiriendo que PR y FL no comparten igual gen de resistencia. La resistencia, por tanto, habría evolucionado de modo independiente en ambas poblaciones, lo que indica un alto potencial de *S. frugiperda* para desarrollar resistencia a toxinas Cry. Por otra parte, se ha sugerido que las poblaciones africanas de *S. frugiperda* probablemente fueran resistentes al maíz Cry1F antes de llegar al continente. Si estas poblaciones potencialmente resistentes llegasen a Europa, el maíz MON 810 podría no controlar eficazmente esta plaga.

Esta tesis proporciona datos originales para la optimización de los planes actuales de IRM a distintas plagas de noctuidos del maíz. Asimismo, aportan información esencial para la toma de decisiones de las autoridades competentes europeas en la elaboración y mejora de los protocolos de seguimiento de la resistencia a cultivos Bt.

# *Abstract*

Genetically modified maize expressing insecticidal toxins of *Bacillus thuringiensis* (Bt maize) is a valuable tool for the control of insect pests. In the European Union (EU) the only cultivated Bt maize varieties are those derived from the transformation event MON 810. These varieties express the toxin Cry1Ab, which controls efficiently the primary pest *Sesamia nonagrioides* and is also effective against the secondary pest *Mythimna unipuncta*, both of which are noctuids. Most of the Bt maize cultivated in the EU is in Spain (around 91%), and more specifically in the Ebro Valley, where over 50% of all maize grown since 2007 is Bt. Resistance evolution is considered as the greatest threat for the long-term sustainability of Bt maize, so that areas where adoption of this technology is high and pest pressure is intense are under a higher risk of resistance development. In this context, the Ebro Valley has been considered as the only hotspot of resistance evolution in the EU. The high-dose/refuge (HDR) strategy applied in Spain is the most commonly adopted insect resistance management (IRM) plan to delay resistance development. This thesis has studied several factors associated with resistance development to Bt maize, with the aim of optimizing IRM strategies for noctuid pests of this crop.

In the EU resistance monitoring is carried out annually considering *S. nonagrioides* populations from different zones within the Ebro Valley. The first objective of this thesis was to assess the variation in susceptibility to Cry1Ab toxin between and within closely sampled populations of the Ebro Valley, to evaluate whether focusing monitoring on those populations could optimize the sampling strategies. No populations with a lower susceptibility to the toxin Cry1Ab have been detected, suggesting that resistance monitoring of *S. nonagrioides* should not concentrate in particular zones of this area. On the other hand, we studied whether susceptibility to the toxin Cry1Ab varied between populations of *M. unipuncta* from areas of high adoption of Bt maize (Ebro Valley) and areas where Bt maize has never been sown (Galicia). The susceptibility to the Bt toxin was low in both populations, suggesting that *M. unipuncta* has had little significant exposure to Cry1Ab in the Ebro Valley, since

this pest has shown a high potential to develop resistance to Bt maize in the laboratory.

Resistance evolution has recently been modeled for *S. nonagrioides* populations in the Ebro Valley, using information on several biological parameters known to affect resistance development. The second objective of this thesis was to improve this model by providing updated and more accurate data on two of these variables. Firstly, we studied the suitability of two cultivated and four wild plants, compared to maize, for larval development and oviposition of *S. nonagrioides*. The results indicate that maize is the most suitable plant for this noctuid. Apart from maize, larvae could only complete their cycle in sorghum, rice and johnsongrass, but they experienced higher mortality, delayed development and lower weight in these hosts. Therefore, they do not comply with the requirements to serve as non-structured refuges of Bt maize. Secondly, female oviposition performance in the phenological stages of maize encountered by $2^{nd}$ (VT, anthesis) and $3^{rd}$ (R4, dough stage) generation adults was estimated and incorporated to the resistance evolution model. Interestingly, the lower fecundity recorded in R4 plants with regards to VT plants suggests that plant phenology greatly affects oviposition performance in *S. nonagrioides*, which has relevant implications for the model.

The third objective was to obtain an updated estimate of the frequency of resistance alleles of *S. nonagrioides* in the Ebro Valley, which is a key parameter of the resistance evolution model. The results of the "$F_2$ screen" indicate that the frequency has increased slightly from the estimate obtained in 2004-2005, although not faster than predicted by the model. Furthermore, this is the first report of the detection of a resistance allele to a Bt crop in the EU. The updated version of the model predicts that control failure will happen in 2047, suggesting that full compliance with refuge requirements of the HDR strategy is essential to ensure the long-term sustainability of Bt maize in the control of *S. nonagrioides* in the Ebro Valley, since the frequency of resistance alleles (0.0036) triples the recommended value (<0.001) for this approach to work.

Inheritance of resistance is known to affect resistance development. Additionally, establishing the genetic basis of resistance in populations from different areas is

crucial to understand whether resistance shares a common origin in the colonies. The fourth objective was to study the inheritance of resistance to Cry1F maize in two resistant populations of *Spodoptera frugiperda* from Puerto Rico (PR) and Florida (FL). The results showed that resistance is inherited similarly in both populations, as a recessive, autosomal and single gene, and that both colonies share a resistance locus. However, recent studies have reported that the mutations responsible for resistance in PR are not present in FL, indicating that the two tested populations do not share the same resistance allele. These results suggest resistance evolved independently in PR and FL, pointing out to a high potential of *S. frugiperda* to develop resistance against Bt toxins. Additionally, *S. frugiperda* populations in Africa were probably resistant to Cry1F maize prior to their arrival to the continent. If these potentially Cry1F-resistant populations arrived to Europe, MON 810 maize would likely be ineffective if used as the main control strategy for this pest.

In summary, this thesis provides novel information that could help to optimize the ongoing IRM plans of different noctuid pests of maize and at the same time might contribute to support the European competent authorities on defining more precise monitoring protocols.

# *Abbreviations*

| | |
|---|---|
| **α** | Significance level |
| **ºC** | Centigrade degrees |
| **$\chi^2$** | Chi-square |
| **Ø** | Diameter |
| **µg** | Micrograms |
| **µl** | Microliters |
| **Ala** | Alanine |
| **ANOVA** | Analysis of variance |
| **Arg** | Arginine |
| **Asp** | Aspartic acid |
| **CABI** | Centre for Agriculture and Biosciences International |
| **cm** | Centimeters |
| **$cm^2$** | Square centimeters |
| **cv** | Cultivar |
| **Cys** | Cysteine |
| **df** | Degrees of freedom |
| **dw** | Dry weight |
| **EFSA** | European Food Safety Authority |
| **EUROPHYT** | European Union Notification System for Plant Health Interceptions |
| **$F_n$** | Generation number n |
| **FAO** | Food and Agriculture Organization of the United Nations |
| **g** | gram |
| **Glu** | Glutamic acid |
| **Gly** | Glycine |
| **h** | Hours |
| **ha** | Hectares |
| **His** | Histidine |
| **INE** | Instituto Nacional de Estadística |
| **Ile** | Isoleucine |
| **kDa** | Kilodaltons |
| **km** | Kilometers |
| **L** | Liters |
| **L:D** | Light:Dark |
| **L1** | First instar |
| **L2** | Second instar |
| **L3** | Third instar |

| | |
|---|---|
| **L4** | Fourth instar |
| **L5** | Fifth instar |
| **L6** | Sixth instar |
| **Leu** | Leucine |
| **Lys** | Lysine |
| **m** | Meters |
| **mM** | Millimolar |
| **MARM** | Ministerio de Medio Ambiente, Medio Rural y Marino |
| **min** | Minutes |
| **ml** | Milliliters |
| **mm** | Millimeters |
| **Met** | Methionine |
| **N/n** | Number of samples or replicates |
| **ng** | Nanograms |
| **nm** | Nanometers |
| **Phe** | Phenylalanine |
| **Pro** | Proline |
| **rh** | Relative humidity |
| **rpm** | Revolutions per minute |
| **SD** | Standard deviation |
| **SE** | Standard error |
| **Ser** | Serine |
| **Thr** | Threonine |
| **Tyr** | Tyrosine |
| **US** | United States of America |
| **v/v** | Volume/volume |
| **Val** | Valine |

# I. General introduction

The combination of a rapidly growing world population and a shift in dietary habits in developing countries towards a higher consumption of animal-based products exerts a remarkable pressure on agriculture for an increase in productivity that guarantees food security (FAO, 2009; Popp *et al*., 2013). An effective control of pests, which cause important yield losses in cropping systems around the world – estimated in over 10% in five major crops at the beginning of this century (Oerke and Dehne, 2004) –, is key to boost crop productivity without further increasing land and water use (Godfray *et al*., 2010).

During the second half of the last century, especially after the advent of synthetic insecticides in the 1940s, the control of insect pests relied heavily on the use of chemical compounds (Casida and Quistad, 1998). However, owing to the concerns arising from the negative effects that the intensive use of insecticides has been reported to have on the environment and human health (Geiger *et al.*, 2010; Pimentel and Burgess, 2014; Park *et al.*, 2015), a shift towards the development and utilization of more specific, safer and environmentally friendly crop protection methods has occurred in the last decades (Popp *et al.*, 2013). In this vein, Integrated Pest Management (IPM) is considered as the most suitable approach for sustainable crop production (FAO, 2018). This strategy combines multiple control methods – such as chemical and biological control, cultural practices and the use of naturally host-plant resistant varieties – with the aim of keeping pest populations below economic injury levels in an economically and ecologically safe manner (Stern *et al.*, 1959; FAO, 1968). A new tool for pest control became available in 1996, when genetically modified crops for insect resistance (Bt crops) were first commercialized. Bt crops are obtained through the genetic modification of crops to express insecticidal toxins of the bacterium *Bacillus thuringiensis*. They represent a form of host plant resistance that is fast, allows for a wider range of traits to be expressed and leads to plants that are highly resistant to pests, and their adoption has implied a shift in insect control towards the use of more specific measures. Insect-resistant crops are generally considered to work within an IPM framework, in combination with other control

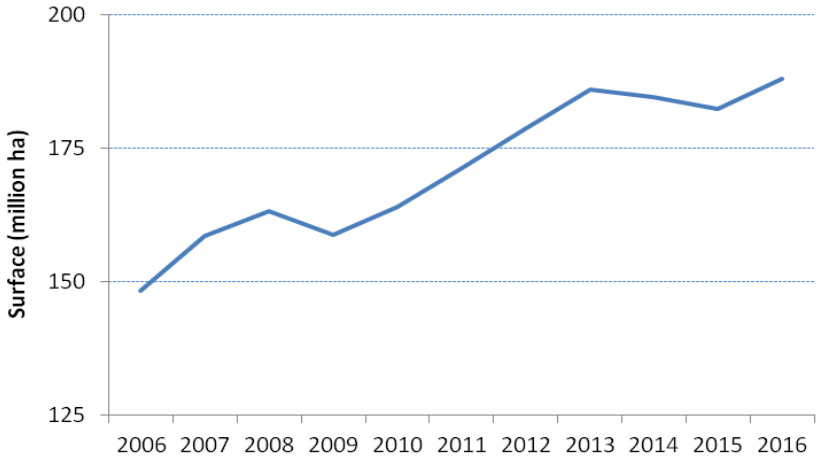
strategies like host plant resistance and conservation biological control (Hellmich *et al.*, 2008; Kennedy, 2008; Shelton *et al.*, 2008).

## *1.1. Maize production*

Maize, *Zea mays* L. (Poales: Poaceae), is a staple crop and the basis of the diet of millions of people in the world, especially in developing countries. It is an annual grass native from Mesoamerica, where it was domesticated from the wild species teosinte around 11,000 years ago (Matsuoka *et al.*, 2002). During the following millennia, maize spread slowly throughout the rest of the Americas, diversifying in hundreds of varieties as it adapted to the different environmental conditions it encountered. The crop was introduced in Europe soon after the arrival of the Europeans to America in 1492, and it rapidly expanded throughout the continent and to the rest of the world (Dubreuil *et al.*, 2006; Mir *et al.*, 2013).

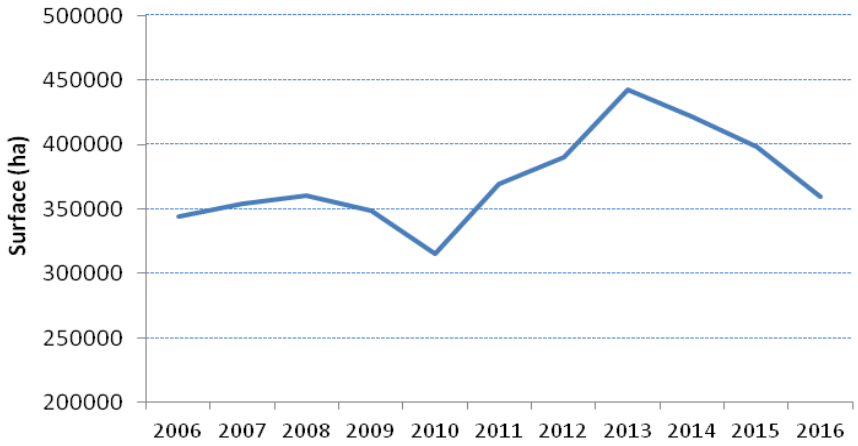
Together with rice and wheat, maize is one of the three most important crops for human consumption at a global scale, although this crop is also used as feed for livestock, in the chemical industry and to produce biofuels, among other uses (Shiferaw *et al.*, 2011). The world surface of this crop has increased remarkably in the last decade and it exceeded 187 million hectares in 2016 (FAOSTAT, 2018) (Fig. 1.1).

**Figure 1.1.** World surface (million ha) of maize between 2006 and 2016. Source: FAOSTAT, 2018.



In Spain, maize is the most economically important summer cereal crop, with nearly 4 million tons of grain maize produced in 353,240 ha in 2016 (Fig. 1.2).

**Figure 1.2.** Surface (ha) of maize in Spain between 2006 and 2016. Source: INE, 2018.




## *1.2. Lepidopteran pests of maize*

Maize is attacked by a wide range of insect pests, including many lepidopteran species that cause significant damage to maize crops worldwide. A great part of these pests are moths (order Lepidoptera) belonging to the families Crambidae and Noctuidae (Table 1.1), whose larvae feed on different parts of the plant. This, along with the fact that many lepidopterans are susceptible to insecticidal toxins from *B. thuringiensis*, has made them the main targets of insect-resistant maize (Bt maize).

For this thesis, we have selected three noctuid pests of maize according to their actual or potential economic importance in the European Union (EU). In first place, the Mediterranean corn borer, *Sesamia nonagrioides* Lefèbvre, is the most prevalent and harmful pest of maize in areas of the Mediterranean basin (Anglade, 1972; Castañera, 1986). The second one, the true armyworm *Mythimna unipuncta* Haworth (also known as *Pseudaletia unipuncta*), is a secondary pest of maize that causes severe damage to maize crops sporadically in some areas of the EU, including Spain (López *et al.*, 2000). Additionally, this pest has proved to have a low susceptibility to Bt maize, and a high potential to develop resistance against it

**Table 1.1.** Lepidopteran pests of maize worldwide.

| Family | Species | Area of economic damage |
|---|---|---|
| Crambidae | *Chilo partellus* Swinhoe | Asia, Africa |
| | *Diatraea crambidoides* Grote | North America |
| | *Diatraea grandiosella* Dyar | North America |
| | *Diatraea saccharalis* Fabricius | America |
| | *Ostrinia furnacalis* Guenée | Asia |
| | *Ostrinia nubilalis* Hübner | Europe, North America |
| Noctuidae | *Agrotis ipsilon* Hufnagel | Worldwide |
| | *Agrotis segetum* Denis & Schiffermüller | Europe, Asia, Africa |
| | *Busseola fusca* Fuller | Africa |
| | *Helicoverpa armigera* Hübner | Worldwide except North America |
| | *Helicoverpa zea* Boddie | America, China |
| | *Mythimna separata* Walker | Asia, Australia |
| | *Mythimna unipuncta* Haworth | Worldwide except Oceania |
| | *Papaipema nebris* Guenée | North America |
| | *Sesamia calamistis* Hampson | Africa |
| | *Sesamia cretica* Lederer | Mediterranean basin, Middle East and Africa |
| | *Sesamia inferens* Walker | Asia |
| | *Sesamia nonagrioides* Lefèbvre | Mediterranean basin, Africa |
| | *Spodoptera frugiperda* J.E. Smith | America, Africa |
| | *Striacosta albicosta* Smith | North America |

(Eizaguirre *et al.*, 2010; Pérez-Hedo *et al.*, 2012; González-Cabrera *et al.*, 2013). Finally, the fall armyworm *Spodoptera frugiperda* J.E. Smith, native from tropical and sub-tropical America, has been included because it has recently arrived to

Africa and rapidly spread throughout the continent (Goergen *et al.*, 2016), raising serious concerns that this pest could expand from Africa to the EU (CABI, 2017; EFSA Panel on Plant Health, 2017), where it is listed as a quarantine pest (EPPO, 2018).

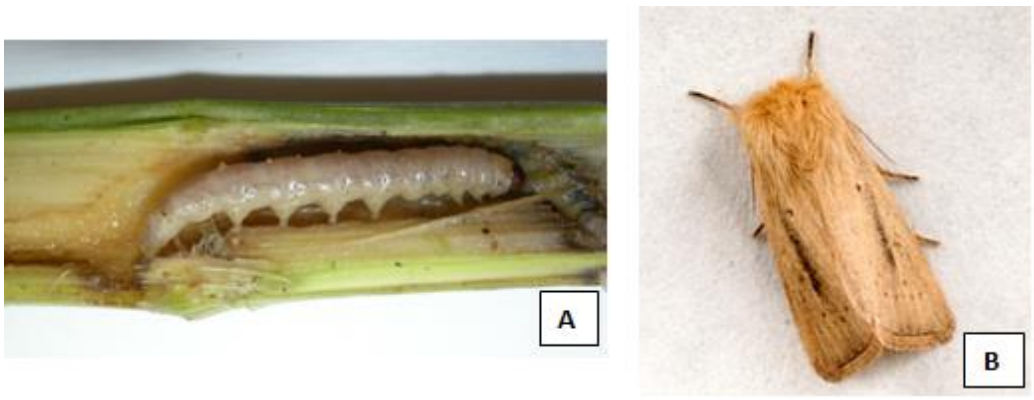
### 1.2.1. *Sesamia nonagrioides*

#### 1.2.1.1. Distribution and description

This species, commonly known as the Mediterranean corn borer, is widely distributed across different regions of the Mediterranean basin south of the 45ºN parallel, including Southern Europe (the Iberian Peninsula, Italy, Greece, France), the Middle East (Turkey, Syria, Iran) and countries in North and Western Africa (Morocco, Ghana) (Eizaguirre and Fantinou, 2012).

Larvae of *S. nonagrioides* are pale yellowish and up to 30-40 mm long when they reach the last larval instar (Anglade, 1972). Pupae are semi-cylindrical and brown, and they are around 20-25 mm long. Adults present a first pair of light brown-colored wings with small dark dots, and a second pair of whitish wings, with an average wingspan of 30 to 40 mm. Female antennae are filiform, whereas those of males are pectinated. Eggs are spherical and yellowish when they are laid, but they turn into a creamy orange as they mature (Fig. 1.3).

**Figure 1.3.** *Sesamia nonagrioides.* Boring behavior of the larvae inside a maize stem (A) and adult female (B).



#### 1.2.1.2. Biology and ecology

The Mediterranean corn borer is considered a polyphagous species that feeds predominantly on maize, although it has been reported in a wide range of plants mainly belonging to the Poaceae and Typhaceae families, including cultivated plants like sugarcane, sorghum and rice, and gramineous weeds (Galichet, 1982; Larue, 1984; Le Rü *et al.*, 2006; Dimou *et al.*, 2007). In northeast Spain it is functionally monophagous on maize because weeds are well-managed in maize fields and other crop host plants are rare (Castañera *et al.*, 2016). It completes a variable number of generations per year depending on the latitude, ranging from two generations in northwest Spain (Cordero *et al.*, 1998), to two generations and a partial third one on maize in southern France and northeast Spain (Alfaro, 1972; Anglade, 1972; Galichet, 1982; Eizaguirre *et al.*, 2002) and up to four in Morocco and Portugal (Hilal, 1977; Figueiredo and Araújo, 1990).

Most of the life cycle of *S. nonagrioides* takes place inside the stem of the maize plants. Gravid females lay their eggs in the leaf sheath of the plants and, soon after the eggs hatch, first instar larvae tunnel inside the stem, where they feed for the rest of the larval stage (Larue, 1984; Farinós *et al.*, 2004). Non-diapausing larvae typically have six instars. Short day length in early August induces diapause in 1$^{st}$ and 2$^{nd}$ instar larvae, in a process that is influenced by temperature and maize phenology (Eizaguirre and Albajes, 1992; Eizaguirre *et al.*, 1994; 2002). Individuals overwinter in maize stubbles as diapausing larvae that feed and undergo several supernumerary molts, and adult emergence takes place from April to June of the following year (Gadenne *et al.*, 1997; López *et al.*, 2001). After emergence, females usually stay in the field where they emerge and they do not usually disperse to other fields until after they have mated (López *et al.*, 1999), whereas males disperse from their emergence fields in their search for females and cover distances reported in up to 400 m (Albajes *et al.*, 2004).

#### 1.2.1.3. Economic importance and management

The Mediterranean corn borer, considered as the most harmful pest of maize in the Mediterranean basin, damages maize plants in different ways. Severe yield

losses, due to larval damage by the first and second generations, have been reported in Spain (Alfaro, 1955). Larvae tunnel inside the stems, restricting the ability of the plants to absorb nutrients and water from the soil, thus leading to lower productions. Additionally, the hollow stalks are more vulnerable to harsh weather conditions and might be broken by the wind or the rain (Delgado de Torres, 1929; Alfaro, 1955). Finally, fungal pathogens often enter maize plants through wounds inflicted in the stem by borers like *S. nonagrioides* (Avantaggiato *et al.*, 2003; Papst *et al.*, 2005). Some of these pathogens produce mycotoxins that can be hazardous for animal and human health, either in a chronic or in an acute way (Bennet and Klich, 2003; Reddy *et al.*, 2010), which has led many countries to establish legal limitations to the mycotoxin content in food products (Wu, 2006; van Egmond *et al.*, 2007).

Traditionally, chemical control of *S. nonagrioides* has been difficult due to the endophyte behavior of the larvae. The efficiency of chemical sprays against this boring pest is greatly limited by the narrow period of time in which larvae can be reached by the compounds before they tunnel into the stem, which may be as low as just a few hours (González-Núñez *et al.*, 2000). Cultural practices such as the removal of crop debris after harvest to eliminate the resources that could be used by overwintering larvae have also been implemented by farmers (Albajes *et al.*, 2002). Host plant resistance appears to be a potentially effective and environmentally friendly means of control. The hydroxamic acid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), present in maize and other cereals, has been implicated in the resistance of maize to *S. nonagrioides* (Gutiérrez and Castañera, 1986; Ortego *et al.*, 1998; Santiago *et al.*, 2017). At present, Bt maize hybrids that express high doses of the toxin Cry1Ab throughout the growing season are the most efficient control strategy in areas with high infestation levels of *S. nonagrioides* (González-Núñez *et al.*, 2000; Castañera *et al.*, 2016).

### *1.2.2. Mythimna unipuncta*

#### 1.2.2.1. Distribution and description

This species, commonly designated as the true armyworm, is a defoliating pest native from the Americas that is now widely distributed in North, Central and

South America, southern Europe, western Asia and central Africa (Poitout and Bues, 1983; CABI, 2018a).

Larvae of *M. unipuncta* can range in color between light greenish and black, and they display dark green, brown and orange stripes along the sides of their bodies, and a speckled yellowish head capsule. Fully developed larvae can be up to 35 mm long. True armyworm pupae are cylindrical and brown and they develop inside a silk case below the ground. Adults are light brown moths, with light-colored mottled forewings that display a white spot in the center and greyish hindwings, and a wingspan that can be up to 40 mm. *Mythimna unipuncta* eggs are spherical and their color turns from white-yellowish when they are laid to grey before they hatch (Cook *et al.*, 2004; Capinera, 2006) (Fig. 1.4).

**Figure 1.4.** *Mythimna unipuncta*. Larva feeding on a maize leaf (A) and adult (B).


#### 1.2.2.2. Biology and ecology

The true armyworm is a polyphagous pest whose larvae attack a wide range of plant species, including both feral species like clover (*Trifolium spp.*) and agricultural crops like maize, barley or wheat (Vieira and Tavares, 1995; Vieira *et al.*, 2003). *Mythimna unipuncta* has been reported to present 4 generations per year in Northeastern Spain, although this number ranges between 2 and 6 depending on the location (López *et al.*, 2000; Rosa and Simões, 2004). This species commonly presents a strong migratory behaviour, in order to avoid the negative effects that extreme temperatures (above 30ºC and below 0ºC) have on

this insect (McNeil, 1987). These migrations can cover thousands of kilometers (Bues *et al.*, 1986), as indicated by the presence of *M. unipuncta* in the Azorean Islands, where this pest species likely arrived in its migration from either North America or continental Europe (Vieira *et al.*, 2003).

Adults of *M. unipuncta* need to feed on nectar or other sugary products in order to mate and oviposit successfully (Capinera, 2006). Mated females lay their eggs in rows, which they place in tight spots such as the gap between the leaf sheath and the stem (Esteban and Balduque, 1983), often in weeds present in maize fields (Naïbo, 1984). Upon emergence, larvae feed on the leaves of the host plant, first avoiding the ribs, and then, when they get larger, devouring the whole foliar tissue (Poitout and Bues, 1983). The larval stage usually comprises six instars, the older of which are highly mobile and can disperse to adjacent plants to avoid competition with other larvae or to feed on more suitable hosts (Eizaguirre *et al.*, 2010; López *et al.*, 2017). When 6$^{th}$ instar larvae are close to pupation, they move downwards in the plant towards the soil, bury themselves and pupate. Adults emerge around 10 days later, and mating begins between 1 and 3 days after adult emergence (Capinera, 2006).

#### 1.2.2.3. Economic importance and management

Population dynamics of this species involve long periods of low pest densities followed by temporary outbreaks that can cause severe damage in maize crops, as reported in regions of North America and Europe (López *et al.*, 2000; Schaafsma *et al.*, 2007). Bt maize varieties derived from the events Bt 176 and MON 810, which express the toxin Cry1Ab, have been proved to be protected from damage by *M. unipuncta* compared to their conventional counterparts (Schaafsma *et al.*, 2007). However, a low susceptibility to the toxin and a high potential to develop resistance against these varieties of Bt maize have been reported in *M. unipuncta* populations from the Ebro Valley (Eizaguirre *et al.*, 2010; Pérez-Hedo *et al.*, 2012; González-Cabrera *et al.*, 2013; García *et al.*, 2015), the area in the EU where Bt maize adoption is highest (EFSA Panel on GMO, 2012).

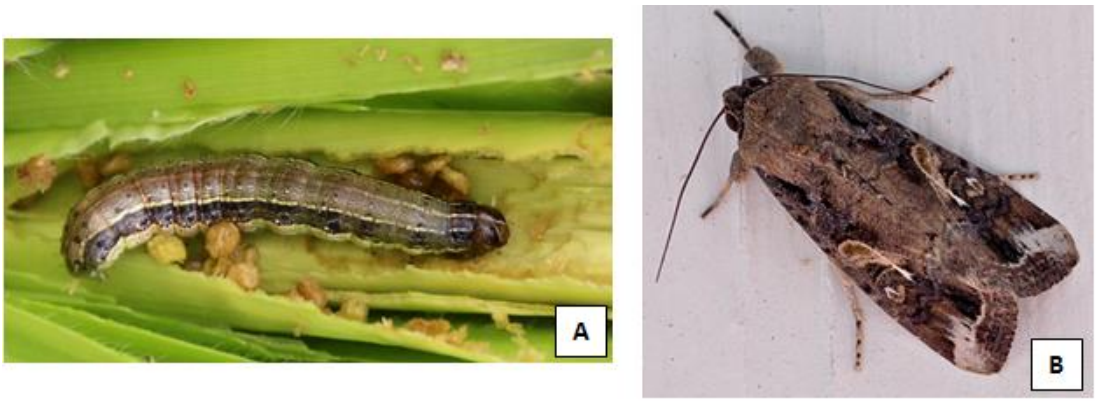
### 1.2.3. *Spodoptera frugiperda*

#### 1.2.3.1. Distribution and description

The fall armyworm, *Spodoptera frugiperda*, is widely distributed across North, Central and South America (Luginbill, 1928; CABI, 2018b), and it has recently become a problem in Africa, where outbreaks were first detected in 2016 and the pest rapidly expanded to most of the countries south of the Sahara (Goergen *et al.*, 2016; CABI, 2017).

Larvae of this species are usually light to dark green with longitudinal stripes and dark spots in their dorsum, but they can vary in color from yellowish to dark brown. Larvae in the late instars are easily recognizable by a dark colored inverted Y in their head capsule. Pupae, which burrow in the soil, are reddish brown and about 15-20 mm long. Adults present sexual dimorphism in this species, with male forewings generally gray and brown with white spots at the center and tip, whereas female forewings are colored more uniformly in light gray and brown. The second pair of wings is silver-whitish and presents a dark line in the border in both sexes. Eggs are spherical and they turn from bright green when they are laid to brown when they are about to hatch (Capinera, 2017; CABI, 2018b) (Fig 1.5).

**Figure 1.5**. *Spodoptera frugiperda*. Larva feeding on a maize whorl (A) and adult (B). Copyright: FAO (A) and Charles Schurch Lewallen (B).



#### 1.2.3.2. Biology and ecology

This pest species is native from tropical and sub-tropical climates in America, and it has a low tolerance to subzero temperatures (Nagoshi and Meagher, 2004). Thus, the number of generations occurring every year in a given area depends on the latitude and the climate, with overlapping generations all year round in tropical areas like some Caribbean islands, northern Brazil or southern Florida, and just one generation in areas of temperate climate like the northernmost states in continental US (Capinera, 2017). Infestations in regions where the insect cannot survive the winter arise from the migration of adults from the overwintering spots, located in tropical areas where the temperature rarely drops below 10ºC (Mitchell *et al.*, 1991; Nagoshi *et al.*, 2012). This long-distance migratory pattern is likely supported by the weather fronts that would carry adults northwards every year (Sparks, 1979). This species has been reported to feed on over a hundred species of plants, although it has a clear preference for gramineous like maize, sorghum and Bermuda grass (Johnson, 1987; Yu *et al.*, 2003).

Female adults usually lay their eggs in clusters of a few hundred on the back of leaves of maize or other grasses (Abrahams *et al.*, 2017). The larval stage usually comprises six instars that feed on foliar tissue, often burrowing into the whorl of young plants where they produce a distinct pattern of perforations and limit their growth potential (Buntin, 2008). Larvae are highly cannibalistic and mobile, and disperse to other plants in peaks of infestation (Chapman *et al.*, 1999; Carroll *et al.*, 2006). Pupation occurs in the soil, just a few centimeters below ground level, and the whole cycle lasts an average of 30 days, depending on the temperature (Capinera, 2017).

#### 1.2.3.3. Economic importance and management

The fall armyworm is a key pest of maize in some tropical areas of America where it is present throughout the year and several crops are sown every season (Sparks, 1979; Siebert, 2008), causing severe yield losses if left untreated (Hruska and Gould, 1997). Additionally, this species has recently become a serious threat to several crops in tropical and subtropical areas of Africa (Goergen *et al.*, 2016;

CABI, 2017). Fall armyworm infestations have been traditionally controlled by chemical insecticides (Carvalho *et al.*, 2013). Some Bt toxins have proved to be effective against *S. frugiperda*, including Cry1F, Cry1Ab and Vip3 (Buntin, 2008; Siebert, 2008; Bernardi *et al.*, 2014). Therefore, Bt maize plants that express one or several of these proteins have been deployed to control insect damage by this pest (Buntin, 2008; Siebert, 2008). However, field-evolved populations resistant to Bt maize expressing different toxins (Cry1Ab and Cry1F) have been reported in different countries (Storer *et al.*, 2010; Huang *et al.*, 2014; Farias *et al.*, 2014a; Omoto *et al.*, 2016; Chandrasena *et al.*, 2017), making *S. frugiperda* the pest species in which a higher number of cases of field-evolved resistance has been detected (Tabashnik and Carrière, 2017).

*Spodoptera frugiperda* is not present in the EU, where it is considered a quarantine pest (EPPO, 2018). However, there is a growing concern that this pest is now more likely to arrive to Europe, owing to the increasing number of detection cases of the pest in imported goods in the last years, the colonizing potential of this species and its recent and rapid expansion throughout the African continent (Johnson, 1987; EFSA Panel on Plant Health, 2017; EUROPHYT, 2018) (Fig. 1.6). The abiotic and biotic conditions of the southernmost part of Europe, including Spain, with warm temperatures and availability of host plants, would be suitable for the establishment of fall armyworm populations (Abrahams, 2017; EFSA Panel on Plant Health, 2017) (Fig. 1.7). Additionally, even if this species could not survive the European winters, it could arrive annually to areas as far up north as central and northern Europe in summer migrations from Africa, causing significant damage to the maize fields located in these regions (CABI, 2017; EFSA Panel on Plant Health, 2017).

**Figure 1.6.** Number of *S. frugiperda* interceptions in imported goods detected in the EU in the last 8 years, according to the origin of the goods. * Interceptions from January to March 2018. Source: EUROPHYT, 2018.

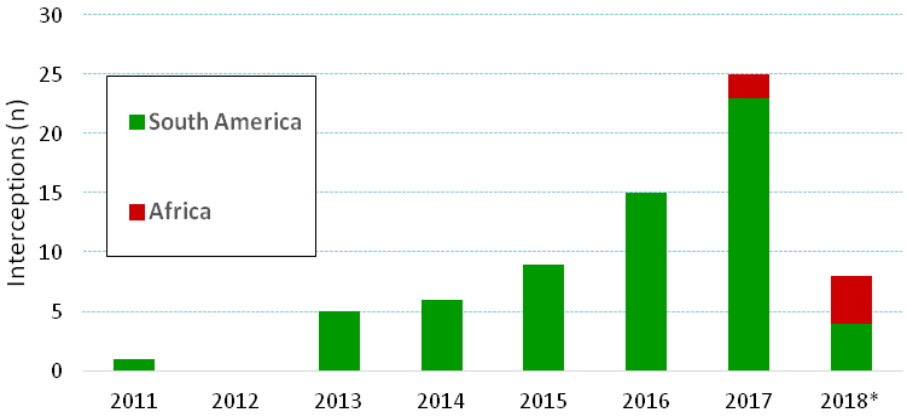
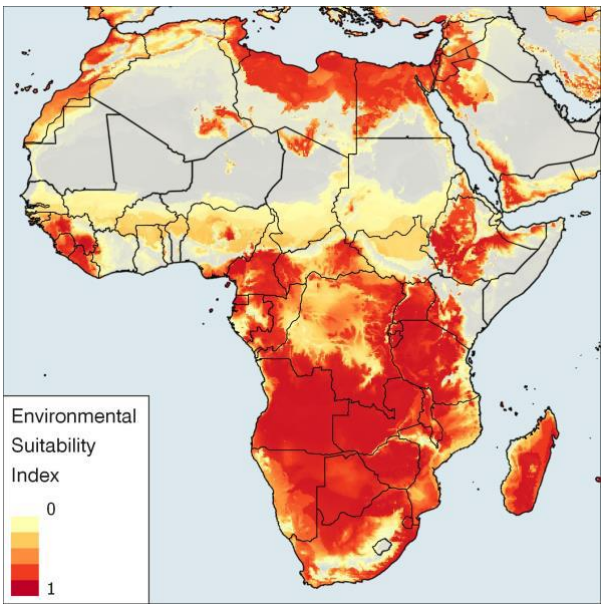



**Figure 1.7.** Forecasted *S. frugiperda* distribution based on climate suitability models. Source: Abrahams *et al.*, 2017.



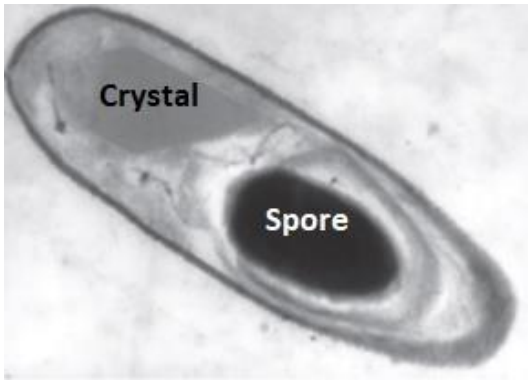

## *1.3. Relevance of Bacillus thuringiensis in crop protection*

### 1.3.1. Biology and ecology of the bacteria

*Bacillus thuringiensis* (Bt) is a gram positive, aerobic bacterium that can be found in a wide range of environments, including soil, plants and insects (Raymond *et al.*, 2010) (Fig. 1.8). Bt was first isolated from infected silkworms in 1901 by the

Japanese researcher Ishiwata Shigetane, although the species was not described and named until 1915, after the German scientist Ernst Berliner isolated the bacteria from the moth *Ephestia kuehniella* (Sanahuja *et al.*, 2011).

**Figure 1.8.** Sporulating *Bacillus thuringiensis* bacterium. Source: Sanchís and Bourguet, 2008.




There are two phases in the life cycle of Bt bacteria: a vegetative phase and a spore formation phase. The vegetative phase, in which the cell divides, takes place when the environmental conditions are favorable and there is nutritional availability. On the other hand, under nutritional shortage or in unfavorable environments the cell shifts to the spore formation phase, giving way to spores which are highly resistant to harsh weather situations and can persist in the environment for long periods (Nicholson *et al.*, 2000; Ibrahim *et al.*, 2010). Insecticidal toxins are produced in both phases: Vip (vegetative insecticidal proteins) toxins are produced by vegetative cells, whereas two kind of δ-endotoxins, Cyt (cytolytic) and Cry (crystal) toxins, are synthesized during sporulation and accumulate in crystal inclusions that make up a remarkable fraction of the weight of the sporulated cells (Palma *et al.*, 2014; Rabinovitch *et al.*, 2017).

Around a thousand Cry, Cyt and Vip toxins have been identified so far, and the list of toxins, designated according to amino acid sequence identity and available at http://www.btnomenclature.info/, grows continuously (Crickmore *et al.*, 2016). The high binding specificity displayed by each family of Bt toxins against one or

a few insect orders make them an attractive option for pest control. For instance, Cry1 toxins have a strong binding affinity for Lepidoptera, whereas Cry2 toxins have a strong affinity for both Lepidoptera and Diptera, Cry3 toxins for Coleoptera, Cry4 toxins for Diptera and Cyt toxins are mostly active against Diptera (van Frankenhuyzen, 2017). Vip toxins, on the other hand, have been reported to be active against insects of the orders Lepidoptera and Coleoptera, depending on the toxin family (de Maagd *et al.*, 2003). Most Bt-based biopesticides and insect-resistant plants that target lepidopteran pests contain Cry toxins.

### 1.3.2. Mode of action of Cry proteins

Each Cry toxin affects a tight group of species in a highly specific manner by binding to particular membrane receptors located in their midgut (Pardo-López *et al.*, 2013; Jurat-Fuentes and Crickmore, 2017). There are several theories on the mode of action of Cry proteins, the most accepted one for Cry1 toxins is described here. Upon ingestion, Cry1 pro-toxins, with a molecular weight of around 130 kDa, are solubilized and activated by proteases in the alkaline lepidopteran midgut, giving way to an activated toxin of around 65 kDa. Then, the activated toxin binds sequentially with different membrane receptors and inserts in the membrane, leading to oligomerization, pore formation and cell lysis. This results in an osmotic shock and insect death, caused by septicemia, starvation or a combination of both (Schnepf *et al.*, 1998; Bravo *et al.*, 2011; Vachon *et al.*, 2012).

### 1.3.3. Bt-based biopesticides

Bt-based biopesticides consist of a mixture of *B. thuringiensis* spores and crystal toxins, formulated as either powders or concentrated liquids, which are sprayed over the plant parts targeted by insect pests (Senthil-Nathan, 2015; Bravo *et al.*, 2017). The first pesticide based on Bt, Sporeine, was commercialized in France in 1938, and the variety and use of Bt-based pesticides increased throughout the century, soon becoming the most prevalent biopesticides in the market (Chandler

*et al.*, 2011). Even though Bt biopesticides are regarded as an environmentally friendly, safe and efficient method for pest management (Popp *et al.*, 2013), their use is hindered by their rapid degradation in the environment, their limited effectiveness against pests that feed inside the plant and their low activity against advanced larval stages, among other factors (Sanahuja *et al.*, 2011).

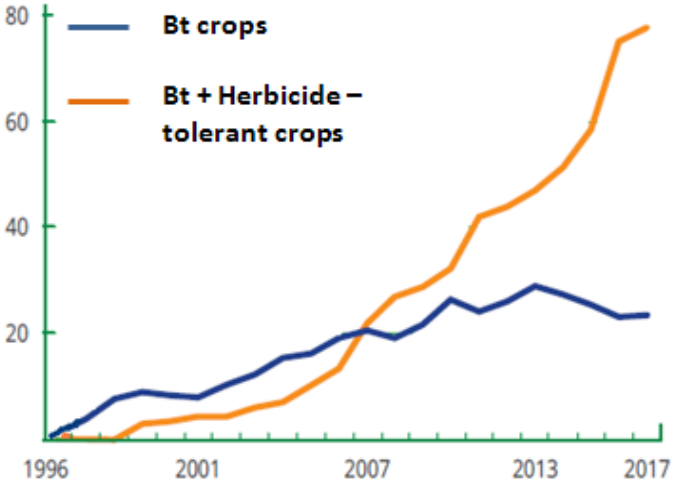
### 1.3.4. Bt crops

The genetic modification of plants to introduce genes of *B. thuringiensis* encoding the expression of toxins has given rise to Bt crops. These plants are designed to produce one or a few toxins constitutively, which confers them protection against the insect pest(s) targeted by these insecticidal proteins (de Maagd *et al.*, 1999). Most Bt genes introduced in Bt plants have been modified for increased toxin expression, so that their efficacy in suppressing target pests is improved (Hilder and Boulter, 1999).

Insect-protected crops that express Bt proteins were not available for commercialization until 1996. On that year, Bt varieties of cotton, maize and potatoes were grown in four countries (the US, Canada, Australia and Mexico), occupying a total area of 1.2 million ha (James, 1997). The number of Bt crops approved for commercialization increased in the following years, and so did the number of countries that grew them, so that by 2017 Bt crops were cultivated in 24 countries of all continents (Fig. 1.9). Most of the Bt events approved for food and feed correspond to cotton and maize (>85% of the total), but there are also Bt varieties of other crops, including rice, eggplant and poplar, although some of them are not commercially available yet and some others are no longer cultivated (ISAAA, 2018). Adoption of Bt varieties has increased steadily with time, so that in 2017 around 101 million hectares were sown with varieties that expressed Bt traits, either alone or in combination with herbicide tolerance traits (ISAAA, 2017) (Fig. 1.10).

**Figure 1.9.** Countries that grew Bt crops in 2017. Source: ISAAA, 2017.

**Figure 1.10.** Adoption of Bt crops in the world between 1996 and 2017, divided by crops that are only insect-resistant and crops that are both insect-resistant and herbicide-tolerant. Source: ISAAA, 2017.




## *1.4. Bt maize*

Bt maize is the most prevalent Bt crop in the world, and the second most important genetically modified (GM) crop, only surpassed by herbicide-tolerant soybean. In 2017, 53 million hectares of Bt maize were sown worldwide, making up to 28% of all the maize sown in the world that year (ISAAA, 2017; FAOSTAT, 2018). The adoption of Bt maize has increased on a steady basis since

it was first marketed in 1996, and this upward trend is expected to continue due to the population growth and the growing demand for grain as feed for livestock as the world population diet changes to a higher reliance on animal products (Popp *et al.*, 2013). According to the data provided in ISAAA (2017), most of the Bt maize sown worldwide in 2017 corresponded to varieties that combined insect resistance with herbicide tolerance (80%), and the US was the top producer of Bt maize in the world, with over 33 million hectares grown that year. Brazil and Argentina are also major producers of Bt maize, growing millions of hectares of varieties derived from several transformation events every year. In the EU around 131,000 hectares of Bt maize were grown in 2017, with Bt maize concentrating largely in Spain.

Over 200 events of genetic modification have been approved for maize, most of which express Bt proteins that target insect pests in a highly specific way. Each event targets specific pests in each area where they are deployed. Varieties derived of the 173 events approved for lepidopteran insect resistance express one or a combination of several Bt toxins, including both natural [Cry1Ab, Cry1A.105, Cry1C, Cry1F, Cry2Ab2, Cry2Ae, Cry9C, Vip3A(a) and Vip2Aa20] and synthetic [Cry1Ab (truncated), moCry1F, Cry1Fa2] forms of toxins. On the other hand, the 122 events approved for coleopteran insect resistance express a different and smaller set of Bt toxins, including Cry3Bb1, Cry34Ab1, Cry35Ab1 and the synthetic form mCry3A. Finally, 34 events express the chimeric δ-endotoxin eCry3.1Ab, that affects both lepidopteran and coleopteran pests (ISAAA, 2018). Up to the date, the maximum number of Bt toxins conferred by a transformation event in maize is six (i.e. SmartStax®, which targets both coleopteran and lepidopteran pests), and maize is the Bt crop in which the highest number of events has been stacked (Parisi *et al.*, 2016).

### 1.4.1. Potential benefits and risks of Bt maize

Several benefits have been associated with the adoption of Bt maize, including increased yields and increased farm income (Brookes and Barfoot, 2014; Klümper and Qaim, 2014), especially in developing countries and in areas where pest pressure is high (Qaim, 2009; Carpenter, 2010; Barrows *et al.*, 2014). The

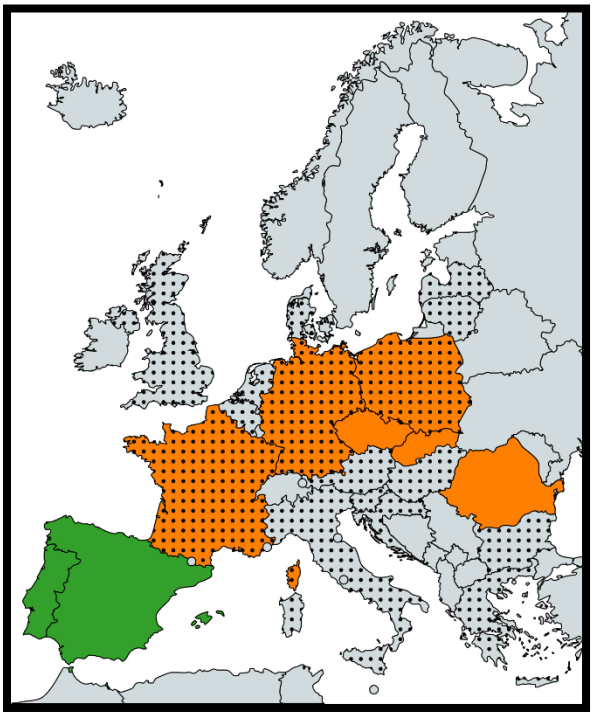
constitutive expression of toxins by Bt plants throughout the growing season removes the need of several insecticide applications per season, often leading to reduced use of chemical insecticides (Cattaneo *et al*., 2006; Klümper and Qaim, 2014), which in turn results in a decrease in the working time allocated to applying these compounds, a reduction in the emission of greenhouse gasses associated with this duty (Barrows *et al*., 2014; Brookes and Barfoot, 2014), and a lower exposure of farmers to these toxic products (Qaim, 2009). Owing to the high specificity of the toxins expressed by Bt maize and their low persistency, this technology is commonly regarded as less harmful to non-target organisms and the environment than traditional synthetic insecticides (Cattaneo *et al.*, 2006; Wolfenbarger *et al.*, 2008). Moreover, the use of this technology can lead to a reduction in the mycotoxin content in plants, since mycotoxin-producing fungi often penetrate the plant via insect wounds, which are less frequent in Bt plants (Wu, 2006). Finally, Bt toxins are considered to have a low toxicity on mammals, including humans (Rubio-Infante and Moreno-Fierros, 2015), and several studies and reviews have found no significant negative effects of Bt food on the health of test animals, livestock and humans (Nicolia *et al.*, 2014; de Vos and Swanenburg, 2018).

In spite of the benefits that Bt maize can bring about, there are also some shortcomings associated with the adoption of this technology. The main threat for the long-term sustainability of Bt maize is the evolution of resistance in target pests, which would render Bt plants ineffective in pest control (Gould, 1998; Zhao *et al.*, 2003). Additionally, the suppression of target pests by Bt crops could lead to an increase in crop damage by secondary pests due to the removal of competition for plant resources or the reduced use of chemical pesticides (Lu *et al.*, 2010). On the other hand, concerns have arisen about the possibility of native relatives getting contaminated by gene flow from Bt plants (Lu and Snow, 2005; Barrows *et al.*, 2014). In an attempt to avoid undesired gene flow, the cultivation of Bt maize is banned in areas where wild relatives are present, and in the EU, Bt fields must be separated from non-Bt fields by a buffer zone, according to both European and national legislation of the member states (EC, 2001; Mendelsohn *et al.*, 2003; Messeguer *et al.*, 2006; Devos *et al.*, 2008; BOE, 2017).

### 1.4.2. Bt maize in the EU

Since Bt maize was first commercialized in the EU in 1998, two transformation events have been approved for cultivation, both of them expressing the toxin Cry1Ab: Bt 176 and MON 810. Maize hybrids derived from the event Bt 176 were grown from 1998 to 2005, and MON 810 maize has been grown since 2003 and up to the date, so that from 2006 all Bt maize sown in the EU, including Spain, is MON 810 (EC, 2007; Farinós *et al.*, 2018). Even though 8 countries in the EU have adopted Bt crops at some point since 1998, only Spain and Portugal, grew a total of around 131,000 ha of Bt maize in 2017, and most of it (around 91%) was in Spain (ISAAA, 2017) (Fig. 1.11).

**Figure 1.11.** EU countries that have adopted Bt maize between 1998 and 2017. Green-shaded countries grew Bt maize in 2017, orange-shaded countries grew Bt maize at some point between 1998 and 2016 and dotted countries have banned Bt maize cultivation in their territories following EU Directive 2015/412.

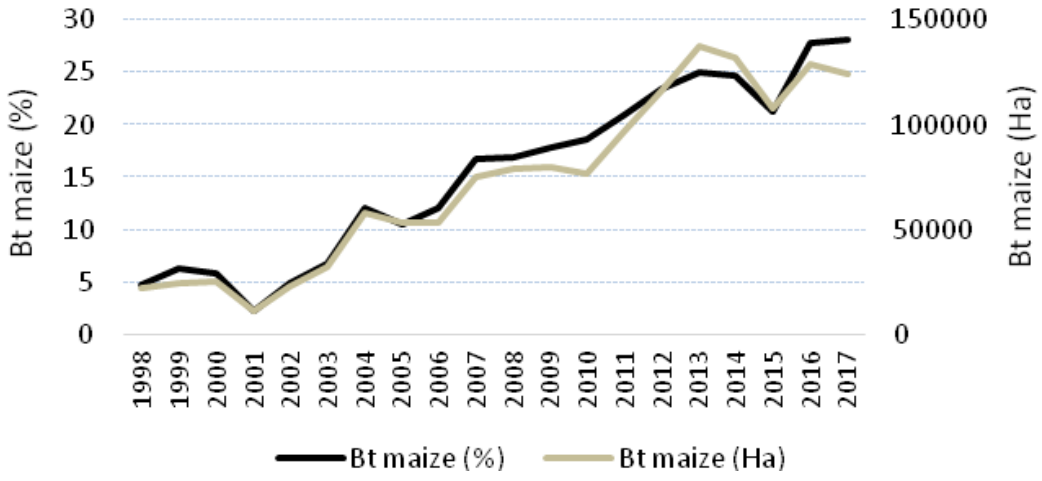


Spain is the only European country where Bt maize has been grown continuously on a large-scale since 1998, to control the stem borers *S. nonagrioides* and *Ostrinia nubilalis*. The adoption of this technology increased steadily in Spain

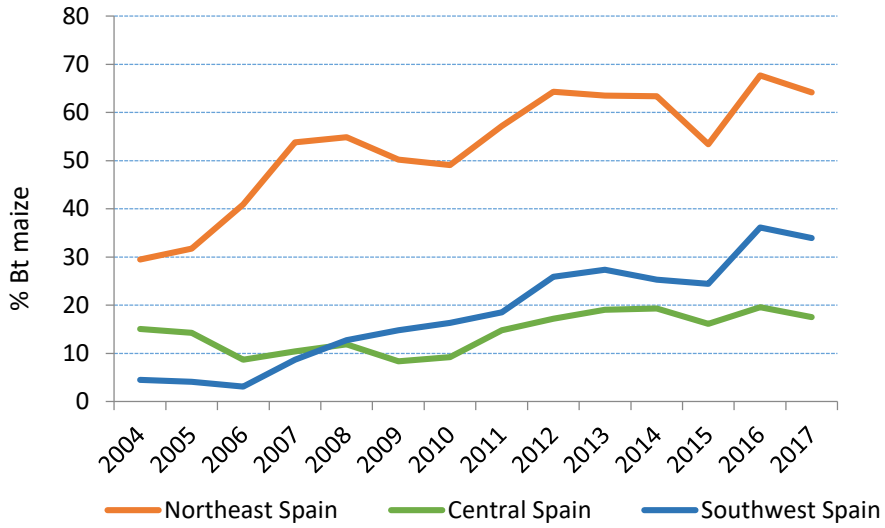
between 1998 and 2013, and from that year it has stabilized in values around 25-30% of all maize grown every year. In 2017, over 124,000 ha of Bt maize were grown in Spain, representing 28% of all maize grown in the country that year (Fig. 1.12A). The distribution of Bt maize in Spain is not homogenous, with regions such as Galicia (northwest Spain) where it has never been grown, areas like Central and Southwestern Spain, where adoption of this Bt crop is moderate (20-30%), and areas of intense Bt maize cultivation, such as the Ebro Valley, where over 60% of all maize cultivated most of the years since 2011 is Bt maize (Castañera *et al.*, 2016) (Fig. 1.12B). These differences are associated with biological and agronomic factors, so that the adoption of Bt maize is higher in areas where the intense maize cultivation and the high pest pressure increase the benefits of adopting this technology in comparison with other areas where pest attack is lower (Gómez-Barbero *et al.*, 2008).

**Fig. 1.12.** Adoption of Bt maize in Spain (A) and in the different maize growing areas (B), where Northeast Spain includes the Autonomous Communities of Aragón, Cataluña and Navarra, Central Spain includes Madrid and Castilla-La Mancha, and Southwest Spain includes Extremadura and Andalucía. Own compilation from data available at www.mapama.es/gob/en/

**(A)**



**(B)**




## *1.5. Insect resistance to Bt maize*

The high and continued expression of toxins by Bt plants exerts a great selective pressure on pests that could lead to rapid resistance evolution, widely considered as the most important threat to the long-term sustainability of Bt crops (Tabashnik, 1994; Gould, 1998). Field-evolved resistance has been defined as a genetically based decrease in susceptibility to the toxin owing to exposure to the pesticide in the field, and it is known as practical resistance if it results in reduced efficacy of the Bt product in suppressing the pest (Tabashnik *et al.*, 2014). Alleles that confer resistance to the toxin are naturally present in pest populations, usually at low frequencies under no-selection conditions, but their frequencies increase under situations of prolonged exposure to the insecticidal protein, ultimately leading to resistance evolution and control failures (Tabashnik *et al.*, 2013).

### 1.5.1. Insect resistance management Programs

The implementation of insect resistance management (IRM) programs is essential to delay resistance evolution, and it is required for the authorization of new varieties in many of the countries where Bt crops are sown (Andow and Ives, 2002; Bates *et al.*, 2005). These programs must rely on the best and most recent available data, including knowledge on the biology of the target pests, the

agricultural practices in the area and the genetic basis of resistance, when possible (Andow, 2008; MacIntosh, 2010; Tabashnik *et al.*, 2013). Furthermore, IRM strategies should be adaptive so that management can change according to the new information (Andow and Ives, 2002; Farinós *et al.*, 2018).

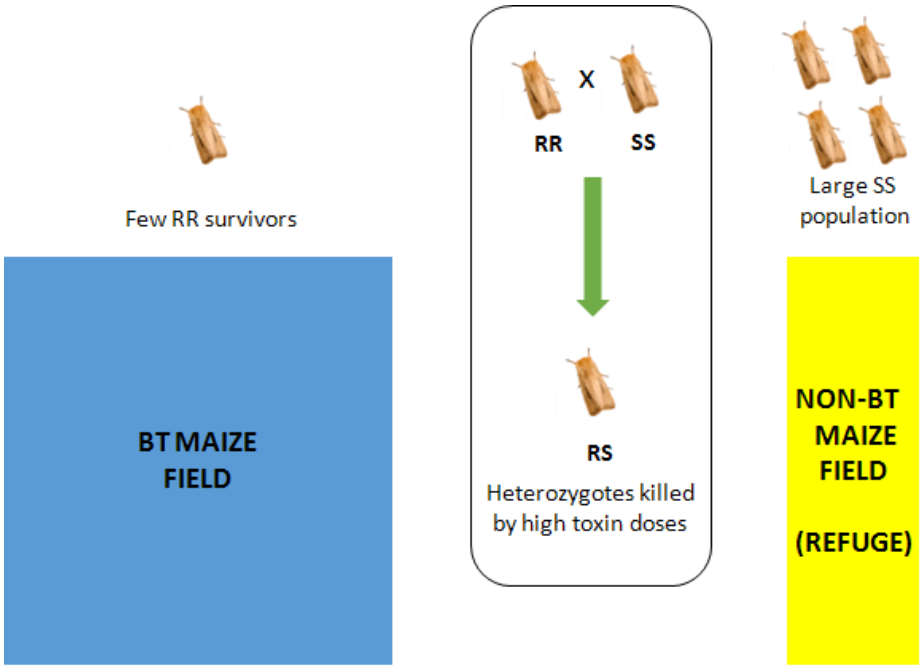
Different approaches have been proposed for IRM in Bt crops, each of them presenting advantages and shortcomings (Bates *et al.*, 2005). However, IRM programs mostly rely on the strategy known as high-dose/refuge (HDR) (Shelton *et al.*, 2000; Zhou *et al.*, 2017), which is described below. On the other hand, the use of pyramided varieties that express different Bt toxins is an additional way to further delay resistance evolution within a HDR context (Zhao *et al.*, 2005; Carrière *et al.*, 2015). Pyramided varieties can control a broader spectrum of insect pests, and they can significantly delay resistance evolution with regards to single-toxin varieties if the stacked traits do not show cross-resistance and all the toxins are high-dose against the targeted pests (Zhao *et al.*, 2003; Brèvault *et al.*, 2013; Gressel *et al.*, 2017).

#### 1.5.1.1. HDR strategy

The HDR strategy involves using Bt varieties that express high toxin doses that kill most individuals of the target pest (>99.99% of susceptible individuals) and allotting a given proportion of the crop to non-Bt varieties that will act as refuges for susceptible individuals (Huang *et al.*, 2011; Carrière *et al.*, 2012). For this strategy to be effective three conditions must be met: (i) mating should be random between individuals from Bt and non-Bt fields; (ii) resistance should be recessive; and (iii) the frequency of resistance alleles should be low, ideally <0.001 (Roush, 1994; Andow and Hutchinson, 1998; Bates *et al.*, 2005). Additionally, farmers' compliance with refuge size and location requirements, which vary according to the Bt crop and the area, is essential for this approach to be effective (Bates *et al.*, 2005; Bourguet *et al.*, 2005). In the EU, farmers planting over 5 ha of Bt maize are required to allocate 20% of their fields to a refuge comprised of non-Bt maize located less than 750 m from the Bt field (EFSA Panel on GMO, 2018). In this scenario, the resistant individuals that would emerge in Bt fields at extremely low frequencies would mate with the more abundant SS individuals arising from the

nearby refuges, resulting in RS offspring that would be susceptible to the toxin expressed by the plants in the Bt field and therefore killed by the Bt plants (Fig. 1.13). Violations of these assumptions have been associated with resistance evolution in the field (Gassmann *et al.*, 2011).

**Figure 1.13.** Schematic representation of the high-dose/refuge strategy.




### 1.5.2. Resistance monitoring

Monitoring for resistance development in field populations of the target pests is a keystone of IRM programs (Marçon *et al.*, 1999; Priesnitz *et al.*, 2016). A first step in resistance monitoring is to determine the baseline susceptibilities to the Bt trait in populations from the geographic range of the target pests where the Bt crop is deployed, using appropriate bioassay techniques (Marçon *et al.*, 1999; Head and Greenplate, 2012). Dose-response bioassays are often used in susceptibility determination, although other methods can also be employed (Huang, 2006). Dose-response assays involve exposing young larvae to different toxin concentrations that cause 10-90% mortality, in order to estimate the concentration that kills or inhibits molting in 50% of the larvae ($LC_{50}$ or $MIC_{50}$, respectively) of the population tested (Siegfried *et al.*, 2007). Once baseline

susceptibilities are established, regular monitoring should be carried out and the susceptibility of the target pests estimated in different areas, with special attention to those areas where the risk of resistance evolution is high (MacIntosh, 2010). Populations of the target pest should be collected in different fields within each area, and the estimated susceptibility of these populations to the Bt trait should be compared with baseline values and/or with susceptible laboratory strains, in order to detect significant shifts in this parameter (MacIntosh, 2010; Farinós *et al.*, 2018). Early detection of resistance evolution would allow for an adaptive response, so that IRM practices can be modified in a timely manner and control failures are further delayed (Andow and Ives, 2002). Additionally, IRM programs should also include other tasks such as growers' education and the establishment of remedial plans in case resistance evolution is detected (Andow and Ives, 2002; MacIntosh, 2010).

#### 1.5.2.1. Resistance monitoring in the EU – the case of Spain

In the EU, the approval of new GM varieties for cultivation is subjected to Directive 2001/18/EC and Regulation 1829/2008 (EC, 2001; 2003), which make post-market environmental monitoring (PMEM) programs mandatory. Resistance monitoring is an essential part of PMEM, and in the case of *S. nonagrioides* it has focused in Spain, the country that concentrates most of the Bt maize in the EU. In this country resistance monitoring has been carried out since Bt maize was first adopted in 1998 (Castañera *et al.*, 2016; Farinós *et al.*, 2018). Firstly, baseline susceptibility to Cry1Ab, the toxin expressed by the transformation events approved in the EU, was determined in four maize growing areas where the target pests were present prior to the adoption of Bt maize: northeast (Ebro Valley), northwest (Galicia), center (Madrid and Castilla La Mancha) and southwest (Extremadura and Andalucía) (González-Núñez *et al.*, 2000). From then on, the resistance monitoring plan established that susceptibility to the Bt protein was to be assessed regularly in areas where adoption of the technology exceeded 20%, on an annual or biannual basis depending on the adoption of Bt maize and the number of generations of the target pest in the area every year (EFSA, 2015). This implied susceptibility had to be assessed every two years in the northeast, center

and southwest maize growing regions. Until 2015, susceptibility assessments relied on dose-response bioassays that used the diet-overlay technique, which has been considered a reliable and consistent method for the evaluation of the susceptibility of corn borer populations to Bt toxins (Farinós *et al*., 2004; 2011). However, in 2016, susceptibility assessment protocols were modified according to the data gathered in over a decade of resistance monitoring and the recommendations of the European Food Safety Authority, so that from that year it is based on diagnostic-dose bioassays, and it is limited to areas where the adoption of Bt maize surpasses 60% and multiple generations of both target pests are present every year (EFSA, 2015; EFSA Panel on GMO, 2016). The data gathered by the resistance monitoring programs indicate that, despite the increasing adoption of Bt maize and the accumulated time of exposure to the toxin, susceptibility to the toxin Cry1Ab did not decrease in populations of any of the target pests from any studied area between 1998 and 2015 (Farinós *et al.*, 2018; Castañera *et al.*, 2016).

### 1.5.3. Field-evolved resistance to Bt crops

The first report of field-evolved resistance to a Bt crop was published in 2006 and it involved populations of *Helicoverpa zea* collected in the US in 2003-2004 in Bollgard® cotton, which showed greatly reduced susceptibility to the toxin Cry1Ac produced by the Bt crop (Ali *et al.*, 2006). From that moment and up to the date, over 15 cases of practical resistance, which according to Tabashnik *et al.* (2014) is "field-evolved resistance that reduces pesticide efficacy and has practical consequences for pest control", have been reported worldwide (Tabashnik and Carrière, 2017). Additionally, there are other cases in which resistance alleles have been detected in the field, but control failure has not occurred (Huang *et al.*, 2012; Zhang *et al.*, 2014; Jin *et al.*, 2015). Reported cases of resistance mainly involve Bt maize and single-toxin events. Resistance has been reported in populations of seven pest species, including six Lepidoptera and one Coleoptera species. It is noteworthy to mention that four out of the six lepidopteran species in which resistance has been detected are noctuids. The first report of field-evolved resistance to Bt maize expressing Cry1Ab protein

corresponded to populations of *Busseola fusca* from South Africa (Van Rensburg, 2007). Field populations of *Spodoptera frugiperda* resistant to Bt maize that expresses the toxin Cry1F were detected in Puerto Rico, Brazil and continental United States (US) just a few seasons after Bt maize hybrids were first deployed (Storer *et al.*, 2010; Farias *et al.*, 2014a; Huang *et al.*, 2014). More recently, field-evolved resistance to Bt sweet maize that expresses different Cry toxins was reported in *Helicoverpa zea* populations from the US (Dively *et al.*, 2016). *Spodoptera frugiperda* is the only species in which resistance has been detected in different countries (insular and continental US, Brazil and Argentina), whereas resistance to different Bt toxins has been also reported in *H. zea*, *Diabrotica virgifera virgifera* and *Pectinophora gossyipiella* (Table 1.2).

Resistance evolution has often been associated with poor compliance of the requirements of the HDR strategy (Table 1.2). In some countries farmers have failed to plant a proportion high enough of refuges or they have failed to plant them as instructed, either by placing them too far from the Bt crop, by not making them as desirable as their transgenic counterpart (for instance by not irrigating them) or by spraying them excessively with insecticides to prevent economic damage, thus killing susceptible individuals (Van Rensburg, 2007; Dhurua and Gujar, 2011; Farias *et al.*, 2014a; Dively *et al.*, 2016; Omoto *et al.*, 2016; Grimi *et al.*, 2018). In most cases of field-evolved resistance, the Bt varieties deployed did not represent a high-dose against the target pest, which sometimes was coupled with non-recessive inheritance of resistance (Gassmann *et al.*, 2011). On the other hand, the climate of some areas along with the cropping regimes adopted could have played a role in some cases (Van Rensburg, 2007; Dhurua and Gujar, 2011; Kranthi, 2015). Thus, in tropical regions where some pests can be found throughout the year with overlapping generations and multiple Bt crops are grown every season, the selective pressure exerted on pests is huge and can lead to early resistance development (Storer *et al.*, 2010; Farias *et al.*, 2014a; Kranthi, 2015; Omoto *et al.*, 2016). Additionally, cross-resistance has been suggested to contribute to resistance evolution in some cases in which a pest became resistant to several Bt toxins in a narrow time period (Dively *et al.*, 2016; Omoto *et al.*, 2016; Zukoff *et al.*, 2016; Grimi *et al.*, 2018).

**Table 1.2.** Reported cases of field-evolved practical resistance. Each case involves a pest species, a Bt toxin and a specific location. (Modified from Tabashnik and Carrière, 2017)

| Pest species | Bt crop | Toxin | Country | Years to resistance[a] | Factors contributing | References |
|---|---|---|---|---|---|---|
| *Busseola fusca* (Lepidoptera: Noctuidae) | Maize | Cry1Ab | South Africa | 8 | 1, 2, 3, 8 | Van Rensburg, 2007; Campagne *et al.*, 2013 |
| *Diatraea saccharalis* (Lepidoptera: Crambidae) | Maize | Cry1F | Argentina | 4 | 3 | Grimi *et al.*, 2018 |
| | | Cry1A.105 | Argentina | 4 | 3, 5 | Grimi *et al.*, 2018 |
| *Helicoverpa zea* (Lepidoptera: Noctuidae) | Maize | Cry1Ab | US | 8 | 1, 3, 6 | Dively *et al.*, 2016 |
| | | Cry1A.105 | US | 6 | 1, 5 | Dively *et al.*, 2016 |
| | Cotton | Cry1Ac | US | 6 | 1 | Ali *et al.*, 2006 |
| | | Cry2Ab | US | 2 | 1, 5 | Ali and Luttrell, 2007; Unglesbee, 2017 |
| *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) | Cotton | Cry1Ac | India | 6 | 1, 3 | Dhurua and Gujar, 2011; Mohan *et al.*, 2016; Nair *et al.*, 2016 |
| | | Cry2Ab | India | 8 | 3, 4, 7, 8 | Fabrick *et al.*, 2015; Kranthi, 2015 |
| *Striacosta albicosta* (Lepidoptera: Noctuidae) | Maize | Cry1Fa | US | 10 | 1 | Eichenseer *et al.*, 2008; DiFonzo *et al.*, 2016; Ostrem *et al.*, 2016 |
| | | | Canada | 10 | 1 | Smith *et al.*, 2017 |
| *Spodoptera frugiperda* (Lepidoptera: Noctuidae) | Maize | Cry1Ab | Brazil | 2 | 1, 3, 4, 5, 6 | Omoto *et al.*, 2016 |
| | | Cry1F | Brazil | 3 | 1, 3, 4 | Farias *et al.*, 2014a; Farias *et al.*, 2016 |
| | | | US | 4 | 1, 4, 8 | Storer *et al.*, 2010; Huang *et al.*, 2014 |
| | | | Argentina | 7 | 1 | Chandrasena *et al.*, 2017 |
| *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) | Maize | Cry3Bb1 | US | 6 | 1, 2, 3 | Gassmann *et al.*, 2011; Andow *et al.*, 2016 |
| | | Cry34/35Ab | US | 7 | 1 | Gassmann *et al.*, 2016; Ludwik *et al.*, 2017 |
| | | mCry3A | US | 4 | 1, 5 | Andow *et al.*, 2016; Gassmann *et al.*, 2014 |
| | | eCry3.1Ab | US | 0 | 1, 5 | Jakka *et al.*, 2016; Zukoff *et al.*, 2016 |

[a] Number of years elapsed between the year when the Bt variety expressing the toxin was marketed and the moment when resistance was first detected.

[b] Factors classified as: 1= Not high dose; 2= Non-recessive inheritance; 3= Low refuge compliance; 4= Tropical conditions allowing multiple crops per season and overlapping generations of the target pest; 5= Cross-resistance between toxins (suspected or known); 6= Delayed development in resistant individuals leading to assortative mating; 7= Deployment of pyramids containing a toxin previously used in the area; 8= Agricultural practices favoring resistance development.

## *1.7. Objectives*

The general aim of this thesis is to optimize the ongoing IRM strategies of Bt maize concerning three noctuid pests of this crop: the primary pest *Sesamia nonagrioides,* the secondary pest *Mythimna unipuncta,* and a potentially invasive pest in the EU, *Spodoptera frugiperda*.

More specifically, we have addressed the following objectives:

– To assess population variation in the susceptibility to the Cry1Ab toxin of *S. nonagrioides* in the Ebro Valley and of *M. unipuncta* in areas with high-adoption (Ebro Valley) or no-adoption (Galicia) of Bt maize.

– To evaluate the performance of *S. nonagrioides* on cultivated and wild host plants and its potential implications for Bt maize resistance management.

– To assess the frequency of resistance alleles to Bt maize in Spanish populations of *S. nonagrioides* using an $F_2$ screen.

– To investigate the genetic basis of resistance to Bt maize in two populations of *S. frugiperda* resistant to Cry1F maize.

# II. Population variation in the susceptibility of *Sesamia nonagrioides* and *Mythimna unipuncta* to the toxin Cry1Ab

## *2.1. Introduction*

Spain is the only European country where transgenic maize expressing the protein Cry1Ab (Bt maize) has been grown continuously since it was first commercialized in 1998, accounting for 91% of the total Bt maize area in the EU in 2017 (ISAAA, 2017). From 1998 to 2013 adoption of Bt maize increased steadily in Spain, and since that year it has stabilized at around 25-30% of all maize grown in the country (estimated from the data available at http://www.mapama.gob.es/en/), with regional differences in adoption (Fig. 1.12B). Continued exposure to the high levels of toxin expressed by the plants exerts a strong selective pressure on pests and could lead to resistance development (Tabashnik, 1994; Gould, 1998), which would threaten the efficacy of Bt maize in suppressing pest damage. The high and continued use of Bt maize in the Ebro Valley and the presence of 2-3 generations per year of the target pests *Sesamia nonagrioides* and *Ostrinia nubilalis* (González-Nuñez *et al*., 2000) render this area as the only hotspot in Europe where resistance has a higher probability of evolving (EFSA Panel on GMO, 2012).

In the European Union (EU), the approval of a genetically modified plant for commercial cultivation is subject to regulation at EU level. Thus, according to the Directive 2001/18/EC and the Regulation (EC) 1829/03, post-market environmental monitoring (PMEM) is a mandatory requirement for commercial release of GM crops (EC, 2001; 2003). With regards to Bt maize, one of the objectives of the PMEM is to assess the potential resistance development of target pests' populations to the protein Cry1Ab expressed in the GM crop, as a consequence of the high selection pressure these populations are subjected to in the field (MARM, 2010). With this purpose, an insect resistance monitoring program was implemented across the EU in order to detect, in a timely manner, shifts in susceptibility of the target pests to the Bt protein that could be indicative of resistance development. This program establishes that monitoring efforts should focus in areas where the ecology of the pest and the high adoption rates of Bt maize make resistance more likely to develop (EFSA, 2015).

A post-market resistance monitoring program for Bt maize was initiated in Spain in 1998 (MARM, 2010). This program allowed for the establishment of the baselines of susceptibility to Cry1Ab toxin of Spanish populations of *S. nonagrioides* and *O. nubilalis* from the most representative regions where Bt maize is grown and several generations of these pests are present per year: Northeast Spain (Ebro Valley), Central Spain and Southwest Spain (González-Núñez *et al*., 2000). Subsequently, between 1999 and 2015, resistance monitoring included biannual samplings in the aforementioned Bt maize growing regions. In each of the three zones, susceptibility of field populations to the protein Cry1Ab was estimated by dose-response bioassays, and the values obtained were compared with baseline susceptibility values determined earlier in the same areas and with the susceptibility of a control laboratory strain, in order to evaluate whether significant changes in this parameter were taking place (EFSA, 2015; Farinós *et al*., 2018). The results indicated that susceptibility of both *S. nonagrioides* and *O. nubilalis* to the protein Cry1Ab has not decreased over time either in the Ebro Valley or in any other areas of Spain between 1999 and 2015 (Farinós *et al.*, 2004; 2011; 2018; Castañera *et al.*, 2016; Thieme *et al.*, 2017). More recently, the regulatory agency of the EU has stated the need to improve resistance monitoring strategies in an attempt to lower detection limits of resistance alleles from frequencies of 5% to 3% (EFSA Panel on GMO, 2016). Gaining knowledge on the smaller-scale variation of susceptibility to Cry1Ab protein in the Ebro Valley, the area in the EU where resistance is more prone to develop, would help optimize sampling strategies.

The secondary noctuid pest *Mythimna unipuncta* has shown a much lower susceptibility to the toxin Cry1Ab expressed in MON 810 Bt maize cultivars. This species has been reported to attack maize fields in the Ebro Valley sporadically causing important yield losses (López *et al.*, 2000; Eizaguirre *et al.*, 2010). Moreover, it was observed that a small percentage of larvae of this pest can survive and complete their development MON 810 maize (Eizaguirre *et al*., 2010; González-Cabrera *et al.* 2013). These findings indicate that the concentration of Cry1Ab expressed by transgenic maize hybrids represents a “low dose” scenario for *M. unipuncta*, favouring the potential development of resistance in field populations (Pérez-Hedo *et al*., 2012; González-Cabrera *et al.*, 2013). Additionally,

they hinted at the risk of resistance evolution in field populations of *M. unipuncta* and the importance of taking this species into consideration in Bt maize resistance monitoring programs (González-Cabrera *et al.*, 2013; García *et al.*, 2015). To our knowledge, no studies have evaluated the susceptibility to Cry1Ab protein of *M. unipuncta* populations from areas where Bt maize has never been grown commercially. Obtaining information on the variation in susceptibility to Cry1Ab protein between areas of high adoption and areas with no history of Bt maize cultivation would help determine whether the high selective pressure that populations from the Ebro Valley are subjected to could have led to lower susceptibility to Cry1Ab protein in comparison with populations from areas where Bt maize has never been sown.

This chapter has two main objectives. The first one is to study the variation in the susceptibility to Cry1Ab protein between (interpopulation variation) and within (intrapopulation variation) populations of the target pest *S. nonagrioides* from the hotspot area of the Ebro Valley, in order to optimize sampling strategies and to improve the resistance monitoring plan by determining whether it should cover large areas or focus in smaller zones where susceptibility is lower. The second objective is to discern whether the susceptibility to Cry1Ab protein varies between field populations of *M. unipuncta* subjected to high or no selective pressure of Bt maize, so as to learn whether this pest could become a threat to the sustainability of the Bt crop.

## *2.2. Materials and methods*

### 2.2.1. Insect collection and rearing

#### 2.2.1.1. *Sesamia nonagrioides*

In November 2014, six populations of *S. nonagrioides* were collected in commercial non-Bt maize fields adjacent to Bt maize fields in two different zones of the Ebro Valley, which were separated geographically by the river Cinca, a tributary of the river Ebro, and by a strip over 10 km wide with no maize fields. Samplings were carried out in three locations per zone: Zone 1 included populations from the municipalities of Vencillón (Huesca), Alfés and Almacelles (Lleida),

whereas Zone 2 included a population collected in the municipality of Bujaraloz (Zaragoza) and two populations from Candasnos (Huesca) (Fig. 2.1; Table 2.1).

**Figure 2.1** Geographical origin of *S. nonagrioides* and *M. unipuncta* populations. Populations of *S. nonagrioides* were collected in six sampling locations ( ▲ ), whereas *M. unipuncta* populations came from two sampling locations (●).

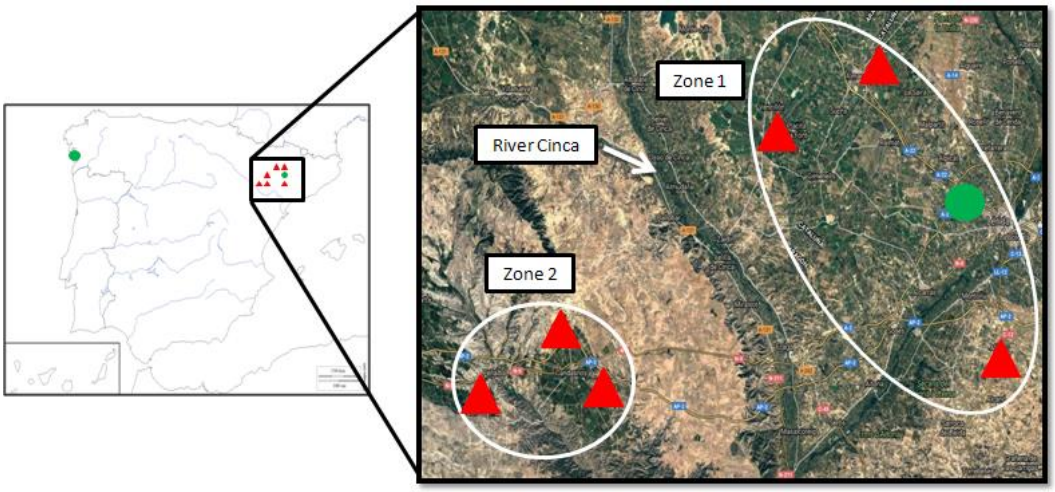



**Table 2.1** Sampling locations of the *S. nonagrioides* populations. All populations were collected in November 2014.

| Zone | Population | Province | GPS coordinates | Initial number of larvae |
|---|---|---|---|---|
| 1 | **Vencillón** | Huesca | N 41º40'55.8" E 0º18'53.3" | 139 |
| | **Alfès** | Lleida | N 41º29'53.4" E 0º37'20.3" | 126 |
| | **Almacelles** | Lleida | N 41º44'27.4" E 0º29'35.4" | 158 |
| 2 | **Bujaraloz** | Zaragoza | N 41º29'00.9" W 0º03'50.4" | 78 |
| | **Candasnos I** | Huesca | N 41º32'31.5" E 0º02'43.4" | 149 |
| | **Candasnos II** | Huesca | N 41º29'20.0" E 0º05'37.6" | 143 |

Each field population included between 78 and 158 larvae that were obtained by slicing up maize stalks with signs of borer damage. Only one larva was collected per plant to avoid collection of siblings, which could bias the results. All larvae were in the last developmental instars when they were collected, and most of them had entered diapause. Individuals from each population were placed in ventilated

boxes in groups of 40-60 larvae and provided with pieces of maize stalk for transportation to the laboratory. Fresh plant material was added daily to guarantee larvae were appropriately fed during the transportation period.

Upon arrival in the laboratory, all larvae were dipped in a 1% bleach solution to eliminate external pathogens and then they were allowed to dry before they were transferred to ventilated plastic boxes (21 x 16 x 4 cm) provided with a meridic diet prepared as described in González-Núñez *et al.* (2000). A thin layer of vermiculite was added to the bottom of each box to facilitate pupation. All boxes were maintained in growth chambers (SANYO MLR-352 PE, Tokyo, Japan) at 16 ± 1 ºC and a 12:12 (L:D) photoperiod. These environmental conditions were aimed to maintain diapause. Every 3-4 days, fresh diet was added to all boxes. When an increase in the pupation rate was observed in a given population, the environmental conditions were shifted to 25 ± 1ºC and a 24:0 (L:D) photoperiod to disrupt diapause. Pupae were removed and transferred to ventilated boxes (Ø 11.5 cm x 4.5 cm high), which were kept in growth chambers at a temperature of 25 ± 1 ºC and a 16:8 (L:D) photoperiod. As adults emerged, they were placed in cages for mating and oviposition, which took place at the same environmental conditions.

To study interpopulation variation in susceptibility to Cry1Ab protein, groups of approximately six pairs of adults of the same population were placed in oviposition cages, consisting of a pot with 8-10 maize seedlings confined by a ventilated methacrylate cylinder closed on top by a mesh cloth adjusted tightly by rubber bands. Between five and ten oviposition cages of this type were set up per population (Fig. 2.2A).

Intrapopulation variation in susceptibility to Cry1Ab protein was studied in three out of the six field populations: Candasnos I, Alfés and Almacelles, the three populations in which the highest number of adults emerged. For this purpose, in each of these populations between 10 and 13 additional oviposition cages containing just three maize seedlings were set up for mating of single pairs of adults.

Seven days later, all adults were removed from both kinds of oviposition cages and the maize stalks were inspected for egg masses. Egg clusters were carefully

removed and placed on top of moistened filter paper in ventilated plastic boxes (Ø 9 cm x 3 cm high), that were kept in growth chambers at 25 ± 1ºC and a 16:8 (L:D) photoperiod until the eggs hatched.

Susceptibility bioassays were performed on neonate larvae (<24 h) of the first generation ($F_1$) of all six field populations, on offspring of both multiple-pairs and single-pair mating. These assays had to be repeated on second generation neonates ($F_2$) of some single adult pairs due to the poor adjustment of the results obtained on $F_1$ larvae to a regression line.

2.2.1.2. *Mythimna unipuncta*

A population collected in Galicia (northwest Spain) was studied as representative from an area where Bt maize had never been sown, whereas one originally from Lleida (northeast Spain) was evaluated as representative from a high Bt maize adoption area (Fig. 2.1).

The Galicia population was started from a lot of 66 L6 larvae and pupae kindly provided by Dr. A. Butrón and Dr. R.A. Malvar, of the Misión Biológica de Galicia (MBG, CSIC) (Pontevedra, Spain). This population was collected in September and October 2015 in an experimental non-Bt maize field located in an area where Bt maize had never been sown commercially, in the MBG (northwestern Spain).

On the other hand, the Lleida population was established from a batch of 92 L6 larvae kindly provided by Dr. M. Eizaguirre (Universitat de Lleida, Lleida, Spain) in October 2015. All individuals were first generation ($F_1$) offspring of adults captured in light traps in the Universitat de Lleida - Institut de Recerca i Tecnologia Agroalimentàries (Lleida, Spain), in September 2015.

All the larvae received from Galicia and Lleida were transferred to 6-well plates (BD Falcon, Erembodegen, Belgium) and reared individually to avoid cannibalism. Larvae were fed with fresh pieces of non-Bt maize leaves until pupation, whereupon pupae were transferred to ventilated boxes (Ø 11.5 cm x 4.5 cm high) for adult emergence. Mating and oviposition took place in ventilated plastic boxes (28 cm x 22 cm x 15 cm) at room conditions. Between 5 and 10 couples of adults were placed

in each box and provided with a solution of watery honey as nourishment and test tubes covered by thin strips of Parafilm M® (Bemis NA, Wisconsin, USA) as a suitable stand for oviposition (Fig. 2.2B). Every 2-3 days, new test tubes were added to the oviposition box and those with egg masses were transferred to plastic boxes (Ø 11.5 cm x 4.5 cm high) with moistened filter paper for egg hatching. Rearing of *M. unipuncta* took place in growth chambers at 23 ± 1 ºC and a 16:8 (L:D) photoperiod.

Susceptibility assays were performed on neonate larvae (<24 h) on the first generation after the arrival of the populations to the laboratory, which corresponded to $F_1$ for Galicia and $F_2$ for Lleida, provided that field collected individuals constituted the parental generation ($F_0$). To study whether susceptibility to Cry1Ab protein of a population from a high Bt maize adoption area decreases after the selection pressure has been removed, larvae of the population collected in Lleida that were not used in the assays performed on $F_2$ neonates were reared in the same fashion for three generations and the assay was repeated on neonates of the $F_5$. For comparison purposes, the same procedure was followed for the population collected in Galicia, in which the susceptibility bioassay was repeated in the $F_4$.

**Figure 2.2** Multiple-pair mating oviposition cages in *S. nonagrioides* (A) and oviposition cages in *M. unipuncta* (B).

### 2.2.2. Source of Cry1Ab protein

The Cry1Ab protein used in the assays was synthesized in Dr. Juan Ferré's laboratory (Universitat de Valencia, Spain) and had a purity of 78.88%. It was obtained from an *Escherichia coli* culture transformed with a plasmid containing the gene Cry1Ab provided by Dr. Ruud de Maagd (Wageningen University, Netherlands).

### 2.2.3. Plant material

Maize leaf tissue was used to assess susceptibility of both noctuid species to Cry1Ab protein. For this purpose, maize plants derived from the event MON 810 expressing Cry1Ab protein (DKC4796YG) and its nearest non-Bt isogenic line (DKC4795) were grown in 8 L pots (Ø 25 cm x 24 cm of height) in the greenhouse, at 25 ± 3 ºC and a 16:8 (L:D) photoperiod. Maize leaves used in the bioassays were obtained from plants that were in the V4-V6 phenological stage.

### 2.2.4. Susceptibility bioassays

#### 2.2.4.1. *Sesamia nonagrioides*

Larval susceptibility to Cry1Ab protein was evaluated by means of diet-overlay bioassays. For this purpose, 50 µl of toxin solution were applied over the surface of the diet (~0.5 ml) contained in each well of a 128-well bioassay tray (Bio-Ba-128, C-D International, Pitman, NJ) and then allowed to dry in a laminar flow cabinet. A neonate larva (<24 h) was then placed in each well and confined using a cover that allowed air circulation (Bio-CV-16, C-D International, Pitman, NJ) (Fig. 2.3). Controls consisted of 50 µl of the buffer where the toxin was diluted, which was applied over the diet following the same procedure. For offspring of multi-pair mating, 4 to 8 replicates of 16 larvae per Cry1Ab protein concentration – 32 larvae in the controls – were tested for each population. Additionally, between 6 and 10 replicates of 16 larvae per toxin concentration – 32 in the controls – were evaluated for offspring of single-pair mating in Candasnos I, Alfés and Almacelles.

Bioassay trays were incubated in a growth chamber at 25 ± 1 ºC and complete darkness. Seven days later, the number of dead larvae and the larval instar of survivors at each toxin concentration were recorded in every replicate. Larvae that did not move when prodded with a fine paintbrush were considered as dead. These data were used to estimate the Cry1Ab dose that killed (lethal concentration, $LC_{50}$) or suppressed molting (molt inhibiting concentration, $MIC_{50}$) in 50% of the larvae. Molt inhibition was calculated based on the number of dead larvae plus surviving larvae that had not molted to second instar.

Eight Cry1Ab protein concentrations were initially prepared on carbonate-bicarbonate buffer (pH 10.5): 0.5, 1, 2, 4, 8, 16, 32 and 64 ng/cm$^2$. These concentrations were subsequently corrected to 4.00, 5.80, 8.41, 12.19, 17.68, 25.64, 37.18 and 53.91 ng/cm$^2$ for better adjustment of the $LC_{50}$ to a regression line, so this parameter was approximately in the middle of the range of concentrations tested.

For comparison purposes, the same method was used to determine susceptibility to Cry1Ab protein of a control laboratory population (CIB, Spain). Three replicates of 16 larvae per toxin concentration – 32 in the controls – were evaluated in this population.

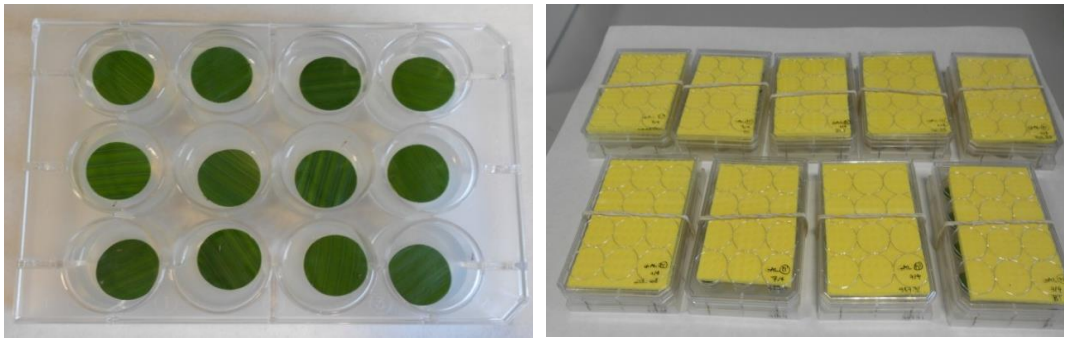
**Figure 2.3** Diet-overlay bioassay tray used to assess susceptibility of *S. nonagrioides* populations to Cry1Ab protein.

Additionally, survival on Bt maize leaf tissue was assessed in each population in the offspring of multiple-pair mating. For this purpose, around 50 $F_1$ neonate larvae (<24 h) of each population were placed in a ventilated plastic box containing moistened filter paper and pieces of fresh Bt maize leaves that were replaced every 2-3 days, and survival was recorded ten days later. The mid-rib was removed from every leaf to avoid larvae reducing exposure to Cry1Ab protein by feeding on this part of the leaf.

#### 2.2.4.2. *Mythimna unipuncta*

Susceptibility to Cry1Ab protein of the two *M. unipuncta* populations was estimated using leaf-disk dipping bioassays, as described in García *et al.* (2015). Disks (Ø 17 mm) were punched out of fresh Bt and non-Bt maize leaves, avoiding the mid-rib, and dipped in a 0.1% solution of the surfactant X-100 Triton. The disks were then air-dried in a laminar flow hood and non-Bt leaf disks were dipped for about five seconds in toxin solutions of concentrations ranging from 0.1 to 1058 µg Cry1Ab/ml in 50 mM carbonate-bicarbonate buffer (pH = 10.5). Non-Bt control disks and Bt leaf disks were dipped in carbonate buffer. All disks were air-dried in a laminar flow hood and then placed individually in each well of 12-wells plates (BD Falcon, Erembodegen, Belgium) over an agar layer for moisture (Fig. 2.4). A neonate larva was placed on each well and plates were shut tightly with rubber bands, placing a plastic screen and a wipe between the cover and the wells to prevent larvae from escaping. Two to five replicates of 12 larvae per toxin concentration were tested per population and generation. The bioassays were conducted in growth chambers at 23 ± 1 ºC and a 16:8 (L:D) photoperiod. Seven days later, larval mortality was recorded. In addition, larval instar and weight of survivors were also recorded to assess the effects of sublethal concentrations of the protein Cry1Ab on larval development and growth of *M. unipuncta*.

**Figure 2.4** Susceptibility assay of *M. unipuncta*. Bioassays were performed using 12-wells plates where treated leaf disks were placed over a thin layer of agar.



### 2.2.5. Statistical analyses

Susceptibility to Cry1Ab protein was estimated for each population based on larval mortality and molt inhibition. For each population and individual pair evaluated, probit analyses were carried out to estimate the $LC_{50}$ and $MIC_{50}$ and their 95% confidence intervals (CI 95%).

In *S. nonagrioides*, variation in susceptibility to Cry1Ab protein was estimated at four different levels: 1) interpopulation variation; 2) between the two studied zones; 3) between each zone and the laboratory strain; and 4) intrapopulation variation between individual pairs belonging to the same population. In *M. unipuncta* variation in their susceptibility to the toxin was first evaluated between the two generations studied in each population ($F_1$ and $F_4$ in the Galicia population; $F_2$ and $F_5$ in the Lleida population). Since no significant differences were observed at that level, data from the two generations of each population were pooled and susceptibility to Cry1Ab protein of the Galicia population was compared with that of the Lleida population.

In both species, lethal and molt inhibition concentration ratios (LCR and MICR, respectively) and their CI 95% were estimated by comparing each pair, generation, population or area's $LC_{50}$ or $MIC_{50}$ with that of the most susceptible one. Confidence intervals that included the value 1 indicated there were no significant

differences between the compared values. The software PoloPC (LeOra Software, 1987) was used in these calculations.

## 2.3. Results

### 2.3.1. *Sesamia nonagrioides*

Interpopulation variation in susceptibility to Cry1Ab protein of the *S. nonagrioides* populations collected in the Ebro Valley is shown in Table 2.2, based on lethal (A) and molt inhibiting concentrations (B). Low variation in susceptibility to Cry1Ab was observed between populations of Zone 1 when either $LC_{50}$ values (ranging from 17 to 32 ng/cm$^2$) or $MIC_{50}$ values (ranging from 9 to 16 ng/cm$^2$) were considered, which resulted in a low LCR [1.9 (1.6-2.2)] and MICR [1.7 (1.5-2.1)] when susceptibility to the Bt protein of the most (Vencillón) and the least (Almacelles) susceptible populations of this zone was compared. Variation in susceptibility to Cry1Ab protein was slightly higher between populations belonging to Zone 2, where $LC_{50}$ values ranged from 13 to 32 ng/cm$^2$, accounting for a LCR of 2.8 (2.3-3.4) between the most (Bujaraloz) and the least (Candasnos I) susceptible populations in this zone. A similar trend was observed in $MIC_{50}$ values, which ranged from 7 to 19 ng/cm$^2$, with a MICR of 2.5 (2.1-2.9) between Bujaraloz and Candasnos I. Given the low variation observed between populations belonging to each zone (< 3-fold in both cases), data from the three populations of Zone 1 were pooled and compared with pooled data of Zone 2 populations. The results indicate susceptibility to Cry1Ab protein did not differ significantly between both zones, neither when $LC_{50}$ [LCR=1.1 (1.0-1.2)] nor when $MIC_{50}$ [MICR= 1.0 (0.9-1.1)] values were considered (Table 2.2).

The values of susceptibility to Cry1Ab estimated in the laboratory colony were of $LC_{50}$ of 18 (12-22) ng/cm$^2$ and a $MIC_{50}$ of 14 (7-18) ng/cm$^2$ (Table 2.2). These values were in the same range of those observed in Zones 1 and 2 based on values of both LCR [1.3 (1.1-1.6) and 1.2 (1.0-1.5) when compared with Zone 1 and Zone 2, respectively] and MICR [0.9 (0.7-1.1) and 0.8 (0.7-1.1) when compared with Zones 1 and 2, respectively].

**Table 2.2** Interpopulation variation in the susceptibility to Cry1Ab of *S. nonagrioides* populations from six locations in two zones of the Ebro Valley. Susceptibility to the Bt toxin was measured by lethal concentrations (A) and molt inhibiting concentrations (B).

| Zone | Population | N[a] | (A) Lethal concentration | | | | | | | (B) Molt inhibiting concentration | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Slope (SE) | $\chi^2$ | df | $LC_{50}$ (CI 95%)[b] | $LC_{90}$ (CI 95%)[b] | LCR ($LC_{50}$) (CI 95%)[c] | LCR ($LC_{50}$) (CI 95%)[d] | Slope (SE) | $\chi^2$ | df | $MIC_{50}$ (CI 95%)[b] | $MIC_{90}$ (CI 95%)[b] | MICR ($MIC_{50}$) (CI 95%)[c] | MICR ($MIC_{50}$) (CI 95%)[d] |
| 1 | Vencillón | 1275 | 2.8 (0.2) | 156 | 62 | 17 (15-20) | 50 (40-67) | 1 | | 3.0 (0.2) | 211 | 62 | 9 (7-11) | 24 (19-33) | 1 | |
| | Alfés | 636 | 2.8 (0.3) | 99 | 30 | 20 (14-26) | 56 (39-112) | 1.1 (0.9-1.4) | 1.1 (1.0-1.2) | 2.8 (0.3) | 141 | 30 | 11 (16-22) | 32 (22-72) | 1.2 (1.0-1.5) | 1 |
| | Almacelles | 1273 | 2.9 (0.3) | 210 | 62 | 32 (26-40) | 90 (64-190) | 1.9 (1.6-2.2)* | | 2.5 (0.2) | 306 | 62 | 16 (11-20) | 52 (38-95) | 1.7 (1.5-2.1)* | |
| 2 | Bujaraloz | 792 | 2.6 (0.2) | 179 | 38 | 13 (10-16) | 41 (30-71) | 1 | | 2.7 (0.2) | 209 | 38 | 7 (4-9) | 20 (15-37) | 1 | |
| | Candasnos I | 956 | 2.8 (0.2) | 205 | 46 | 32 (26-43) | 95 (64-191) | 2.8 (2.3-3.4)* | 1 | 2.3 (0.2) | 331 | 46 | 19 (14-26) | 66 (42-161) | 2.5 (2.1-2.9)* | 1.0 (0.9-1.1) |
| | Candasnos II | 1115 | 2.0 (0.1) | 369 | 54 | 22 (16-31) | 96 (57-276) | 1.8 (1.5-2.1)* | | 2.5 (0.2) | 367 | 54 | 12 (9-15) | 39 (28-70) | 1.7 (1.4-1.9)* | |
| | Laboratory | 480 | 3.9 (0.5) | 63 | 22 | 18 (12-22) | 37 (29-64) | - | - | 4.0 (0.6) | 79 | 22 | 14 (7-18) | 29 (22-60) | - | - |

[a] Number of neonate larvae tested, including controls.

[b] 50% and 90% lethal concentrations ($LC_{50}$ and $LC_{90}$) and molt inhibiting concentrations ($MIC_{50}$ and $MIC_{90}$) and their 95% confidence intervals (CI 95%) are expressed in ng $cm^{-2}$

[c] $LC_{50}$ and $MIC_{50}$ are significantly different ($p < 0.05$) if the 95% confidence interval of the lethal concentration ratio (LCR) or the molt inhibiting concentration ratio (MICR) does not include 1. Within a zone, asterisks in a population indicate susceptibility to Cry1Ab protein significantly lower from that of the most susceptible population, marked in that column by the number 1.

[d] Data of the three populations in each zone were pooled to calculate $LC_{50}$ and $MIC_{50}$ per zone. $LC_{50}$ and $MIC_{50}$ are significantly different ($p < 0.05$) if the 95% confidence interval of the LCR or the MICR does not include 1. No significant differences in susceptibility to the toxin Cry1Ab were obtained between the most susceptible zone, marked in that column by the number one, and the other zone.

Regarding intrapopulation variation in susceptibility to Cry1Ab (Table 2.3), in Alfés, $LC_{50}$ values ranged from 9 to 42 ng/cm$^2$ and $MIC_{50}$ values ranged from 5 to 31 ng/cm$^2$, which translated into a LCR of 4.6 (2.5-8.5) and a MICR of 6.3 (3.8-10.4) between the most and the least susceptible pairs. Similar variation in susceptibility to Cry1Ab protein was observed between pairs of adults from Almacelles when lethal concentration values were considered ($LC_{50}$= 11-42 ng/cm$^2$), although a slightly higher variation was observed when molt inhibition was considered ($MIC_{50}$= 2-35 ng/cm$^2$). This resulted in a LCR of 3.7 (2.7-5.0) and a MICR of 14.4 (2.7-77.2) between the most and the least susceptible pairs. The lowest variation in susceptibility to Cry1Ab protein was observed between pairs of adults of Candasnos I, either when lethal ($LC_{50}$= 11-27 ng/cm$^2$) or molt inhibition ($MIC_{50}$= 9-21 ng/cm$^2$) was considered. The differences in susceptibility to Cry1Ab protein between the least and the most susceptible pair in this population were below 3-fold [LCR= 2.5 (2.0-3.2); MICR= 2.4 (1.9-3.1)].

Finally, no survival or molting were recorded in neonates of the laboratory population or in larvae of any of the field populations after eight days feeding on leaf tissue of Bt maize plants.

### 2.3.2. *Mythimna unipuncta*

In the Galicia population, results of the concentration ratio analyses show there were no significant differences in susceptibility to the Bt protein between the first and the fourth generations, both when the LCR [1.2 (0.3-5.5)] and the MICR [1.3 (0.5-3.6)] were considered. Therefore, data of the two generations tested were pooled to obtain one set from Galicia. Similar results were observed in Lleida, in which no significant differences in susceptibility to Cry1Ab protein were observed between the second and the fifth generations, as indicated by their LCR [1.6 (0.2-12.8)] and their MICR [3.5 (0.9-13.9)]. Given these results, data were pooled into one set from Lleida (data of the probit analyses not shown).

Susceptibility to Cry1Ab protein in the populations from Galicia and Lleida, measured by their $LC_{50}$ and $MIC_{50}$ values, is shown in Table 2.4. Similar values of

**Table 2.3** Intrapopulation variation in susceptibility to Cry1Ab protein of three populations of *S. nonagrioides* from the Ebro Valley. Susceptibility was tested in the offspring of single pairs of adults, and measured by lethal concentrations (A) or molt inhibiting concentrations (B).

| Zone | Population | Parental pair | N[a] | (A) Lethal concentration | | | | | | (B) Molt inhibiting concentration | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Slope (SE) | $\chi^2$ | df | $LC_{50}$ (CI 95%)[b] | $LC_{90}$ (CI 95%)[b] | LCR ($LC_{50}$) (CI 95%)[c] | Slope (SE) | $\chi^2$ | df | $MIC_{50}$ (CI 95%)[b] | $MIC_{90}$ (CI 95%)[b] | MICR ($MIC_{50}$) (CI 95%)[c] |
| 1 | Alfés | P1[d] | 317 | 3.4 (0.4) | 8 | 6 | 9 (7-11) | 22 (17-33) | 1 | 3.9 (0.5) | 14 | 6 | 8 (5-11) | 17 (13-31) | 1.6 (1.2-2.1)* |
| | | P2 | 160 | 4.2 (0.8) | 3 | 6 | 22 (18-27) | 44 (35-68) | 2.4 (1.8-3.2)* | 7.8 (1.7) | 5 | 6 | 12 (10-14) | 18 (15-24) | 2.4 (1.9-3.2)* |
| | | P3 | 159 | 5.7 (1.1) | 5 | 6 | 32 (27-37) | 53 (43-77) | 3.4 (2.7-4.4)* | 6.3 (1.1) | 6 | 6 | 20 (17-13) | 32 (27-42) | 4.0 (3.1-5.2)* |
| | | P4 | 160 | 6.1 (1.1) | 7 | 6 | 30 (25-37) | 49 (39-81) | 3.3 (2.5-4.2)* | 6.4 (1.1) | 5 | 6 | 25 (22-29) | 40 (34-54) | 5.1 (4.0-6.6)* |
| | | P5 | 160 | 2.2 (0.5) | 7 | 6 | 42 (28-117) | 163 (74-2500) | 4.6 (2.5-8.5)* | 2.0 (0.4) | 7 | 6 | 31 (21-64) | 135 (65-1059) | 6.3 (3.8-10.4)* |
| | | P6 | 159 | 3.1 (0.5) | 11 | 6 | 11 (7-16) | 29 (20-76) | 1.2 (0.9-1.7) | 6.4 (1.5) | 1 | 6 | 5 (4-6) | 8 (7-11) | 1 |
| | Almacelles | P1 | 160 | 3.3 (0.5) | 8 | 6 | 11 (8-15) | 28 (20-56) | 1 | 4.8 (0.9) | 6 | 6 | 7 (6-9) | 14 (11-23) | 3.0 (0.6-16.4) |
| | | P2 | 159 | 6.2 (1.4) | 1 | 6 | 42 (36-51) | 67 (54-112) | 3.7 (2.7-5.0)* | 5.7 (1.1) | 2 | 6 | 35 (30-42) | 59 (48-91) | 14.4 (2.7-77.2)* |
| | | P3 | 160 | 4.1 (0.7) | 2 | 6 | 28 (23-34) | 57 (44-89) | 2.4 (1.8-3.3)* | 4.2 (1.0) | 5 | 6 | 19 (14-23) | 38 (30-61) | 7.6 (1.4-41.7)* |
| | | P4 | 159 | 3.1 (0.5) | 9 | 6 | 20 (14-28) | 51 (34-124) | 1.7 (1.2-2.5)* | 3.9 (0.6) | 3 | 6 | 10 (8-13) | 22 (18-32) | 4.3 (0.8-23.0) |
| | | P5 | 160 | 4.1 (0.6) | 5 | 6 | 14 (12-16) | 28 (23-39) | 1.2 (0.9-1.6) | 3.9 (0.6) | 3 | 6 | 8 (7-10) | 18 (14-26) | 3.3 (0.6-18.0) |
| | | P6 | 157 | 2.8 (0.6) | 13 | 6 | 18 (10-28)[e] | 51 (32-187)[e] | 1.6 (0.9-2.8) | 4.0 (0.8) | 9 | 6 | 7 (4-10) | 15 (10-38) | 2.9 (0.5-16.2) |
| | | P7 | 159 | 3.3 (0.5) | 10 | 6 | 12 (8-17) | 29 (20-67) | 1.2 (0.8-1.7) | 3.1 (1.2) | 4 | 6 | 2 (1-4)[e] | 6 (5-9)[e] | 1 |
| | | P8 | 159 | 2.9 (0.4) | 12 | 6 | 13 (9-21) | 37 (23-124) | 1.1 (0.7-1.5) | 2.3 (0.4) | 8 | 6 | 7 (4-10) | 25 (16-74) | 2.9 (0.5-16.1) |
| | | P9[d] | 313 | 4.4 (0.8) | 32 | 14 | 24 (15-32) | 48 (36-109) | 2.1 (1.5-3.0)* | 4.9 (1.0) | 66 | 14 | 20[f] | 37[f] | 8.1 (0.2-325.7) |
| | | P10[d] | 317 | 7.9 (1.8) | 2 | 6 | 17 (14-18) | 24 (21-31) | 1.5 (1.1-1.9)* | 7.2 (1.4) | 6 | 6 | 14 (11-16) | 21 (18-29) | 5.8 (1.1-30.9)* |
| 2 | Candasnos I | P1[d] | 320 | 5.3 (0.6) | 17 | 6 | 11 (8-14) | 19 (15-33) | 1 | 7.9 (1.2) | 7 | 6 | 9 (7-10) | 13 (11-16) | 1 |
| | | P2 | 159 | 3.7 (0.5) | 8 | 6 | 20 (15-27) | 46 (33-92) | 1.9 (1.4-2.4)* | 6.1 (1.0) | 3 | 6 | 16 (14-19) | 26 (22-35) | 1.9 (1.5-2.3)* |
| | | P3 | 160 | 3.1 (0.5) | 5 | 6 | 16 (12-20) | 40 (30-66) | 1.4 (1.0-1.9) | 3.2 (0.6) | 7 | 6 | 9 (5-12) | 22 (15-49) | 1.0 (0.7-1.5) |
| | | P4[d] | 315 | 4.6 (0.9) | 22 | 14 | 18 (12-22) | 34 (27-56) | 1.6 (1.2-2.1)* | 3.9 (0.6) | 21 | 14 | 14 (9-18) | 29 (23-46) | 1.6 (1.2-2.1)* |
| | | P5[d] | 318 | 3.6 (0.7) | 19 | 14 | 20 (14-24) | 45 (35-80) | 1.8 (1.4-2.3)* | 5.7 (1.1) | 21 | 14 | 15 (11-18) | 25 (21-36) | 1.7 (1.4-2.1)* |
| | | P6[d] | 314 | 4.4 (0.9) | 4 | 6 | 27 (22-32) | 54 (44-80) | 2.5 (2.0-3.2)* | 4.3 (0.8) | 5 | 6 | 21 (17-24) | 41 (34-56) | 2.4 (1.9-3.1)* |

[a] Number of neonate larvae tested, including controls.

[b] Lethal concentrations ($LC_{50}$ and $LC_{90}$) and molt inhibiting concentrations ($MIC_{50}$ and $MIC_{90}$) and their 95% confidence intervals (CI 95%) are expressed in ng $cm^{-2}$

[c] $LC_{50}$ and $MIC_{50}$ are significantly different ($p < 0.05$) if the 95% confidence interval of the lethal concentration ratio (LCR) or the molt inhibiting concentration ratio (MICR) does not include 1. Within a population, asterisks in a parental pair indicate susceptibility to Cry1Ab protein is significantly lower from that of the most susceptible pair, marked in that column by the number 1.

[d] Susceptibility to Cry1Ab protein was evaluated in neonates of the $F_2$.

[e] CI 95% could not be estimated because the coefficient g was > 0.5 at the 95% probability level. CI 90% are shown in the table.

[f] Neither CI 95%, nor CI 90% could be estimated.

susceptibility to the Bt protein were obtained in both populations when this parameter was measured by either the $LC_{50}$ (137 and 123 µg Cry1Ab/ml, respectively) or the $MIC_{50}$ (54 and 69 µg Cry1Ab/ml, respectively). Additionally, in both the Galicia and the Lleida populations, the values of $LC_{90}$ and $MIC_{90}$ values were more than 19 times larger than those of $LC_{50}$ and $MIC_{50}$. No significant differences in susceptibility to Cry1Ab protein were detected between the two populations, as pointed out by the values of the LCR [1.1 (0.2-6.1)] and the MICR [1.3 (0.1-22.6)]. The high similarity in $LC_{50}$ and $MIC_{50}$ values obtained in the populations from Galicia and Lleida is shown graphically by the probit lines representing mortality and molt inhibition per toxin concentration in these populations (Fig. 2.5). In this figure it can be observed that both populations displayed a probit mortality and molt inhibition of 5, corresponding to the $LC_{50}$ and $MIC_{50}$, respectively, at similar values of Cry1Ab protein concentration.

**Table 2.4** Interpopulation variation in susceptibility of *M. unipuncta* populations from Galicia and Lleida to Cry1Ab protein, measured by lethal concentrations (A) and molt inhibiting concentrations (B).

**(A) Lethal concentrations**

| Population | N[a] | Slope (SE) | $\chi^2$ | df | $LC_{50}$ (CI 95%)[b] | $LC_{90}$ (CI 95%)[b] | LCR ($LC_{50}$) (CI 95%)[c] |
|---|---|---|---|---|---|---|---|
| **Galicia** | 1202 | 0.9 (0.1) | 80 | 34 | 137 (68-257) | 4472 (1517-47610) | 1.1 (0.2-6.1) |
| **Lleida** | 1032 | 0.7 (0.2) | 160 | 66 | 123[d] | 9388[d] | 1 |

**(B) Molt inhibiting concentrations**

| Population | N[a] | Slope (SE) | $\chi^2$ | df | $MIC_{50}$ (CI 95%)[b] | $MIC_{90}$ (CI 95%)[b] | MICR ($MIC_{50}$) (CI 95%)[c] |
|---|---|---|---|---|---|---|---|
| **Galicia** | 1202 | 1.0 (0.1) | 47 | 34 | 54 (33-80) | 1032 (584-2487) | 1 |
| **Lleida** | 1032 | 0.9 (0.3) | 153 | 66 | 69[d] | 2018[d] | 1.3 (0.1-22.6) |

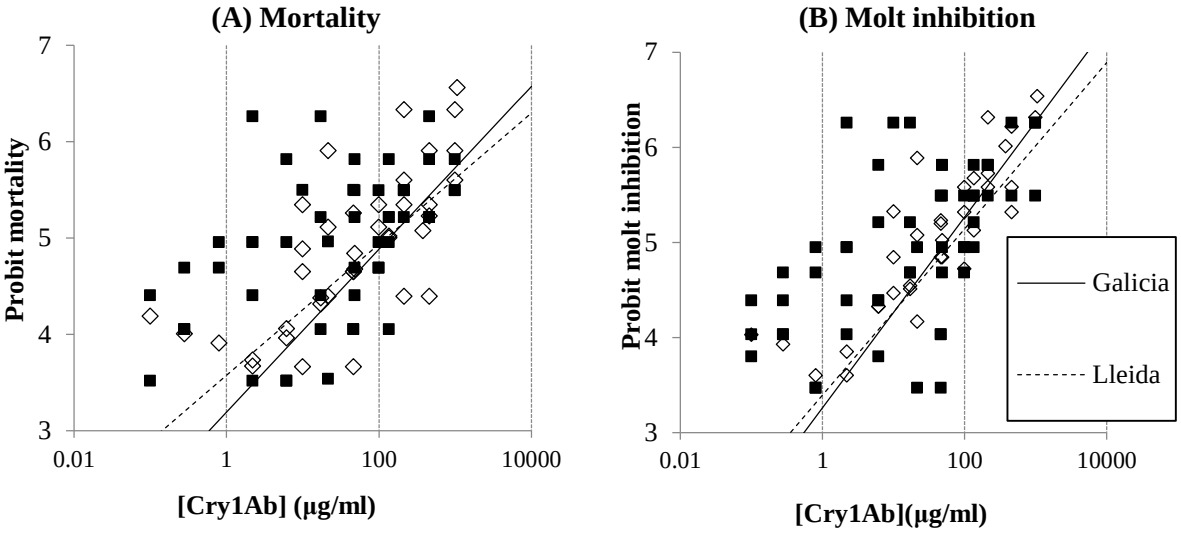
[a] Number of neonate larvae tested in leaf disks dipped in different toxin concentrations, including controls.

[b] Lethal concentrations ($LC_{50}$ and $LC_{90}$) and molt inhibiting concentrations ($MIC_{50}$ and $MIC_{90}$) and their 95% confidence intervals (CI 95%) are expressed in µg Cry1Ab $ml^{-1}$

[c] $LC_{50}$ and $MIC_{50}$ are significantly different ($p < 0.05$) if the 95% confidence interval of the lethal concentration ratio (LCR) or the molt inhibiting concentration ratio (MICR) does not include 1. No significant differences were found.

[d] Neither CI 95%, nor CI 90% could be estimated.

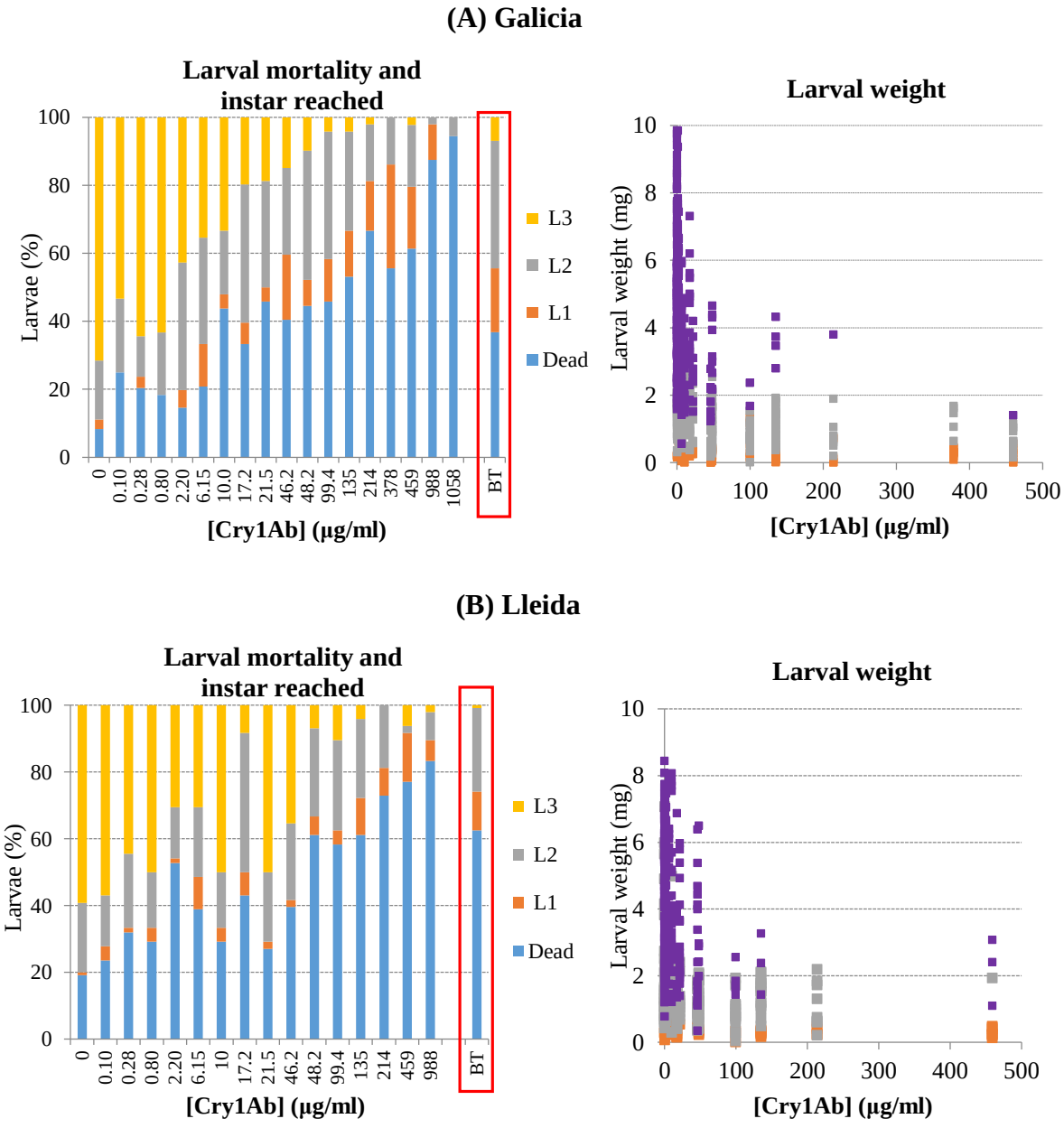
**Figure 2.5.** Mortality (A) and molt inhibition (B) per Cry1Ab protein concentration (µg/ml) in *M. unipuncta* populations from Galicia (■) and Lleida (◇).




Delayed development and growth inhibition associated with increasing toxin concentrations were recorded in survivors of the assays in both the Galicia and the Lleida populations, following a similar trend (Fig. 2.6). The delay in larval development was indicated by the low proportion of larvae that had reached the third larval instar after the 7-day bioassay, which was lower than 20% for concentrations over 21.5 µg Cry1Ab/ml in larvae from the Galicia population and in doses over 48.2 µg Cry1Ab/ml in larvae from the Lleida population, whereas 72% (Galicia) and 59% (Lleida) L3 larvae were recorded in the controls. Additionally, the lower larval weight recorded in L3 larvae of both populations with increasing concentrations of Cry1Ab protein suggested survivors of the assay suffered growth inhibition, although a high variability was observed in this parameter. Regarding larvae fed with Bt leaf disks for 7 days, mortality values similar to those obtained at intermediate Cry1Ab doses were recorded in the Galicia population (37%), whereas higher mortality, close to values recorded at high Cry1Ab concentrations, was observed in the population from Lleida (63%). In addition, larvae of both populations that survived after 7 days feeding on Bt maize suffered a delay in larval development, with a low proportion of L3 larvae in the populations from Galicia (7%) and Lleida (1%). Given the low number of larvae that reached the third instar in this treatment, their weights could not be compared. However, growth inhibition was noticeable in L2 larvae of both populations, as

indicated by the lower mean weights recorded in larvae fed with Bt maize (0.87 ± 0.05 mg in the Galicia population; 0.95 ± 0.08 mg in the Lleida population) in comparison with those recorded in the controls (1.57 ± 0.10 mg and 2.00 ± 0.33 mg in the Galicia and Lleida populations, respectively).

**Figure 2.6** Larval mortality, larval instar and weight after seven days' exposure to non-Bt maize leaf disks dipped in different concentrations of Cry1Ab protein or to Bt maize leaf disks of *M. unipuncta* populations from Galicia (A) and Lleida (B).



## *2.4. Discussion*

Programs for monitoring corn borers resistance to Bt maize have been in place in Spain since 1998 (Castañera *et al.*, 2016). Recently, resistance monitoring guidelines were modified according to EFSA's recommendations, so that from 2016 monitoring is undertaken on a yearly basis and it focuses on the Ebro Valley, where several populations of *S. nonagrioides* are collected in different zones of intense and regular Bt maize cultivation (EFSA, 2015; EFSA Panel on GMO, 2017). The modification of the sampling procedure is an attempt to focus resistance monitoring in those areas where resistance is more likely to develop. The information provided by this study is important for the development of more effective resistance monitoring programs because it underpins the accuracy with which shifts in susceptibility of *S. nonagrioides* populations indicative of resistance evolution can be identified.

Our findings indicate there is low inter and intrapopulation variation in susceptibility to Cry1Ab protein in field populations of *S. nonagrioides* from the two areas surveyed in the Ebro Valley, as pointed out by the low resistance ratios observed between and within populations, both when lethal (<5-fold) and molt inhibiting (commonly <10-fold) concentrations were considered. This variation in susceptibility to Cry1Ab toxin is in the same range of that observed in Spanish *S. nonagrioides* populations at a larger scale, between field populations from different maize growing regions (Farinós *et al.*, 2004). Additionally, similar variation in susceptibility to Cry1Ab protein was reported in field populations of the noctuid *Helicoverpa zea* collected in different states in the US, ranging between 3 and 5-fold depending on whether $LC_{50}$ or $MIC_{50}$ values were considered (Siegfried *et al.*, 2000). On the other hand, the absence of survival and molting in neonate larvae fed with Bt maize leaf tissue for 10 days reported in this study agrees with the results obtained in a study in which over 8,000 larvae of *S. nonagrioides* populations collected in the Ebro Valley were subjected to the same assay (Farinós *et al.*, 2018). The results reported here suggest that, given that no populations with a significantly lower susceptibility to the protein Cry1Ab were detected, monitoring should

continue to consider different zones within the Ebro Valley, since we have not detected any particular zone where resistance development is more likely.

The secondary maize pest *M. unipuncta* has been reported to cause yield losses sporadically in the Ebro Valley, where selection pressure due to the intense cultivation of Bt maize is high (Eizaguirre *et al.*, 2010). A study reported that a population of *M. unipuncta* from the Ebro Valley was able to develop resistance to MON 810 maize after 5-12 generations of laboratory selection, so that 22-57% of the larvae completed their life cycle on Bt maize plants (González-Cabrera *et al.*, 2013). In addition, low susceptibility to Cry1Ab toxin has been reported for this species in populations collected in the Ebro Valley (Eizaguirre *et al*., 2010; Pérez-Hedo *et al*., 2012; González-Cabrera *et al*., 2013). However, these field data were obtained long after the deployment of commercial cultivation of Bt maize. Thus, a decrease in susceptibility to the toxin could have occurred in *M. unipuncta* populations collected in the Ebro Valley with respect to populations from other areas without Bt maize. The data presented here reveal that, even though Bt maize has never been sown commercially in Galicia, susceptibility to Cry1Ab protein of *M. unipuncta* populations from this area ($LC_{50}$ = 137 µg Cry1Ab/ml) was not significantly different from that of populations collected in Lleida ($LC_{50}$= 123 µg Cry1Ab/ml), where Bt maize has been sown on a steady basis for the last twenty years with an increasing adoption that exceeded 50% of all maize every year of the last decade. Ingestion of either Bt maize or maize leaf disks treated with different concentrations of Cry1Ab resulted in a similar delay in larval development and growth inhibition in both populations. Delayed larval development associated with exposure to sublethal doses of Cry1Ab protein has also been observed in other species of noctuids, including *Spodoptera littoralis* (Dutton *et al.*, 2005), *Spodoptera frugiperda* and *H. zea* (Chilcutt *et al.*, 2007). However, in accordance with the low susceptibility of this species to Bt maize, Pérez-Hedo *et al.* (2012) found no differences in weight gain between larger (L6) *M. unipuncta* larvae fed on a diet containing Bt maize leaves and larvae fed on a diet without this Bt component. There was a remarkable difference between $LC_{50}$ and $LC_{90}$ values and $MIC_{50}$ and $MIC_{90}$ values (>19-fold in both cases) in both the Galicia and the Lleida populations, in comparison with the highly susceptible species *S. nonagrioides*,

where this range is usually below 5-fold. This contrast would support the low susceptibility of this secondary pest to the toxin Cry1Ab, since some individuals were not killed by the highest concentrations tested in the bioassays. Taken together, these results indicate that resistance to Bt maize has not evolved in Spanish field populations of *M. unipuncta*. Indeed, the fact that only the second generation (out of four) of *M. unipuncta* has been observed to produce yield losses in maize fields in Lleida (López *et al*., 2000), would reduce its exposure to Bt maize. Nevertheless, the naturally low susceptibility to Cry1Ab protein of this species and its potential to develop resistance to Cry1Ab protein rapidly (González-Cabrera *et al.*, 2013) suggest that this secondary pest should not be overlooked in the Bt maize resistance monitoring program in Spain. Interestingly, given that this is the first report of the susceptibility of *M. unipuncta* populations from Galicia to Cry1Ab, the $LC_{50}$ value estimated in this population could be considered a baseline susceptibility of *M. unipuncta* to Cry1Ab protein in forthcoming IRM programs.

# III. Performance of *Sesamia nonagrioides* on cultivated and wild host plants: implications for Bt maize resistance management

## *3.1. Introduction*

Host selection is a key factor that affects survival and performance of phytophagous insects. Selection of suitable host plants for oviposition is especially important in Lepidoptera, given that neonate larvae are usually relatively immobile and tend to remain in the plant where they emerged (Thompson and Pellmyr, 1991; Renwick and Chew, 1994). Accordingly, the "preference-performance" hypothesis predicts that female adults will lay their eggs preferentially in hosts that are optimal for the development of their offspring (Jaenike, 1978). However, oviposition on hosts of low quality or unsuitable for larval development has often been reported (Scheirs *et al.*, 2000; Liu *et al.*, 2011), and different theories have been put forward to explain this fact (Thompson, 1988; Mayhew, 2001). Gaining knowledge on the range of hosts that can be used by insect pests and their preference among them is essential for pest management, including that of pests affecting crops that express *Bacillus thuringiensis* toxin genes (e.g. Bt maize). Furthermore, insect resistance management (IRM) strategies should also consider this issue, as resistance evolution is expected to occur faster in monophagous pests feeding on Bt crops, which are subjected to a high selective pressure in comparison with polyphagous pests, which can feed on a wider range of plant species (Georghiou and Taylor, 1977; Gould, 1988).

The Mediterranean corn borer, *S. nonagrioides,* is a major pest of maize (Castañera, 1986), sorghum (Dimou *et al.*, 2007; Mantzoukas *et al.*, 2015) and rice (Ntanos and Koutroubas, 2000; Esfandiari *et al*., 2015) in the Mediterranean area. In Spain it is highly specialized in maize, being considered its primary host. Nevertheless, it is a polyphagous species, since it has been recorded in a wide range of cultivated and wild host species of the Poaceae, Cyperaceae and Thyphaceae families (Kfir *et. al.*, 2002; Le Rü *et al.*, 2006). Moreover, because it is a multivoltine species, *S. nonagrioides* can switch from maize to some of their potential wild hosts (e.g. *Sorghum halepense*, *Typha domingensis*, *Phragmites australis*, *Arundo donax*), which are commonly found within or close to maize fields in Spain. Recently, a resistance evolution model for *S. nonagrioides* was developed by our group taking into account more than 20 variables known to affect the rate of resistance

development, including aspects of the pest biology and genetics and the agronomic practices in the area (Castañera *et al*., 2016). This model considered *S. nonagrioides* as a functional monophagous on maize in the Ebro Valley, which would therefore be subjected to a high selective pressure by the high adoption rate of Bt maize in this area. Determining the potential range of non-Bt alternative crops and/or weeds or grasses where *S. nonagrioides* can complete its life cycle and establishing its preference among them would help to improve this resistance evolution model and optimize the ongoing IRM strategies in this area.

In this context, the use of alternative hosts as unstructured refuges that foster susceptible populations of target pests has been proposed as a strategy to delay resistance development (MacIntosh, 2010). Some authors have argued that using natural refuges could reduce and sometimes even suppress the need of planting structured refuges composed of non-Bt varieties (Qiao *et al.*, 2010). In principle, refuges can be non-Bt varieties of the Bt crop or any other plant, provided it hosts sufficiently large pest populations mating randomly with populations from Bt fields (Leniaud *et al*., 2006).

On the other hand, multivoltine species such as *S. nonagrioides* are subjected to variations in the nutritional and morphological quality of the host plant in the time interval between generations, which could influence oviposition, larval survival and the size of the population exposed to Bt toxins. The latter parameter has been proved to affect the rate of resistance evolution to Bt crops, so that having a realistic estimate on the size of the pest population exposed to the Bt crop is essential to generate a reliable resistance evolution model (Sisterson *et al.*, 2004). *Sesamia nonagrioides* presents two generations and a partial third in the Ebro Valley every year (Eizaguirre *et al.*, 2002), but no estimates on the actual size of each generation are available. Generation size was estimated for the *S. nonagrioides* resistance evolution model by means of female fecundity of each generation, given the important and positive association known between the two variables. For this purpose, fecundity of females reared in the laboratory under the photoperiods experienced by larvae and adults of the different generations in the field was used as a proxy (Castañera *et al.*, 2016). However, photoperiod is not the only variable

that changes between generations, the phenology of maize plants is also different in the three generations, owing to the aging of the plants (Castañera *et al*., 2016). Acquiring more information on the variation of female fecundity according to the age of maize plants could give us a better estimate of the size of each generation of *S. nonagrioides*, and therefore help us improve the resistance evolution model for this pest species.

This chapter has two aims. First, to investigate whether cultivated and wild plant species reported to be potential hosts of *S. nonagrioides* are suitable for larval development and oviposition of this pest, as well as its preference among the cultivated species. These data will help to determine whether these plant species could act as unstructured refuges. Second, to evaluate the oviposition performance of *S. nonagrioides* females at the two phenological stages of maize met in the field by females of the 2$^{nd}$ and 3$^{rd}$ generation of this pest, anthesis (VT) and dough stage (R4), respectively. The information obtained will help to improve the management of resistance of *S. nonagrioides* to Bt maize.

## *3.2. Material and methods*

### 3.2.1. Plant material

We tested the host suitability for oviposition and larval performance of *S. nonagrioides* of three crop plants – *Zea mays* (maize), *Oryza sativa* (rice) and *Sorghum bicolor* (sorghum) – and four wild host plants that are frequently found within maize fields – the weed *Sorghum halepense* (johnsongrass) – or close to them: *Typha domingensis* (cattail), *Phragmites australis* (common reed) and *Arundo donax* (giant reed) . All plant species belong to the family Poaceae, except cattail, which belongs to the family Typhaceae. Additionally, maize plants were used to study the oviposition performance of *S. nonagrioides* female at the two plant phenological stages mentioned above.

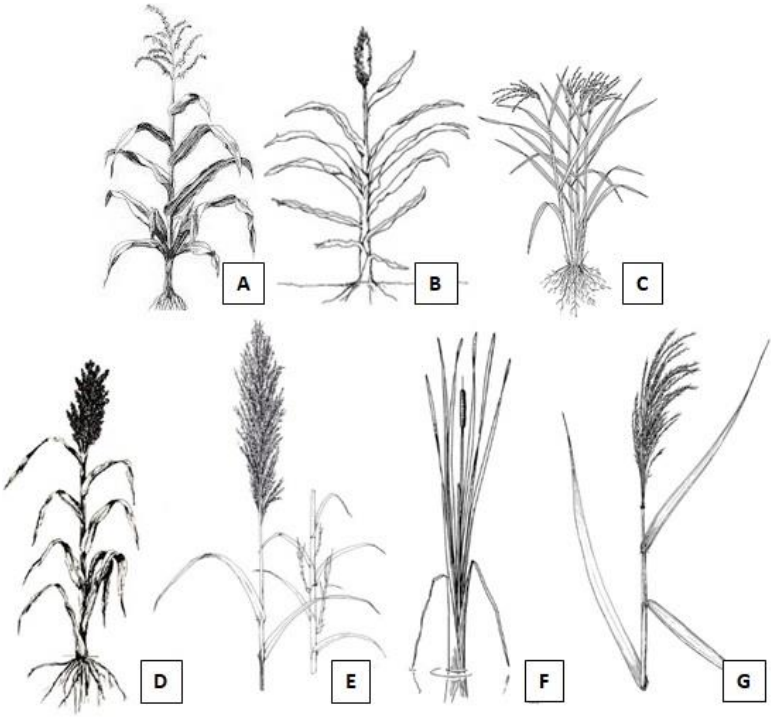
Germinated seeds of maize (cv. DKC4795) were sown in 28-wells seedbed trays using Compo Sana Universal® soil (Compo Agricultura SL, Barcelona, Spain) and kept in a growth chamber for approximately 14 days. When seedlings reached the V2 stage, they were transferred to 8 L pots (Ø 25 cm x 24 cm high) and moved to

a greenhouse to continue their development. Sorghum plants (cv. Express Rojo) were grown following the same procedure. Germinated rice seeds (cv. Gleva) were sown in 28-wells seedbed trays containing a mixture of 63.5% peat (CompoAgricultura SL, Barcelona, Spain), 36.5% vermiculite (Distribuciones Quimebora SL, Madrid, Spain) and 0.63 g of $CaCO_3$ (Manuel Riesgo S.A., Madrid, Spain) per liter of soil and maintained in growth chambers (SANYO MLR-350 H, Tokyo, Japan) for around three weeks. Once the stem had branched, plants were transferred individually to 2.5 L pots (Ø 16 cm x 15 cm high) containing the same soil mixture and a tablet of fertilizer, with a composition of 15% nitrogen, 10% phosphorus, 12% potassium and 2% MgO (Osmocote®, KB, France), and moved to a greenhouse.

All the wild species were grown in the greenhouse in 8 L pots (Ø 25 cm x 24 cm high). Johnsongrass plants were collected in an experimental field located in San Fernando de Henares (Madrid, Spain), whereas cattail plants were collected in the river Pantueña, in Valverde de Alcalá (Madrid). Upon arrival to the laboratory, all plants of both species were carefully checked to remove insects and other fauna before transferring them to the greenhouse. On the other hand, common reed plants were obtained from Ecodena S.L. (Sevilla, Spain), and giant reed plants were grown from rhizomes kindly provided by Dr. Cristina Chueca (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain). Johnsongrass and giant reed were grown in Compo Sana Universal® soil (Compo Agricultura SL, Barcelona, Spain), whereas a mixture of 50% Compo Sana Universal® soil (CompoAgricultura S.L., Barcelona, Spain) and 50% river sand was used to grow cattail and common reed (Fig. 3.1).

All plants were grown in a greenhouse at a temperature of 25 ± 3 ºC, a 75 ± 10 % rh and a 16:8 (L:D) photoperiod.

**Figure 3.1.** Plant species used: Maize (A), sorghum (B), rice (C), johnsongrass (D), giant reed (E), cattail (F) and common reed (G). Plants are not scaled. Copyright: Maize - Protein Abundance Database (http://pax-db.org); Sorghum – Humanity Development Library (http://www.nzdl.org); Rice and johnsongrass – Gramene Archive (http://archive.gramene.org/); Giant reed, common reed and cattail – University of Florida IFAS (https://plants.ifas.ufl.edu/plant-profiles/).



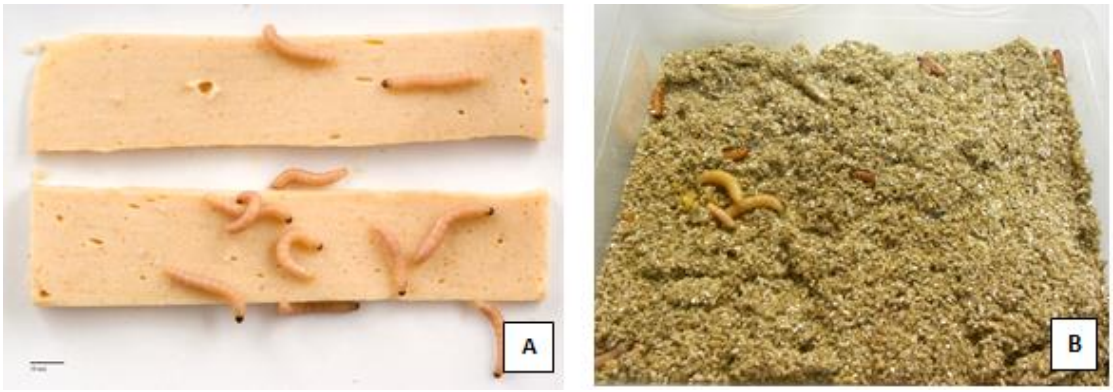

All assays to assess larval performance and feeding and oviposition preference of *S. nonagrioides* were performed with V6-V8 plants of all plant species except rice, which was used when the plants reached the panicle formation phase. On the other hand, maize plants at the phenological stages of VT (anthesis) and R4 (dough) were used to study female oviposition performance according to plant age.

### 3.2.2. Insect rearing

Insects used in all assays came from a laboratory colony of *S. nonagrioides* that had been reared in the laboratory for at least 2 generations before the experiments were performed. Rearing took place in ventilated plastic boxes containing filter paper and thin strips of a meridic diet (González-Núñez *et al.*, 2000) that was renewed every 2-3 days. Larval instars first to third were reared in groups of approximately 100 individuals in Ø 11.5 cm x 4.5 cm high boxes, and when they reached the fourth

instar, larvae were transferred to larger boxes (21 x 16 x 4 cm) in groups of 50-60 individuals. To facilitate pupation, a layer of vermiculite was added to the bottom part of the boxes when the last instar was reached (Fig. 3.2). Pupae were collected and their sex was determined using a stereomicroscope (Leica M125, Leica Microsystems, Germany). Groups of 10 pupae of the same sex were kept in ventilated plastic boxes (Ø 11.5 cm x 4.5 cm height) until adults emerged, whereupon 8-10 pairs of adults were placed in oviposition cages consisting of a pot with 8-10 maize seedlings and a ventilated metachrylate cylinder (Ø 11 cm x 29.5 cm height). Seven days later, egg clusters were collected and placed on top of moistened filter paper in ventilated plastic boxes for egg hatching (Ø 8.9 cm x 2.3 cm height). The whole rearing process took place in growth chambers at a temperature of 25 ± 0.3 ºC and a 16:8 (L:D) photoperiod.

**Figure 3.2.** Rearing of *S. nonagrioides* in the laboratory. Larvae feeding on meridic diet (A) and last instar larvae burrowing in vermiculite to pupate (B).

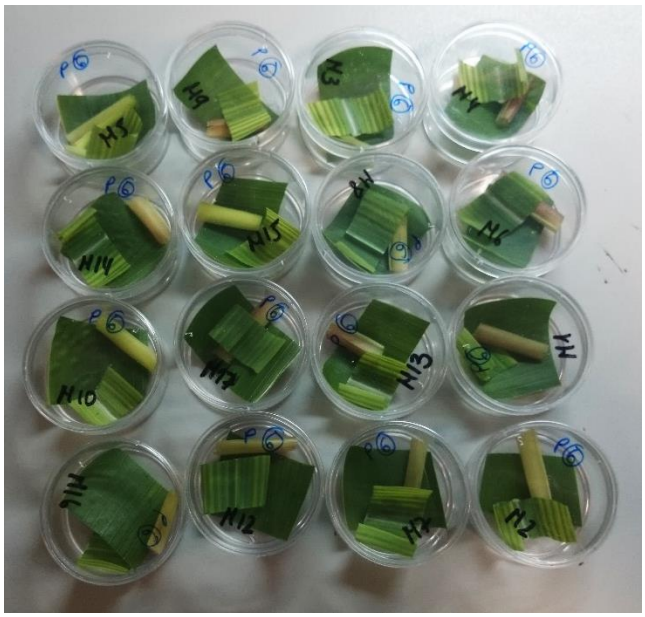



### 3.2.3. Performance of *S. nonagrioides* on different host plants

A preliminary no-choice test using 10-20 replicates per plant species, each of them consisting of three confined pairs of *S. nonagrioides* per arena, was performed to confirm that the seven hosts selected were suitable for this study. The results showed that females laid a significant number of fertile eggs in all the plants (on average, more than 400 eggs per replicate) (data not shown). Therefore, larval performance was assessed on all seven hosts in two ways: by using excised parts of leaves and stems and using whole plants.

In the first case, a neonate larva (<24 h) was confined in a plastic box and fed with fresh pieces of leaves and stems of each plant species for its whole larval development (Fig. 3.3). All boxes were examined daily, and the dates of molting, pupation and adult emergence, as well as pupal weight, were recorded. To compare whether feeding on alternative hosts during the larval cycle had an effect on adult performance, when adults emerged they were set up in individual pairs and placed in pots containing three V3 maize seedlings confined by a ventilated cylinder (Ø 5.4 cm x 15.5 cm high) for mating and oviposition. Egg clusters were collected seven days later and the number of eggs was estimated using a stereomicroscope. Eggs were placed on top of moistened filter paper in plastic boxes for hatching and their viability was recorded, considering those eggs that did not hatch after seven days as non-viable. Between 60 and 144 larvae were used for each plant species. A control was set up with 102 replicates, each of them containing one larva fed with the same meridic diet used to rear the *S. nonagrioides* laboratory population. These experiments were carried out in growth chambers at 25 ± 0.3 ºC and a 16:8 (L:D) photoperiod.

**Figure 3.3.** Bioassay of larval performance using parts of plants (maize, in this photography).

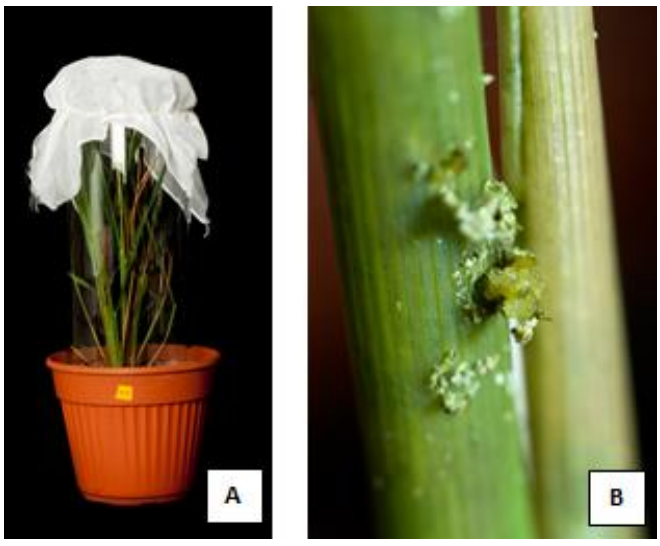


Additionally, the standardized growth index (SGI) was estimated for each replicate of each potential host species tested as described in Amer and El-Sayed (2014):

$$SGI = \text{Pupal weight (mg)} / \text{Larval period (days)}$$

In the assays that used whole plants, each plant was infested with six neonate (<24 h) unfed larvae of *S. nonagrioides*, by placing two neonates on the leaf sheaths of leaves 3, 4 and 5 of each plant, with the exception of common reed, in which only one neonate larva per leaf was used due to the narrow diameter of the stem in this species. The main stem of rice plants was considered for infestation. Plants were then confined within a ventilated methacrylate cylinder to prevent larvae from escaping and watered regularly during the running time of the experiment (Fig. 3.4). Plants were dissected 25-27 days after the experiment started, the larvae and pupae were recovered and their weight and larval stage were recorded. These assays were performed in a greenhouse, using 7-22 plants per species, at 25 ± 3 ºC and a 16:8 (L:D) photoperiod.

**Figure 3.4.** Larval development in whole plants. Rice plant confined by a plastic cylinder at the time of infestation (A) and feeding damage observed at the end of the assay (B).



### 3.2.4. Oviposition preference on cultivated hosts

Two-choice assays were carried out to determine the oviposition preference of females between the primary cultivated host (maize) and rice or sorghum. The three host species were sown at the same time and exposed to *S. nonagrioides* females when maize plants reached the V8 phenological stage. For each replicate, three newly emerged adults (<24 h), two males and a female, were confined in a ventilated plastic box and maintained at 25 ± 0.3 ºC and a 16:8 (L:D) photoperiod to promote mating. Twenty-four hours later, the moths were released in the choice arena, consisting of two plants, one maize plant and either a rice or a sorghum plant, confined with a mosquito net that gave the moths enough space to move freely (Fig. 3.5). The adults were released inside this area in a point equidistant from both plants. Twenty replicates of each option (maize-rice or maize-sorghum) were set up. These assays took place in a greenhouse at a temperature of 25 ± 3 ºC and a 16:8 (L:D) photoperiod. Female moths were dissected to check their mating status, and only replicates in which a mated female was recovered were considered as valid. The oviposition response was estimated as the total number of eggs laid per female and plant. The number of eggs in each plant species was recorded in each replicate, egg clusters were placed in ventilated boxes on top of moistened filter paper and egg viability was estimated a week later as previously described.

**Figure 3.5.** Oviposition choice assays between a maize plant and either a sorghum or a rice plant.

### 3.2.5. Larval feeding preference

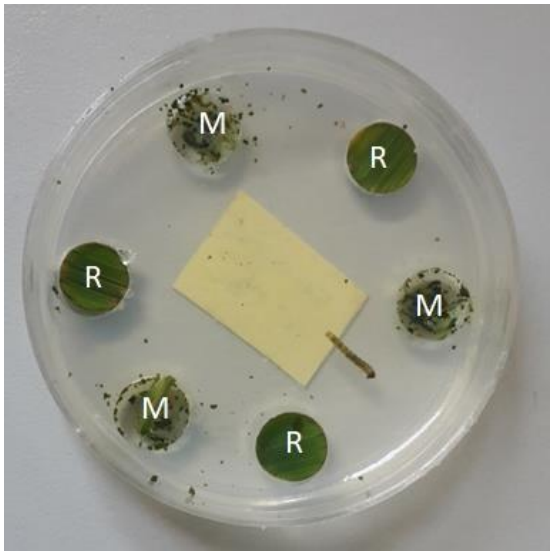
Since maize is the primary host of *S. nonagrioides* in Spain, this species was used as the reference host in two-choice assays.

*Two-choice tests*

Two-choice assays were conducted to examine feeding preferences of *S. nonagrioides* larvae between maize and the other two cultivated hosts (rice and sorghum). The choice arena consisted of a Petri dish (Ø 60 mm x 5 mm high) coated on its bottom half with a 2.5% agar solution. Leaf disks (Ø 8 mm) containing the mid-rib were excised from V6-V8 rice, maize and sorghum plants with a cork borer No. 4 and fit into the holes punched in the agar layer, alternating leaf disks of two species (maize-rice, maize-sorghum, sorghum-rice) in the agar arena. Twenty replicates of each combination of plant species were evaluated. In each replicate, a recently molted (<24 h) second instar larva weighing 0.50-1.25 mg (mean ± 1 SD) after a 6 hour-starvation period was placed in the center of the dish (Fig. 3.6). All dishes were carefully sealed and placed in a growth chamber at 25 ± 0.3 ºC and complete darkness for the duration of the assay. The experiment concluded when

larvae in an external control that only contained maize disks had consumed approximately 50% of the plant material. Both the initial and final fresh weights of larvae and leaf disks were recorded, separately for each plant species. When the experiment ended, larvae were killed and dried in an oven at 60ºC. Uneaten leaf disks were cleaned of frass and oven-dried following the same procedure. The dry weight of both larvae and leaf disks was recorded 48 h later.

**Figure 3.6.** Larval feeding two-choice assays 72 h after the experiment started, where M = maize and R = rice.




The nutritional indices described by Farrar *et al.* (1989) were calculated on a dry weight (dw) basis. The relative consumption rate (RCR) was estimated separately for the two plant species in each two-choice assay:

$$\text{Relative Consumption Rate (RCR)} = (DW_i - DW_f) / (LW_i \times D)$$

where $DW_i$ = initial dw of leaf disks (mg), $DW_f$ = final dw of leaf disks (mg); $LW_i$ = initial larval dw (mg), and D = duration of the feeding period (days). Initial dry weight of leaf disks was calculated from their fresh weight using an equation that relates both parameters, obtained for each plant species by weighing ten batches of six freshly excised leaf disks and weighing them again after 48 hours at 60 ºC. $LW_i$ was calculated similarly using an equation obtained by weighing 373 second instar

larvae in the same weight range that those used in the assays after the starvation period. All weights were determined using an analytical balance (Mettler Toledo AX205, Mettler-Toledo International Inc., Columbus, OH, USA).

Additionally, the preference index proposed by Kogan and Goeden (1970) was calculated as a measure of larval preference:

$$\text{Preference Index (C)} = (2 \times A) / (M + A)$$

where A = consumption of alternative host (% dw), and M = consumption of primary host (% dw).

The preference index C can range between 0 and 2, so that C = 1 indicates larvae do not feed preferentially on either plant, whereas values lower than 1 point to a preference for the primary host and values higher than 1 indicate larvae feed preferentially on the alternative host.

*No-choice tests*

No-choice assays were conducted to evaluate whether larval performance was related to their feeding on leaf disks of each species. These tests were performed similarly to two-choice assays, but all disks in the agar arena corresponded to the same plant species. The number of replicates tested was 17 for maize, 16 for sorghum and 8 for rice. In this case, the experiment concluded when larvae in the maize assay had consumed approximately 75% of leaf disks.

Three nutritional indexes were estimated in no-choice feeding assays: RCR, the relative growth rate (RGR) and the efficiency of conversion index (ECI) (Farrar *et al.*, 1989), so that:

$$\text{Relative Growth Rate (RGR)} = (LW_f - LW_i) / (LW_i \times D)$$

where $LW_f$ = final larval dw (mg), $LW_i$= initial larval dw (mg), and D = duration of feeding period (days); and

$$\text{Efficiency of Conversion Index (ECI)} = RGR / RCR$$

*Free sugar and free amino acid content of the cultivated hosts*

Maize, sorghum and rice leaves used in the feeding assays were used to assess their free amino acid and soluble sugar content, two factors that have been proved to stimulate feeding in some lepidopteran species (Beck and Hanec, 1958; Hedin *et al.*, 1990).

To estimate the quantity of free amino acids in each species, the extraction method described in Hacham *et al.* (2002) was followed. Three samples, each of them containing 20-30 leaf disks, were analyzed per plant species. With this purpose, disks of maize, sorghum or rice excised from the same leaves used in the feeding assays were frozen at -80ºC and grinded using a mortar to obtain about 100 mg of leaf material per sample. The extraction began by adding 600 µl of extraction buffer, composed of water:chloroform:methanol (3:5:12 v/v), to each sample and mixing carefully. The samples were then centrifuged at 4ºC and 14,000 rpm for 4 min, after which the supernatant of each sample was transferred to a new tube and the pellet was re-suspended in 600 µl of extraction buffer and centrifuged again as described above. The supernatant was collected and pooled with that obtained in the previous centrifugation, the pellet was discarded and 300 µl of chloroform and 450 µl of double distilled water were added to the pooled supernatants of each sample. This was followed by a final 2-minute centrifugation of the samples at the same temperature and speed, resulting in a solution with two clearly distinguishable parts, in which methanol and water made up the top layer and contained the amino acids. This part of the solution was carefully separated and transferred to a new tube, which was placed in a SpeedVac Concentrator Savant SVC-100H (ThermoFisher scientific, Wilmington, DE, USA) overnight. When all the solvent was evaporated, the samples were taken to the Protein Chemistry Service at the CIB (CSIC, Madrid), where their amino acid content was determined using a Biochrom 30 Amino Acid Analyser (Biochrom, USA). For this purpose, the samples were first resuspended in 100 µl of sodium citrate loading buffer at pH 2.2 (Biochrom, USA), and 10 µl of each sample were injected in the analyzer. The free amino acid content in each sample was estimated on a dry weight basis.

Determination of the plants' soluble carbohydrates content was performed on dry plant material according to the method described by Maness (2010). Leaf disks excised from the leaves used in the assays were oven-dried at 75ºC for 48 h and then grinded with a pestle in a mortar until a fine powder was obtained. Three samples were considered for each species, and approximately 3 mg of leaf powder were used per sample. To extract the free sugars in the plant material, the sample was homogenized in 650 µl of 95% ethanol and heated at 80ºC for 20 min, followed by centrifugation at 10,000 rpm for 10 min and collection of the supernatant in a clean tube. This process was repeated two more times. The supernatants of each sample were pooled together and then divided in two 750 µl replicates, which were dried in a SpeedVac Concentrator Savant SVC-100H for approximately 12 h. The samples were then re-suspended in 500 µl of double-distilled water and 1 ml of a solution of 0.2% anthrone in 95 % sulfuric acid (v/v) was added to each of them. After a 15-minute incubation period at 90ºC, the absorbance of each sample at 630 nm was measured in a VERSAmax microplate reader (Molecular Devices Corp., Sunnyvale, USA).

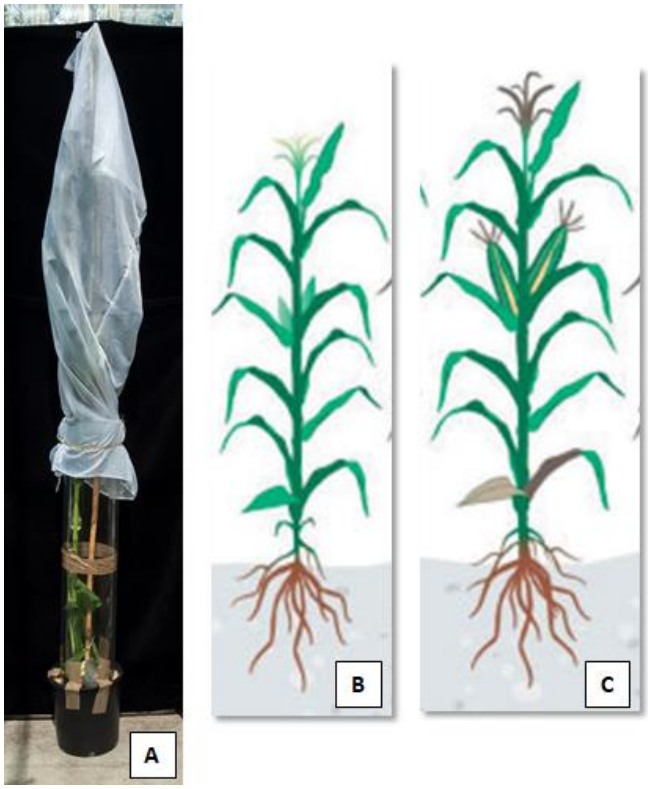
### 3.2.6. Oviposition of *S. nonagrioides* on maize at two phenological stages

Greenhouse assays were carried out to determine the fecundity (number of eggs per mated female) and fertility (egg viability, %) of *S. nonagrioides* adults at two phenological stages of maize plant coincident with female field flights of the 2$^{nd}$ and 3$^{rd}$ generations of this pest: VT (tasseling or anthesis) and R4 (dough stage), respectively (Castañera *et al.*, 2016)

When maize plants reached VT, three pairs of newly emerged adults of *S. nonagrioides* from a laboratory population were placed inside an oviposition cage, consisting of a potted maize plant confined by a plastic cylinder and sealed on top by a fine mesh fabric that allowed air circulation (Fig. 3.7). Females were marked with a red dot on a wing to make them easily distinguishable from males. The assays were run for seven days, after which the adults in each replicate were recovered and female genitalia were dissected using a stereomicroscope to check females' mating status. Only cases in which all females were recovered and at least one of them was

mated were considered as valid. In each replicate the number of egg clusters and their distance to the base of the plant was registered. Egg clusters were collected and maintained in the same conditions described in section 3.2.4., and egg viability was estimated seven days later. The assay was run again following the same procedure in the succeeding generation of the *S. nonagrioides* population, when maize plants sown at the same time than those used the previous generation were in R4. Thirty replicates were studied at each of the two experimental times. All the assays were performed in a greenhouse at a temperature of 25 ± 3 ºC and a 16:8 (L:D) photoperiod.

**Figure 3.7.** Oviposition performance of *S. nonagrioides* according to maize phenology. Oviposition cage (A), maize plant in VT (B) and R4 maize plant (C).




### 3.2.7. Statistical analysis

Prior to the statistical analysis of the results, normality and homocedasticity were checked in all variables, and those that did not comply with these requirements were transformed, to arc sin $\sqrt{x}$ in the case of percentages and to log (x + 1) in the case of continuous variables.

In larval development assays using parts of plants one-way ANOVA analyses followed by either a Dunnett's t test (when variances were homogenous) or a Dunnett's T3 test (when variances were not homogenous) were carried out to study whether length of the larval cycle, pupal weight, adult longevity and SGI in the different plant species differed significantly from the values recorded in maize. Both post-hoc Dunnett's multiple comparisons tests compare means from several experimental groups against a control group mean, maize in our case, to see if there are significant differences (Shingala and Rajyaguru, 2015). A Student's t test was carried out to check for differences between host species in fecundity and fertility for adult pairs resulting from larvae fed on maize and sorghum, the only two species in which *S. nonagrioides* completed its cycle and adult pairs could be set up for mating and oviposition. In assays that considered whole plants, one-way ANOVA analyses followed by the multiple comparisons tests mentioned above were used to study whether larval recovery rate and mean weight per instar were different in the alternative hosts in comparison with maize.

Differences in fecundity and fertility between maize and the alternative host were analyzed by paired Student's t tests. This test compared the values resulting from subtracting the value of the variable measured in the alternative host (sorghum or rice) from the value of the variable measured in maize.

Similarly, differences in RCR between species in larval feeding two-choice assays were analyzed with paired Student's t tests.

In no-choice larval feeding assays, one-way ANOVA analyses followed by a Dunnett's t test if the variable was homocedastic, and a Dunnett's T3 test if it was not, were performed to determine if host species had a significant effect on RCR, RGR and ECI.

Differences between potential hosts and maize in their free sugar and total free amino acid content were analyzed with one-way ANOVA analyses followed by Dunnet t tests.

Finally, to study whether the phenology of maize plants affects *S. nonagrioides* oviposition, Student's t tests were carried out to check for differences in the

fecundity, fertility, mean number of eggs per cluster and mean distance of egg clusters to the base of the plant.

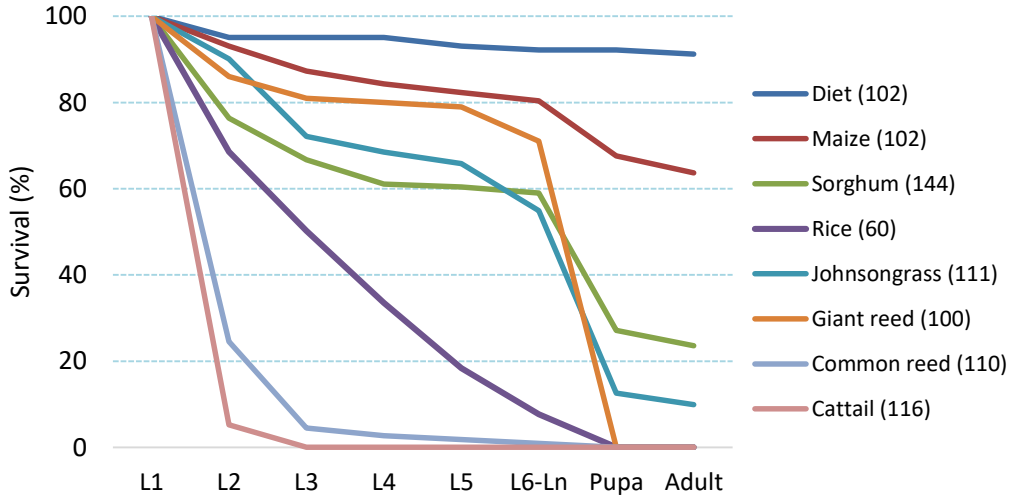
A significance level of $\alpha = 0.05$ was considered, and all analyses were performed using the statistical software SPSS (SPSS Statistics 24.0, IBM, USA).

## *3.3. Results*

### 3.3.1. Performance of *S. nonagrioides* on different host plants

*Sesamia nonagrioides* only reached the adult stage when larvae were fed with pieces of three out of the seven tested plant species: maize, sorghum and johnsongrass, with survival rates to the adult stage of 63.7, 23.6 and 9.9%, respectively. Larvae fed on common reed and cattail died mostly during the early larval stages, while larvae fed on rice and giant reed died at more advanced larval stages, so that no pupae were obtained in either of these four species (Fig. 3.8). Supernumerary molts were common in larvae fed on the alternative hosts, with a maximum of 13 molts recorded on a larva fed on rice, but most of these larvae did not complete their larval cycle. The *S. nonagrioides* population used in the experiments was suitable, as indicated by the high survival rate to adult (91.2%) recorded when larvae were fed on an artificial diet as control.

**Figure 3.8.** Survival rates of the different stages of *S. nonagrioides* on seven host plants, where L1-L6 are larval instars and Ln indicates supernumerary molts. Number of replicates per plant species is shown in brackets.



Focusing on the three plant species where *S. nonagrioides* completed its development, this noctuid had a better performance when fed with maize than when fed with either sorghum or johnsongrass, as indicated by the shorter duration of the larval development and the higher adult longevity and pupal weight observed in larvae fed on maize compared to both sorghum and rice. Increased growth was observed in larvae fed on maize with regards to both sorghum and johnsongrass, as indicated by the higher values of SGI obtained in the former host. Additionally, fecundity and fertility were significantly higher in adult pairs derived from larvae fed with maize (an average of 539 eggs per pair with a mean fertility of 88%) than in those fed with sorghum (125 eggs, 42% of which were fertile). Since females and males emerged at different times, no couples could be set up for mating and oviposition in adults resulting from larvae fed with johnsongrass (Table 3.1).

**Table 3.1.** Performance of *S. nonagrioides* fed on different plant species (mean ± SE). Only those species in which *S. nonagrioides* completed its life cycle are shown.

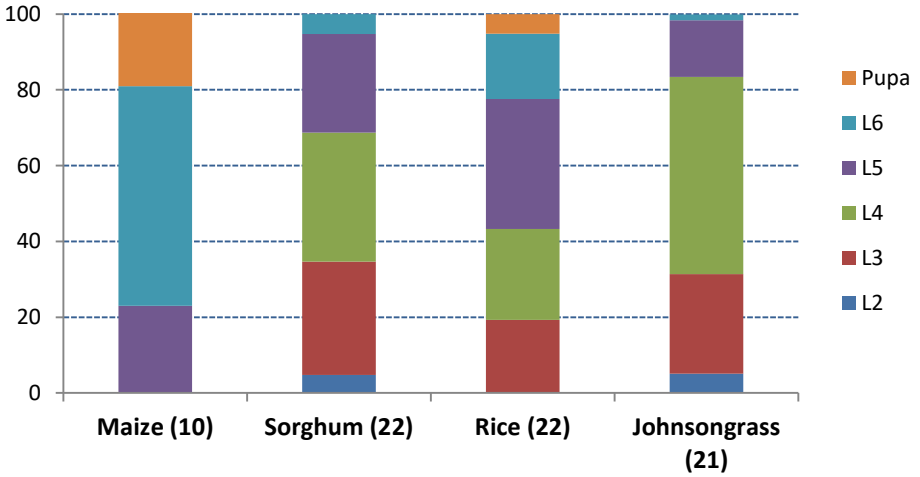
| Host | Larval development (days) | Pupal weight (mg) | SGI[d] (mg/day) | Adult longevity (days) | Fecundity | Fertility (%) |
|---|---|---|---|---|---|---|
| **Maize**[a] | 30.9 ± 0.4 | 188 ± 5 | 4.6 ± 0.1 | 8.7 ± 0.3 | 539 ± 32 | 88.2 ± 1.8 |
| **Sorghum**[b] | 48.3 ± 1.2 * | 128 ± 3 * | 2.7 ± 0.1 * | 5.1 ± 0.2 * | 125 ± 31 * | 42.1 ± 14.0 * |
| **Johnsongrass**[c] | 46.1 ± 1.7 * | 113 ± 5 * | 2.0 ± 0.1 * | 5.1 ± 0.5 * | - | - |
| ***F* / *t* (p)** | 162.22 (<0.001) | 56.19 (<0.001) | 66.89 (<0.001) | 10.40 (<0.001) | 108.13 (<0.001) | 3.04 (0.011) |

Means were compared by one-way ANOVA ($p < 0.05$)
* Significantly different from values obtained in maize (Dunnett t test, $p < 0.05$).
[a] Sixty-five adults emerged from larvae fed on maize; 16 couples were set up for mating and oviposition.
[b] Thirty-four adults emerged from larvae fed on sorghum; 10 couples were set up for mating and oviposition.
[c] Eleven adults emerged from larvae fed on johnsongrass. Female and male adults emerged at different times, so no couples could be set up.
[d] Standardized Growth Index.

In the assays using whole plants, individuals of *S. nonagrioides* were recovered 25-27 days' post-infestation in six out of the seven plant species tested, whereas no larvae were recovered from giant reed. However, only four of these species, maize, sorghum, rice and johnsongrass, yielded a recovery rate that exceeded 10% of the

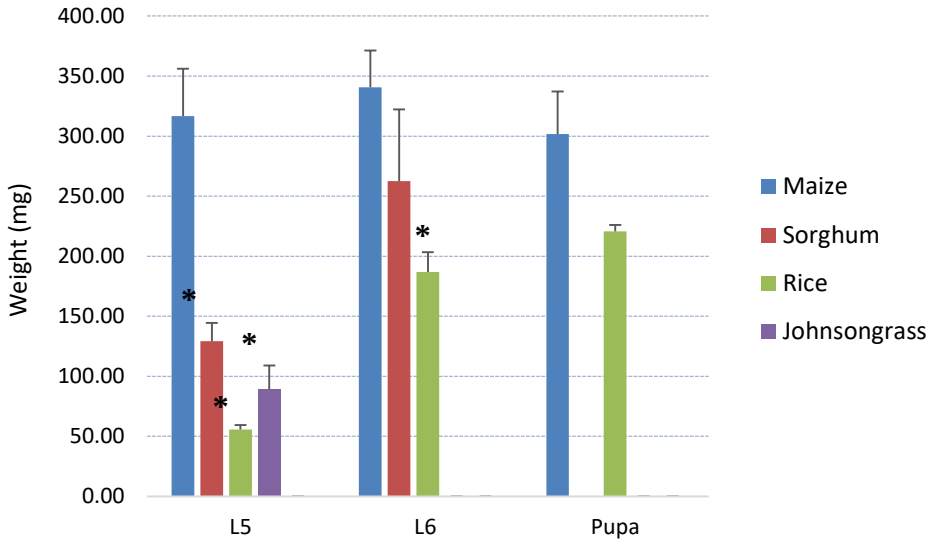
initial number of larvae and were considered in the analyses. When the experiment was stopped, nearly four weeks' post-infestation, all larvae should be at least L5, since the average duration of the larval stage in *S. nonagrioides* larvae reared at 25 ºC and a 16:8 photoperiod was estimated in 32.5 days (López *et al.*, 2001). However, only in maize plants 100% of the larvae recovered were L5 or bigger, and most of the larvae recovered from these plants were L6 (57.5%). Nearly 95% of the larvae recovered from sorghum were L2 to L5, and around 5% were L6 larvae. Likewise, over 75% of the larvae recovered from johnsongrass were L3 and L4 larvae, and around 17% were L5 and L6 larvae. Finally, most of the individuals recovered from rice plants were L3 to L6 larvae (34.3% L5, 23.9% L4, 19.3% L3 and 17.2% L6), and 5.2% were pupae (Fig. 3.9).

**Figure 3.9.** Frequency of the different stages of the life cycle in *S. nonagrioides* recovered from infested plants. Number of replicates per plant species is shown in brackets.




Average larval weight was significantly higher in L5 larvae recovered from maize than in those recovered from sorghum, rice and johnsongrass. Sixth instar larvae were also heavier in maize plants with regards to those from rice plants, whereas the difference was not significant in the case of L6 larvae recovered from sorghum. Finally, the average weight of pupae found in maize plants did not differ significantly from that of pupae recovered from rice ($p = 0.054$) (Fig. 3.10).

**Figure 3.10.** Larval and pupal weight per host species (mean ± SE).




Means were compared by one-way ANOVA
* Significantly different from values obtained in maize (Dunnett t test, $p < 0.05$).

### 3.3.2. Oviposition preference on cultivated hosts

The average adult recovery rate per replicate was 80 ± 7 % in maize-sorghum assays and 93 ± 3 % in maize-rice assays. The average number of eggs laid on maize plants was significantly higher than that recorded in sorghum or rice, but no significant differences were observed between hosts regarding fertility in maize-sorghum or maize-rice assays (Table 3.2).

**Table 3.2.** Fecundity and fertility (mean ± SE) of *S. nonagrioides* in choice assays between maize-sorghum and maize-rice.

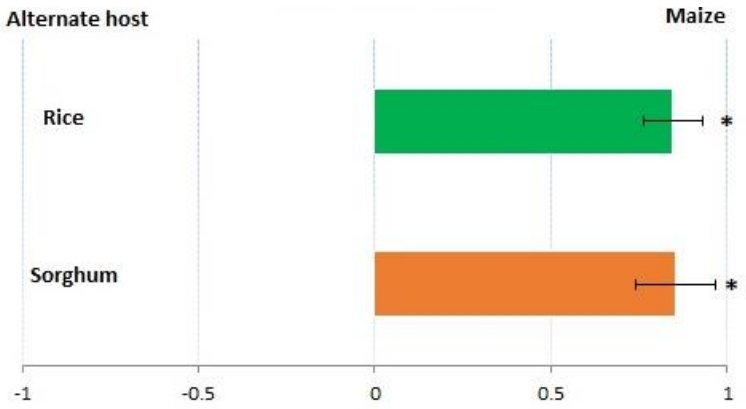
| Host pair | N | Fecundity | t (p) | Fertility (%) | t (p) |
|---|---|---|---|---|---|
| **Maize** | 18 | 387 ± 43 | 6.90 (<0.001) | 96.4 ± 1.1 | -0.85 (0.486) |
| **Sorghum** | | 25 ± 16 * | | 99.4 ± 0.4 | |
| **Maize** | 15 | 449 ± 50 | 7.35 (<0.001) | 88.7 ± 6.8 | 0.45 (0.671) |
| **Rice** | | 32 ± 15 * | | 96.2 ± 1.6 | |

Means were compared by paired Student's t test ($p < 0.05$).
* Significantly different from values obtained in maize

The strong preference for maize as oviposition substrate in comparison with the two alternative hosts observed in both choice assays was confirmed by the high

oviposition index in maize-sorghum (0.85 ± 0.11) and in maize-rice (0.85 ± 0.08) assays (Fig. 3.11).

**Figure 3.11.** Oviposition index of *S. nonagrioides* in choice assays considering maize versus sorghum or rice plants.




* Fecundity was significantly higher in maize.

### 3.3.3. Larval feeding preference

*Two-choice assays*

The relative consumption rate was significantly higher in maize compared to rice, whereas this index had a significantly higher value in sorghum when larvae could choose between this species and either maize or rice (Table 3.3).

**Table 3.3.** Relative consumption rate (mean ± SE) of *S. nonagrioides* on the three cultivated plant species using two-choice assays.

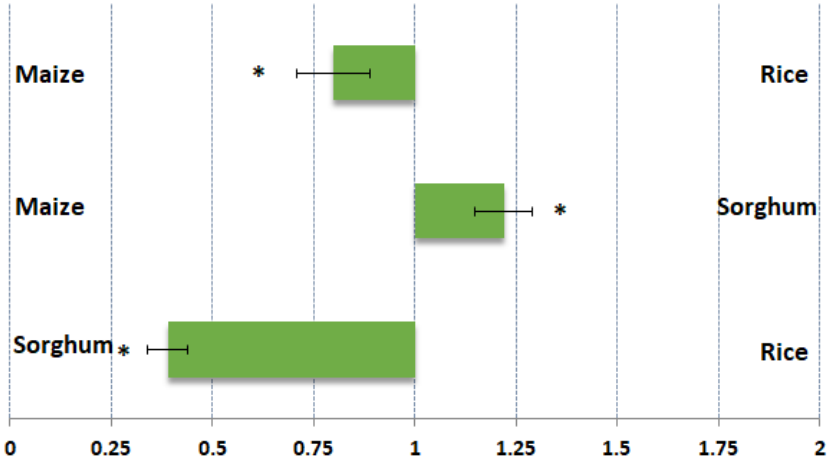
| Choice assay (N) | RCR maize | RCR rice | RCR sorghum | *t* (p) |
|---|---|---|---|---|
| **Maize - Rice (19)** | 3.83 ± 0.46 | 2.02 ± 0.25 | - | 3.78 (0.001) * |
| **Maize - Sorghum (20)** | 2.74 ± 0.26 | - | 4.17 ± 0.47 | - 2.33 (0.031) * |
| **Sorghum - Rice (18)** | - | 1.21 ± 0.18 | 6.04 ± 0.62 | 8.02 (<0.001) * |

Means were compared by paired Student's t test ($p < 0.05$).
* RCR differs significantly in the two species tested in each two-choice assay.

These results are supported by the values of the feeding preference index (C), which indicated that *S. nonagrioides* larvae preferred maize to rice in rice-maize assays

(0.80 ± 0.09), whereas sorghum was the preferred foliar tissue in both maize-sorghum (1.20 ± 0.10) and sorghum-rice (0.48 ± 0.10) assays (Fig. 3.12).

**Figure 3.12.** Feeding preference index (C) of *S. nonagrioides* for the three cultivated plant species in two-choice assays.




* RCR differs significantly in the two species tested in each two-choice assay, as shown in Table 3.4 ($p < 0.05$).

*No-choice assays*

Relative consumption (RCR) of leaf disks was significantly higher in maize compared with sorghum and rice. However, the relative growth (RGR) of larvae in sorghum was higher than in maize assays, resulting in a significantly higher ECI in this species (51.6 ± 8.3 %) compared to maize (18.7 ± 1.0 %). On the other hand, RGR and ECI were higher in maize in comparison with rice (Table 3.4).

**Table 3.4.** Nutritional indices (mean ± SE) of *S. nonagrioides* in no-choice assays on the three cultivated hosts.

| Host (n) | RCR | RGR | ECI (%) |
|---|---|---|---|
| **Maize (17)** | 4.85 ± 0.37 | 0.88 ± 0.06 | 18.7 ± 1.0 |
| **Sorghum (16)** | 2.69 ± 0.36 * | 1.19 ± 0.08 * | 51.6 ± 8.3 * |
| **Rice (8)** | 2.98 ± 0.40 * | 0.13 ± 0.02 * | 4.7 ± 0.8 * |
| ***F* (p)** | 10.4 (<0.001) | 46.0 (<0.001) | 94.6 (<0.001) |

Means were compared by one-way ANOVA ($p < 0.05$).
* Significantly different from values obtained in maize (Dunnett t or T3 test, $p < 0.05$).

*Free sugar and free amino acid content of the cultivated hosts*

Free sugar content was significantly higher in rice leaf tissue compared to maize, whereas no differences were observed between maize and sorghum. On the other hand, the total free amino acid content was significantly higher in both sorghum and rice plants with regards to maize, where the lowest free amino acid content was observed (Table 3.5).

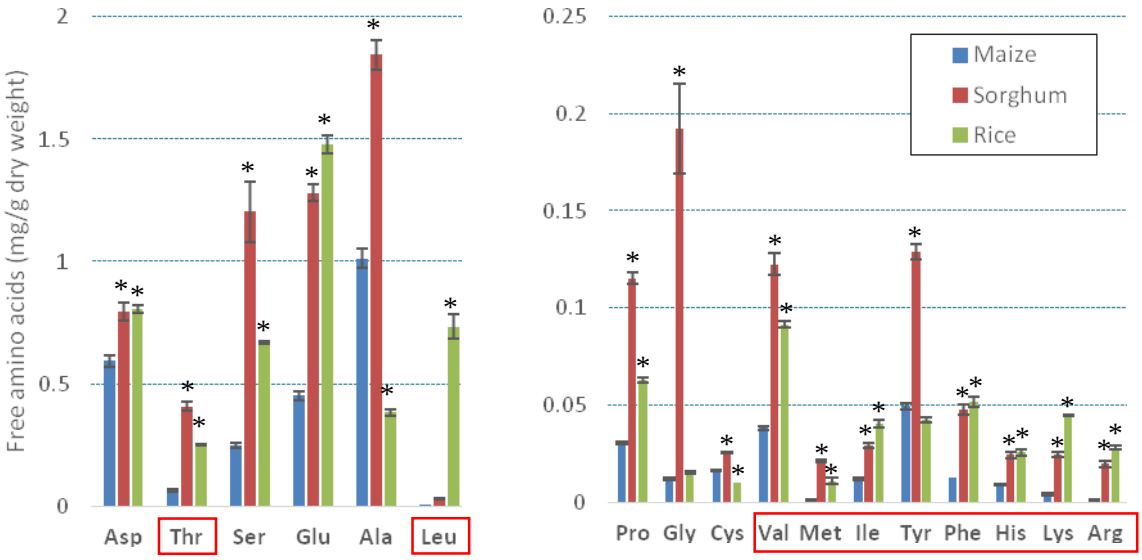
**Table 3.5.** Free sugars and free amino acid content (% of dw) of maize, sorghum and rice leaf tissue.

| Host | Free sugars (%) | Free amino acids (%) |
|---|---|---|
| **Maize** | 3.63 ± 0.32 | 0.256 ± 0.008 |
| **Sorghum** | 3.86 ± 0.30 | 0.631 ± 0.030 * |
| **Rice** | 5.14 ± 0.28 * | 0.475 ± 0.001 * |
| ***F* (p)** | 6.86 (0.028) | 90.28 (<0.001) |

Means were compared by one-way ANOVA.
* Significantly different from values obtained in maize (Dunnett t test, $p < 0.05$).

Significantly different levels of each individual amino acid were obtained in the three plant species ($F \geq 15.6$; $p < 0.05$). In line with the higher percentage of total free amino acids obtained in sorghum leaf disks in comparison with the other two plant species tested, sorghum leaves were richer in most amino acids when compared with maize leaf tissue, except in the case of leucine, the levels of which did not differ statistically between both species. Additionally, amino acid levels were commonly higher in rice in comparison with maize, except in the case of alanine and cysteine, which were higher in maize leaf tissue, whereas tyrosine and glycine content did not differ between the two plant species (Fig. 3.13).

**Figure 3.13.** Free amino acid content (mg/g of dw leaf disk) in the maize, sorghum and rice plants used in the larval feeding assays. Amino acids which are essential for noctuids are framed in a red box.




Means were compared by one-way ANOVA.
* Significantly different from the values obtained in maize (Dunnett t test, $p < 0.05$).

### 3.3.4. Oviposition of *S. nonagrioides* on maize at two phenological stages

Fecundity and fertility per mated female was significantly higher when maize plants were at VT stage, compared to females which laid eggs on plants at R4 stage ($t$ = 4.13; $p < 0.001$). Fertility was also lower at R4 phenological stage ($t$ = 3.97; $p$ = 0.001). However, the number of eggs per cluster did not vary between the two tested phases ($t$ = 2.926; $p$ = 0.094). Finally, females laid the eggs at a lower position in the stem in R4 plants (at an average of 30.6 ± 3.7 cm from the base of the plant) compared to females which laid their eggs in plants in anthesis (53.0 ± 3.8 cm) (Table 3.6).

**Table 3.6.** Variation in fecundity and fertility, mean number of eggs per cluster and egg location in maize plants at the phenological stages of VT and R4 (mean ± SE).

| Phenological stage (n) | Fecundity | Eggs per cluster (n) | Fertility (%) | Location of egg clusters in the plant (cm)[b] |
|---|---|---|---|---|
| **VT (24)** | 396 ± 26 | 86 ± 5 | 98.6 ± 0.5 | 53.0 ± 3.8 |
| **$R_4$ (25)** | 219 ± 28 | 77 ± 8 | 72.2 ± 7.3 | 30.6 ± 3.7 |
| **t (p)** | 4.13 (<0.001)* | 2.926 (0.094) | 3.97 (0.001)* | 4.13 (<0.001)* |

Means were compared by Student t test ($p < 0.05$).
[b] Mean distance of egg clusters to the base of the plant.

## *3.4. Discussion*

Here we provide information about the importance of alternative host plants for *S. nonagrioides* populations and their role in the development of resistance. We have determined the host preference of this primary maize pest by comparing its oviposition preference among wild and cultivated plants, as well as the larval performance on each host. When no other choice was available, adult females laid eggs on all the tested wild and cultivated hosts. These data suggest that *S. nonagrioides* females will lay eggs on any hosts, regardless of the quality of host plants, if no other hosts are available, as already observed in this and other noctuid species (Sparks, 1979; Benda *et al.*, 2011; Dimotsiou *et al.*, 2014). This indicates that, even though chemical and olfactory stimuli from plants play a role in host location and selection (Renwick and Chew, 1994), they do not seem to be essential cues for oviposition of *S. nonagrioides*. Once landed, females evaluate the surface of the plant with the antennae and the abdomen tip. This prospection step ends when the abdominal tip makes contact with the slit of the leaf sheath, the abdominal tip is introduced into the slit and oviposition begins (Robert and Frerot, 1998). In this vein, two other noctuid species, *Busseola fusca* and *Mythimna unipuncta,* have been observed to lay eggs on structures that resemble the tight gap provided by the leaf sheath in maize plants, where these pests commonly oviposit, emphasizing the important role of sensory and physical cues in eliciting oviposition in these species (Calatayud *et al.*, 2007; González-Cabrera *et al.*, 2013).

To analyze the plant "preference-performance" hypothesis, we have focused on survival, development times of immature stages, and pupal weight. We used whole plants and excised pieces of leaves to limit uncontrolled variation among whole plants within species, so that *S. nonagrioides* consumption and developmental rates could be fairly assessed across host plant species. We have found that *S. nonagrioides* was able to complete the larval cycle in the three cultivated hosts, maize, sorghum and rice, as well as on the weed johnsongrass. Rice was clearly the least preferred and least suitable cultivated host for *S. nonagrioides*, as indicated by the low efficiency of conversion of leaf tissue of this host into body mass (4.7% in rice, compared with the 18.7% and the 51.6% recorded in maize and sorghum leaf tissue, respectively). Additionally, even though a high proportion of larvae was recovered from infested rice plants, these larvae were developmentally delayed and their growth was restricted in comparison with larvae recovered from maize plants. Interestingly, the performance of *S. nonagrioides* larvae was significantly worse in any of the alternative hosts in comparison with maize, as indicated by the higher mortality, delayed developmental time, reduced standardized growth index, smaller larval and pupal size, shorter adult lifespan and reduced fecundity and fertility. The only wild species in which the Mediterranean corn borer could complete its cycle, johnsongrass, shares remarkable similarities with sorghum, given that it is a hybrid of *S. bicolor* and *S. propinquum* (Ejeta and Grenier, 2005). Common reed, giant reed and cattail were low quality hosts for larval development of *S. nonagrioides*, though it has been reported to feed on these plant species (Vieira, 2002; Govender *et al.*, 2011; Moyal *et al.*, 2011; Goftishu *et al.*, 2018). This might be due to the differences in the nutritional quality of these plants with regards to maize. An adequate nutritional composition is essential for growth, development and life processes of insects. In addition, leaf toughness or plant chemical defenses, are among the plant characteristics proved to affect performance and survival of first instar larvae (Zalucki *et al.*, 2002) and could partially explain the results obtained.

Among the cultivated host plants, females of *S. nonagrioides* displayed a clear oviposition preference for maize, as indicated by the more than ten times higher fecundity recorded in maize plants in comparison with rice and sorghum plants in two-choice experiments. These results agree with those observed in *S. nonagrioides*

by Dimotsiou *et al.* (2014), who also reported higher fecundity in maize than in sorghum plants. This positive relationship between adult preference and larval performance has been observed in other lepidopteran species (reviewed in Gripenberg *et al.*, 2010), whereas a mismatch between both parameters has been proved in other cases (Berdegué *et al.*, 1998; Panthi *et al.*, 2016), as a result of the complex set of ecological and genetic variables that affect adult preference and larval performance (Thompson, 1988; Mayhew, 2001).

Larval feeding experiments were performed using leaf disks as an appropriate proxy to understand feeding preferences of *S. nonagrioides* larvae, since the use of stems produced unclear results. Sorghum was the preferred host when larvae were subjected to two-choice tests with leaf tissue of this species and either maize or rice. Sorghum and maize plants are phylogenetically very close and they share remarkable similarities in their architecture (Swigoňová *et al.*, 2004). However, they differ in their nutritional composition, as indicated by the significantly higher content of free amino acids detected on sorghum leaf tissue compared with maize. Free amino acids have been proved to stimulate feeding in lepidopteran pests (Beck and Hanec, 1958; Hedin *et al.*, 1990), which could explain the feeding preference recorded for sorghum. Moreover, this might be in part responsible for the high efficiency of conversion recorded in larvae feeding of sorghum leaf disks, which nearly tripled that recorded in maize. No feeding preference was recorded for rice despite the significantly higher levels of free sugars obtained in this host. In addition, the low growth rates observed in larvae fed with rice leaf disks (0.13 versus 0.88 and 1.19 recorded in maize and sorghum plants, respectively) suggest a low nutritional quality and digestibility of rice for *S. nonagrioides*. Therefore, these results indicate that free amino acid and free sugar content were not major drivers of *S. nonagrioides* larval performance.

Altogether our results suggest that the potential wild hosts tested here cannot be used as natural unstructured refuges for Bt maize within the HDR strategy, because, even if these plant species are present and abundant near or within (johnsongrass) Bt maize fields in Spain, they do not comply with the requirements that must be met by unstructured refuges, i.e. that they are able to host a large population of high

quality moths and that no asynchrony exists between these moths and those emerging from Bt fields (Ravi *et al.*, 2005; Gryspert and Grègoire, 2012; Van den Berg, 2017). Natural hosts have been proved to contribute to delay resistance development to Bt cotton in China and the US in the polyphagous and highly dispersive pests *Helicoverpa armigera*, *Helicoverpa zea* and *Heliothis virescens* (Head *et al.*, 2010; Brèvault *et al.*, 2012; Jin *et al.*, 2015; Abney *et al.*, 2017). On the other hand, they were not considered as effective refuges for Bt maize for *Ostrinia nubilalis* in France and in the US, and for several stem-boring pests of maize in Africa (Bourguet *et al.*, 2000a; Losey *et al.*, 2001; Van den Berg, 2017). In relation to the cultivated hosts, our data suggest that only sorghum could be considered as a potential refuge for *S. nonagrioides* in areas where this crop is cultivated close to Bt maize fields, but only if the agronomic management of sorghum fields guaranteed that *S. nonagrioides* moths from this crop would emerged synchronized with those from Bt maize fields.

On the other hand, we have found that the maize phenology affected the oviposition performance of *S. nonagrioides*, so that fecundity was nearly cut in half in plants at the R4 stage (219 eggs per female) in comparison with that recorded at anthesis (396 eggs per female). These findings are in line with those reported by Fantinou *et al.* (2004), which recorded similar female fecundities when adults were subjected to the photoperiods experienced by the second and third generations of *S. nonagrioides* in the Ebro Valley [an average of 372 eggs per female in 16:8 (L:D) photoperiod and 218 eggs per female in a 10:14 (L:D) photoperiod] (INE, 2016). These results indicate *S. nonagrioides* fecundity seems to be closely related to both photoperiod and maize plant phenology. Interestingly, the model of Castañera *et al.* (2016) used for the third generation the fecundity value (218 eggs/female) obtained by Fantinou *et al.* (2004), identical to the value estimated in this study (219 eggs/female), which supports the validity of this parameter in the model. Additionally, a higher proportion of females did not mate when confined in more mature maize plants (55% unmated females versus 21% in plants in anthesis). This might explain the lower average fertility recorded in R4 plants (72.2%) in relation to VT plants (98.6%). It is likely that *S. nonagrioides* females recognize plants in an advanced reproductive stage as a low quality host for their offspring, decreasing

the frequency of mating and causing a reduction in the number of viable eggs. Similar findings have been reported in other lepidopteran species, like *B. fusca*, *Plutella xylostella* or *H. virescens*, which had a lower fecundity as the age of their host plants increased (Navasero and Ramaswamy, 1993; Ndemah *et al.*, 2001; Campos *et al.*, 2003). Lepidopteran females have been observed to reduce their fecundity or the nutritious content of eggs on poor quality hosts, and in some extreme cases they might even reabsorb the eggs to obtain more energy, which they would use to extend their life span and increase the probabilities of finding a more suitable host (Awmack and Leather, 2002). Egg batch size, which has been positively associated with fecundity and avoiding egg desiccation (Courtney, 1984; Clark and Faeth, 1998), was not affected by maize phenology.

All the data reported here provide insights that will allow to fine-tune the predictions of the resistance evolution model of *S. nonagrioides* to Bt maize in the Ebro Valley (Castañera *et al*., 2016) and to optimize the current resistance management strategy. The suitable hosts of the Mediterranean corn borer tested here cannot constitute an unstructured refuge that would contribute to delay resistance development, since they have proved to be unable to foster large populations of high quality *S. nonagrioides* moths that emerged at the same time than adults from Bt crops. Refuges for susceptible individuals should therefore continue to be composed of non-Bt maize plants.

# IV. Frequency of resistance alleles to Bt maize ($F_2$ screen) in Spanish populations of *Sesamia nonagrioides*

## *4.1. Introduction*

Genetically engineered (GE) crops are considered the fastest agricultural technology adopted by farmers worldwide recently (ISAAA, 2017). Currently, the only GE crop allowed for cultivation in the EU is MON 810 maize, which expresses the *Bacillus thuringiensis* toxin Cry1Ab (Bt maize). Although Bt maize was sown in two EU countries in 2017, most of it was in Spain (91%), the only European country where Bt maize has been grown steadily since 1998 (Castañera *et al.*, 2016; ISAAA, 2017). More specifically, Bt maize farming is largely concentrated in the Ebro Valley, located in northeast Spain, where over 50% of all maize sown since 2007 expresses the toxin Cry1Ab (Farinós *et al.*, 2018). The intensive cultivation of Bt maize coupled with the presence of several generations per year of the target pests *S. nonagrioides* and *O. nubilalis*, render the Ebro Valley as the only hotspot in Europe where resistance might evolve (EFSA Panel on GMO, 2012). Owing to this, use of adequate insect resistance management strategies is key to ensuring the long-term sustainability of Bt maize in Spain.

The high-dose/refuge (HDR) strategy has been widely adopted to delay evolution of resistance to Bt crops (Bates *et al.*, 2005; Tabashnik *et al.*, 2013). For the HDR approach to be effective mating should be random between individuals coming from refuges and Bt fields, inheritance of resistance should be recessive and the frequency of resistance alleles should be very low, ideally < 0.001 (Gould, 1998; Tabashnik *et al.*, 2004; Huang *et al.*, 2011).

Since Bt crops were first commercialised in 1996, several cases of field-evolved resistance have been reported worldwide (Tabashnik and Carrière, 2017). As reviewed in Table 1.2. (Chapter I. General Introduction), the evolution of resistance has often been the result of failure to meet the requirements of the HDR strategy, including poor refuge compliance (Van Rensburg, 2007; Gassmann *et al.*, 2011; Farias *et al.*, 2014a; Dively *et al.*, 2016), use of non-high dose events (Gassmann *et al.*, 2011) and non-recessive inheritance of resistance (Gassmann *et al.*, 2011; Campagne *et al.*, 2013). Additional environmental factors and agricultural practices may have promoted the evolution of resistance in the field

(Van Rensburg *et al.*, 2007; Storer *et al.*, 2010; Dhurua and Gujar, 2011; Farias *et al.*, 2014a).

Verifying that the frequency of resistance alleles is within the limits required for the HDR approach to be effective is key to make sure this strategy is suitable for resistance management (Andow and Alstad, 1995). Several methods have been used to estimate the frequency of resistance alleles, although some of them involve testing unfeasible quantities of insects and others render indirect or poor estimates (Andow and Alstad, 1998; Andow and Bentur, 2010). For instance, in species in which a laboratory-selected resistant population is available, directed mating can be performed between the resistant population and the studied field population, and the frequency of resistance alleles can then be estimated by the screening of the $F_1$ and $F_2$ offspring of these crosses on Bt plant or toxin (Gould *et al.*, 1997). Alternatively, the method known as the $F_2$ screen is considered as the most suitable method for the detection of rare recessive resistance alleles (Andow and Alstad, 1998; Venette *et al.*, 2000). In this method, field collected individuals are mated individually in the parental generation ($F_0$), and the offspring of each pair is reared separately as an isofemale line which is sib-mated in the $F_1$. $F_2$ offspring of each line are screened to test their susceptibility to the toxin or the Bt plant, so that the frequency of resistance alleles can be inferred from the number of lines that tested positive in the screen and the total number of lines tested (Andow and Alstad, 1998). This method has been applied successfully to estimate the frequency of alleles conferring resistance to Bt toxins in populations of pest species like *O. nubilalis* (Andow *et al.*, 2000; Bourguet *et al.*, 2003), *Spodoptera frugiperda* (Huang *et al.*, 2014; Farias *et al.*, 2016) or *Helicoverpa armigera* (Dourado *et al.*, 2016).

A study carried out in 2004-2005 used an $F_2$ screen to estimate the frequency of resistance alleles to Cry1Ab maize in populations of *S. nonagrioides* from the Ebro Valley, where it is the most harmful maize pest, and Greece (Andreadis *et al.*, 2007). Previous works had suggested this stem borer species was a single panmictic unit in Southern Europe (De la Poza *et al.*, 2006), leading to an estimated expected frequency of resistance of 0.0015, very close to the low

frequencies required for the HDR strategy to be effective (Andreadis *et al.*, 2007). Given that this value was based on a relatively small number of samples, and that the adoption of Bt maize in the Ebro Valley increased steeply in the last decade, from 35% in 2005 to 74% in 2016 (Farinós *et al.*, 2018), it is important to re-examine the frequency of resistance in this area. Furthermore, re-evaluation of the frequency of resistance alleles in the Ebro Valley would help assess the accuracy of the predictions of the *S. nonagrioides* resistance evolution model, which forecasted the frequency of resistance alleles would not exceed 0.5 in this area until 2050 (Castañera *et al.*, 2016).

The aim of this study was to estimate the frequency of resistance alleles in *S. nonagrioides* from the Ebro Valley, in order to assess if the assumption of low resistance alleles' frequency for the HDR strategy still holds.

## *4.2. Materials and methods*

As outlined in Andow and Alstad (1998), an $F_2$ screen is a four-step method, consisting of 1) sampling of individuals in the field and establishment of isofemale lines; 2) rearing of the $F_1$ and sib-mating of the adults in each line; 3) testing susceptibility of each line to Bt toxins by screening the $F_2$ neonates, and 4) statistical analysis of results. Given that each mated female carries four gametic haplotypes, if one of the parents carried a resistance allele, 1/16 (6.25%) of the $F_2$ offspring would be expected to be homozygous resistant and test positive in the screen.

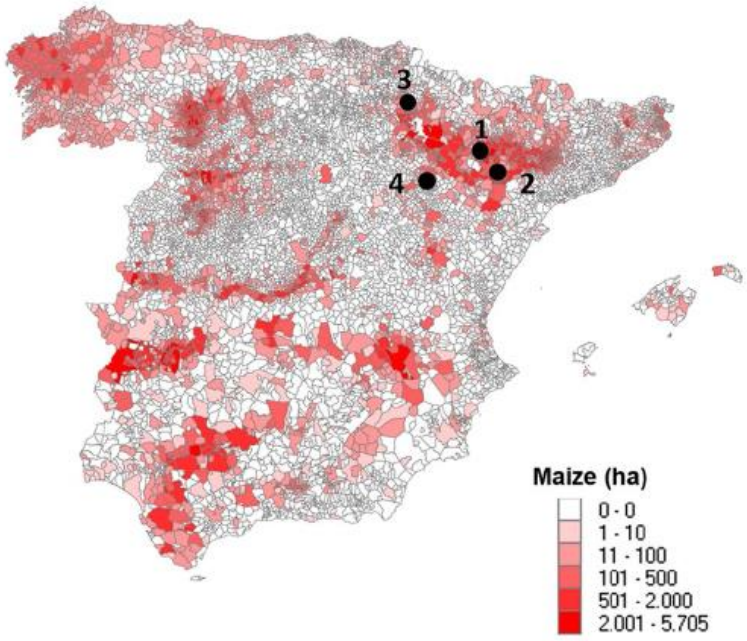
### 4.2.1. Insect collection and establishment of isofemale lines

Frequency of resistance alleles was assessed in *S. nonagrioides* populations from the Ebro Valley, in Spain. As a geographic unit, the Ebro Valley includes parts or all of different Autonomous Communities in northeast Spain, the largest of which are Aragón and Cataluña. These two also are the major producers of maize in the Ebro Valley, where adoption of Bt maize reached 76.9% during 2016. Recently, adoption of Bt maize has increased dramatically in Navarra, an Autonomous Community upstream and to the west of Aragón. The proportion of Bt maize in

Navarra nearly tripled in the last decade, from 21% in 2004-2005 to 58% in 2017. Consequently, in this study, we have included Navarra as a part of the Ebro Valley. Navarra contributes ~8 % (2017) of the maize area in the Ebro Valley.

Fifth and sixth instar larvae of *S. nonagrioides*, most of which had entered diapause, were collected in September and October of 2016 in four different regions of the Ebro Valley (Fig. 4.1): Los Monegros and Bajo Cinca in the province of Huesca (12-13 September and 20-21 September, respectively), Tafalla in the province of Navarra (3-5 October) and Valdejalón in the province of Zaragoza (20 October). Larvae were collected in 1-4 non-Bt maize fields at each region by dissecting damaged maize stalks, and placed in plastic boxes containing fresh pieces of maize stalks for transportation to the laboratory.

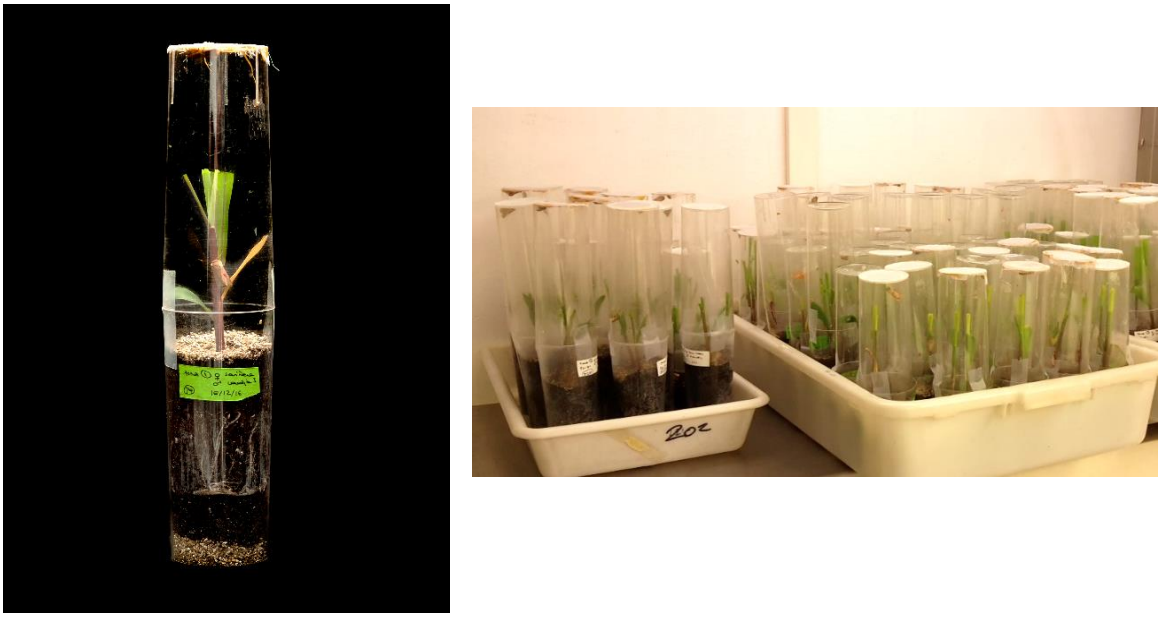
**Figure 4.1.** Cultivated surface (ha) of maize in municipalities of Spain (INE, 2009). Sampling locations are indicated by dots: Los Monegros (1), Bajo Cinca (2), Tafalla (3) and Valdejalón (4).




Upon arrival to the laboratory, larvae were surface sterilized by dipping them in a 1% bleach solution for approximately 30 seconds and then allowed to dry. Groups of ≈50 larvae from the same field were transferred to plastic boxes (21 x 16 x 4 cm) and reared on a meridic diet (González-Núñez *et al.*, 2000), on top of filter

paper and a bottom layer of vermiculite to facilitate pupation. Boxes were stored in growth chambers (SANYO, MLR-352 PE, Japan) at a temperature of 16 ± 0.3 ºC and a 12:12 (L:D) photoperiod to maintain diapause. Every 3-4 days, fresh diet was added and every box was examined for pupae. When an increase in pupation was observed, environmental conditions were shifted to 25 ± 0.3 ºC and continuous light to promote rupture of diapause. Pupae were separated according to their sex and field of collection and kept in plastic boxes (Ø 11.5 cm x 4.5 cm high) until adult emergence. Each emerging adult was paired individually with an adult of the opposite sex originating from the same location, but not necessarily from the same field. Mating and oviposition took place at 25 ± 1 ºC and a 16:8 (L:D) photoperiod, in cages that consisted of two maize seedlings placed in a cylindrical plastic cup and confined by a ventilated plastic cup on top (Fig. 4.2). Egg masses were collected seven days later and placed on top of moistened filter paper in plastic boxes (Ø 8.9 cm x 2.3 cm height).

**Figure 4.2.** Oviposition cages for mating of a pair of adults of the parental generation ($F_0$).

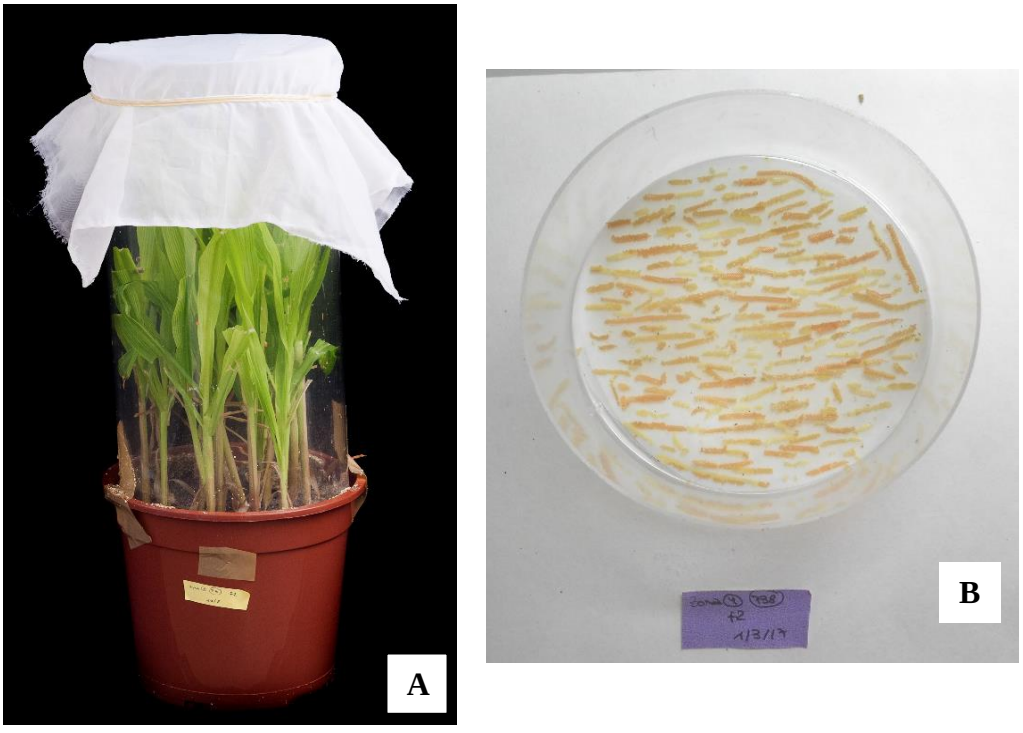


### 4.2.2. Rearing and sib-mating of the $F_1$

The offspring of each two-parent family defined one line and were reared separately on meridic diet, first in 11.5 cm diameter x 4.5 cm height boxes and

later in 23 x 21 x 5 cm boxes, containing a maximum of 200 larvae per line. When the last larval stage was reached, vermiculite was added to the bottom part of the boxes for pupation. Pupae were collected and their sex was determined. Upon adult emergence, a single oviposition cage consisting of a pot with 25 maize seedlings confined by a ventilated plastic cylinder (Ø 20 cm x 45 cm height) was set up per isofemale line for sib-mating of the $F_1$ adults, and the number of females and males placed in each cage for 2-3 consecutive days was recorded (Fig. 4.3A). Egg masses were collected seven days later and placed on moistened filter paper (Fig. 4.3B). In each line, the number of eggs laid in both the parental and first generations was estimated. Rearing and mating of the $F_1$ generation took place under a temperature of 25 ± 0.3 ºC and a photoperiod of 16:8 (L:D).

**Figure 4.3.** Oviposition cage for sib-mating of the $F_1$ of each isofemale line (A) and $F_2$ egg masses collected from an oviposition cage (B).




### 4.2.3. Plant material

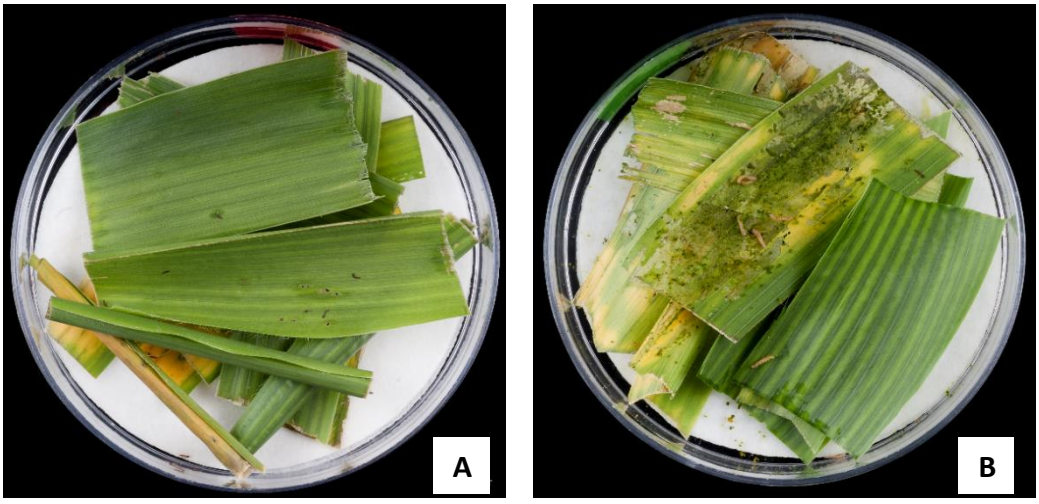
Plants of Bt maize expressing Cry1Ab toxin (DKC4796YG) and its closest non-Bt hybrid (DKC4795) were grown in 8 L pots in the greenhouse, at a temperature of 25 ± 3 ºC, a relative humidity of 75 ± 10 % and a photoperiod of 16:8 (L:D).

Before the susceptibility assays were performed, the presence of Cry1Ab protein was confirmed in every plant using lateral flow test strips (ImmunoStrip® for Bt-Cry1AbProtein/Bt-Cry1Ac-Protein, AGDIA Biofords, France). Leaves were excised from plants at the V6-V10 phenological stage.

### 4.2.4. Testing susceptibility to Cry1Ab maize

In each line 60-250 neonates (<24 h) were placed in ventilated plastic dishes (Ø 8.9 cm x 2.3 cm high) containing 4-5 pieces of Bt maize leaves from which the mid-rib had been removed, to prevent larvae from tunneling inside and avoiding or reducing exposure to Cry1Ab toxin. A maximum of 85 neonates were placed in each dish. Moistened filter paper was added to the dishes to keep leaves turgid and every 2-3 days, fresh leaves were added to each dish. Larval survival and leaf damage were evaluated on day 5 of the screen. Larvae that did not move when touched with a fine hair brush were considered dead. A line was considered positive if extensive feeding damage and second instar larvae were detected in Bt maize assay dishes on day 5. Additionally, control dishes with 20-50 neonate larvae from every line were screened on conventional maize and mortality was recorded 8 days later (Fig. 4.4). All screens were performed at 25 ± 0.3 ºC and a 16:8 (L:D) photoperiod.

**Figure 4.4.** Screen of $F_2$ neonates. Larvae were screened on leaves of Bt (A) or conventional (B) maize in which the mid-rib had been removed.



Lines that tested positive in the $F_2$ screen were rescreened following the same procedure in the next generation to confirm the presence of a resistance allele. For this purpose, larvae that were not used in the $F_2$ screen were reared to produce the third generation. A line that tested positive in both the second and the third-generation screens was considered to be a true positive and thus to carry a resistance allele. Therefore, in this study, resistance was defined as the ability to molt to second instar and cause significant feeding damage on Bt leaf tissue for at least two consecutive generations.

### 4.2.5. Statistical analyses

Bayesian statistics, which allow statistical inferences about the studied population, were used to analyze the data. The expected frequency of resistance alleles [E(q)] and its 95% credibility intervals were calculated according to equations in Andow and Alstad (1998, 1999) and Stodola and Andow (2004). Mathematica 8.0 (Wolfram Research, 2011) was used to calculate these parameters.

Variation in female fecundity between generations was studied to evaluate possible inbreeding depression. The number of eggs laid per female in the first and second generations of each line was estimated from photographs, counting eggs individually in the parental generation and using GIMP 2.8.20 software for the first generation. In the latter case, the digital images were processed to distinguish eggs from the whitish background. For this purpose, the blue channel of a split RGB image was used, and the number of pixels with an intensity between 0 and 98 (0 = black; 255 = white) represented eggs. The number of eggs corresponding to a given number of pixels was estimated by regression, and the regression equation was used to estimate egg numbers for each female. As the distribution of fecundities was non-normal, a Mann Whitney U test was used to compare per capita fecundity between generations.

The probability of missing a resistance allele that was present in an isofemale line (false negative, $P_{No}$) was estimated for each line as a function of the number of $F_1$ males and females that produced the $F_2$ neonates, the number of screened neonates per $F_1$ female, and the average control mortality on conventional maize

(μ) (Stodola and Andow, 2004). Calculations were performed using R 3.4.0. Detection probability of each line was calculated as $1\text{-}P_{No}$, and the overall detection probability of the experiment was estimated as an average of the values of all lines.

To ascertain if the expected frequency of resistance estimated from this sample differed from the initial estimate obtained from 2004-2005 (Andreadis *et al.*, 2007), the joint probability density function for the two estimates was calculated as described in Wenes *et al*. (2006). When $p > 0.05$, the estimates were considered not statistically different. Mathematica 8.0 (Wolfram Research, 2011) was used for this analysis.

The *S. nonagrioides* resistance evolution model (Castañera *et al.*, 2016) was updated to include data on Bt adoption rate for the years 2014-2016. Two simulations of 300,000 runs were conducted to predict the resistance frequency in 2016: first initializing the R allele frequency randomly from the posterior beta distribution of the 2004-2005 estimate (Andreadis *et al.*, 2007) and second, initializing it with the expected value for 2004-2005 (Castañera *et al.*, 2016). Finally, the *S. nonagrioides* resistance evolution model was updated to use the 2016 estimated resistance frequency as the initial value and recalculate the number of years to resistance failure. Large differences compared with the previous estimate (Andreadis *et al.*, 2007) would indicate resistance is not evolving as originally predicted. Mathematica 8.0 (Wolfram Research, 2011) was used to perform these simulations.

## *4.3. Results*

A total of 1,327 fifth and sixth instar larvae of *S. nonagrioides* were collected in non-Bt maize fields at four locations of the Ebro Valley in September and October of 2016 (Table 4.1). Upon emergence, 385 pairs of adults were confined in single-pair cages for mating and egg-laying. One hundred and fifty-four of these pairs (40.0% of the initial number) produced enough fertile eggs and went on to establish isofemale lines, whereas the remaining pairs did not mate or produced few viable eggs. Sib-mating of the $F_1$ occurred in 143 of the lines (37.1%) out of

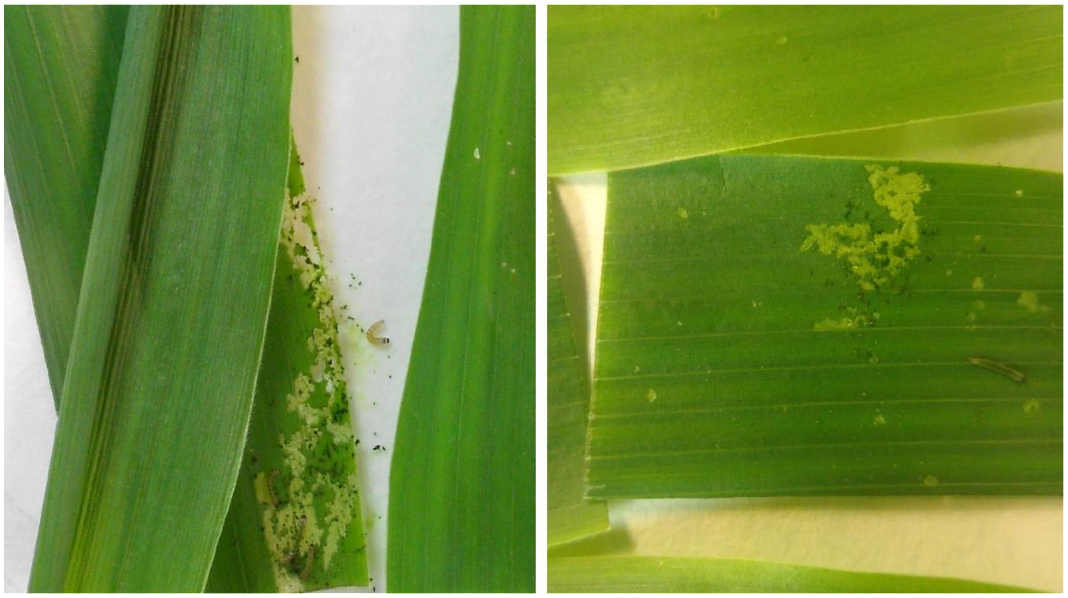
the 147 that produced adults in the $F_1$, and 137 of these lines (35.6%) produced enough viable offspring as to allow for an $F_2$ screen (Table 4.1). The average $F_1$ family size in the lines that were screened was 27.4 ± 1.0 females and 27.2 ± 1.0 males, and an average of 11.4 ± 0.6 neonates per $F_1$ female and line were screened on Bt leaf tissue. Average fecundity per female was 668.4 ± 14.2 in the parental generation and 230.5 ± 8.0 in the $F_1$ generation. A Mann-Whitney U test was carried out to determine if differences in fecundity between generations were significant. Results of this analysis showed that there was a significant reduction in *per capita* fecundity between the parental generation and the $F_1$ generation in the 137 lines that were subjected to an $F_2$ screen ($U = 240$; $p < 0.001$).

**Table 4.1.** Number of larvae collected in each location and number of isofemale lines progressing through each step of the $F_2$ screen per location.

| Location | Number of larvae collected | $P_0$ lines established | Lines that produced $F_1$ larvae | Lines that produced $F_1$ adults | $F_2$ screened |
|---|---|---|---|---|---|
| **Los Monegros** | 410 | 126 | 55 | 52 | 50 |
| **Bajo Cinca** | 493 | 147 | 63 | 61 | 60 |
| **Tafalla** | 387 | 101 | 33 | 32 | 26 |
| **Valdejalón** | 37 | 11 | 3 | 2 | 1 |
| **TOTAL** | **1,327** | **385** | **154** | **147** | **137** |

Larval survival after 5 days of exposure to Bt maize was observed in 104 of the 137 screened lines (75.9% of the lines tested). However, molted larvae and extensive leaf damage due to larval feeding were only detected in line P350, which consequently was considered the only potentially positive line. To confirm the presence of a major resistance allele, this line was rescreened using 1000 $F_3$ neonates. Substantial feeding damage and second instar larvae were detected on fresh Bt leaves on day 5 of the rescreen (Fig. 4.5), rendering P350 as a true positive line. Control mortality in conventional maize dishes was 13.2 ± 0.8 % in the $F_2$ screen, and 2.3 % in the F3 screen in line P350.

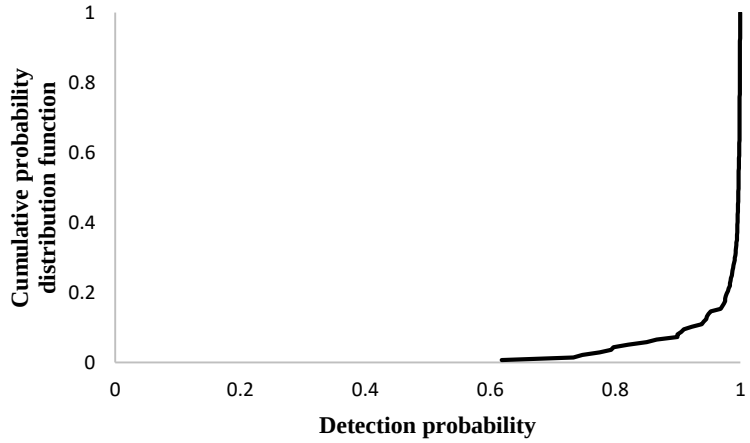
**Figure 4.5.** Leaf damage in Bt leaf tissue caused by larval feeding in the $F_3$ screen. Extensive larval feeding and larvae of the 2$^{nd}$ instar were detected in Bt maize on day 5 of the rescreen of the $F_3$ of line P350.



Considering that one true positive line was detected (S = 1) after screening N = 137 lines, the expected frequency of major resistance alleles (q) was estimated to be E(q) = 0.0036, with a 95% credibility interval between 0.0004 and 0.0100.

As shown in Fig. 4.6, the probability of detecting a resistance allele (1-$P_{No}$) was >95 % in 86.1% of the lines tested, and only 4.4% of the lines had a detection probability < 80%. The experiment-wise detection probability was 97.5%, meaning that if a resistance allele was present, it would have been detected 97.5% of the times the experiment was performed. This value might be an underestimate of detection probability, since control mortality was recorded at day 8 and it is likely lower values would have been detected if it had been recorded at day 5, just like mortality in Bt maize was.

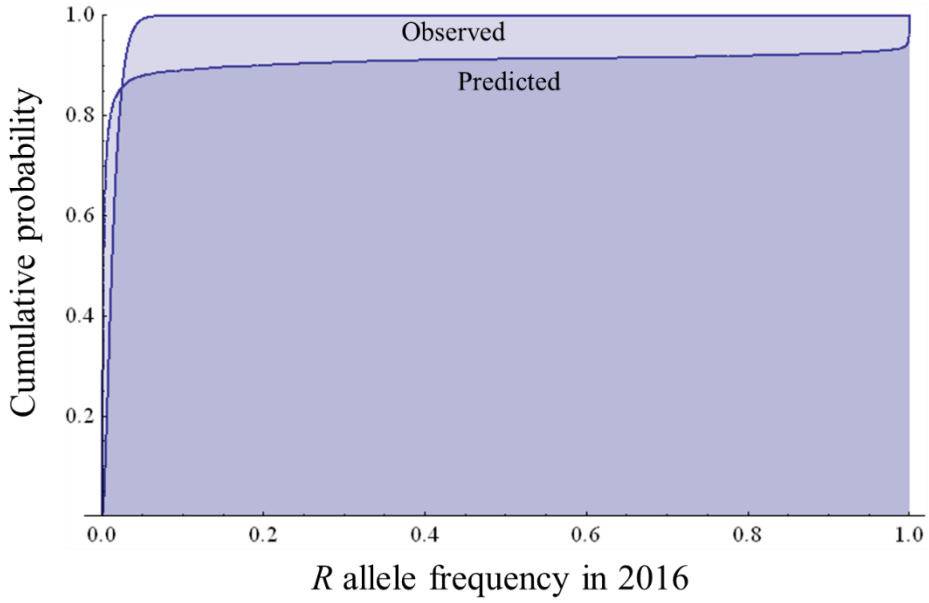
**Figure 4.6.** Cumulative probability distribution function (CDF). Cumulative probability of detecting a resistance allele, calculated as $1\text{-}P_{No,}$ where $P_{No}$ is the type II error.




To assess whether expected frequency of the resistance allele (R) had changed since it was estimated in *S. nonagrioides* populations in 2004-2005 (Andreadis *et al.*, 2007), the joint probability density function of these estimates was calculated. Results of this test indicate the two estimates were not statistically different, although the probability that they were the same was only $p = 0.21$.

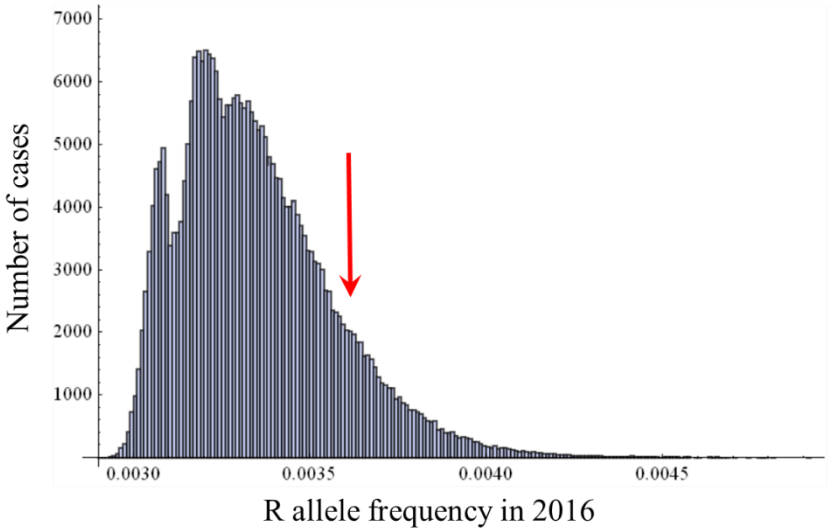
When random samples from the estimated probability distribution of the initial R allele frequency were used to initialize the evolutionary model (Andreadis *et al.*, 2007), they poorly predicted the probability distribution of the R allele in 2016 (Fig. 4.7). This indicated that the probability distribution of the initial R allele frequency was poorly estimated.

**Figure 4.7.** Predicted and observed cumulative probability distributions for the R allele frequency in 2016. The predicted distribution has about 5% of all cases with the R allele going to fixation by 2016 and about 10% of the cases the frequency is >0.5. It also overpredicts the probability of a low R allele frequency in 2016.




We then used the expected value of the initial R frequency (Andreadis *et al.*, 2007) to predict the 2016 R frequency. The observed expected frequency (0.0036) was not significantly different from the predicted frequency (0.0033, p = 0.12), although it was higher than predicted (Fig. 4.8). Consequently, the evolutionary model was reinitialized with the 2016 R allele frequency to predict the number of years to resistance from 2016. The updated model predicted resistance would occur in 31 years from 2016, which was 2.8 years earlier than predicted with the 2004-2005 R allele frequency (Castañera *et al.*, 2016).

**Figure 4.8.** Histogram of the predicted probability distribution for the R allele frequency in 2016. The mean of this distribution is 0.0033. Red arrow is the R allele frequency estimated in this work (0.0036).




## *4.4. Discussion*

For two consecutive generations larvae of line P350 molted to second instar and caused substantial feeding damage after 5 days on Bt leaf tissue, indicating that this line carried a resistance allele. To our knowledge, this is the first time a resistance allele has been detected in a field population of *S. nonagrioides*. Moreover, no previous works have reported a reduction in susceptibility to Cry1Ab toxin in field populations of this species, either in the Ebro Valley or elsewhere (Castañera *et al.*, 2016; Farinós *et al.*, 2018).

The ability of neonate *S. nonagrioides* of line P350 to provoke a light defoliation on Bt leaves is "incipient resistance" as defined by Tabashnik *et al.* (2014). Susceptible larvae are unable to cause light defoliation on Bt plants. This light defoliation could reduce Bt plant efficacy and have practical consequences for pest control. Newly hatched larvae of this species only need to eat a small amount of maize leaves, because they can then tunnel into the stalk where they feed until pupation. In stalks, the expression of the toxin is lower (Székács *et al.*, 2010). Once they reach the stalk, they could feed on the stem and cause yield losses that

could be particularly damaging in the case the first generation larvae, when maize is in the early stages of development.

The sharp decrease in female fecundity between the parental and the first generation probably did not bias the R allele frequency estimates. If lines carrying a resistance allele were selectively lost or retained prior to the screen, our 2016 estimate would be biased (Andow and Alstad, 1998). However, even the reduced number of $F_1$ eggs was more than sufficient to provide a high detection probability (97.5%). The decrease probably was not due to inbreeding because the effects of inbreeding would only occur during the $F_2$ generation. The low values of mortality on conventional maize observed in both the $F_2$ and $F_3$ screens further indicate the absence of strong inbreeding depression in the isofemale lines. Instead, the decrease in fecundity was most likely related to the high larval density experienced by larvae in the $F_1$, which has been observed to be associated with smaller pupae and lower fecundity in *S. nonagrioides* (Fantinou *et al.*, 2008). Moreover, the parental generation comes from larvae that were in diapause, and diapausing larvae of *S. nonagrioides* undergo several supernumerary moults without pupating (Eizaguirre *et al.*, 1994; López *et al.*, 1995), resulting in larger and consequently more fecund adults compared with non-diapausing adults (Fantinou *et al.*, 2004).

The results of our experiments suggest that the frequency of resistance increased slightly from 2004-2005 to 2016 in the Ebro Valley. Our model predicts that resistance frequency should increase only slightly by 2016 from the initial estimate during 2004-2005 (Castañera *et al.*, 2016). This small increase is probably related to the strong selective pressure posed by the high adoption rate of Bt maize in the Ebro Valley for most of the last decade (Farinós *et al.*, 2018). Additionally, use of Bt varieties containing Event 176 between 1998 and 2005, which expressed a lowering toxin titer as the season progressed, could have accelerated the evolution of resistance (Onstad and Gould, 1998; Castañera *et al.*, 2016). Overall, these results emphasize the importance of continued careful monitoring of resistance evolution in the Ebro Valley and the need to consider using additional strategies to slow down this process.

Although the frequency of R has increased slightly, resistance is not evolving faster than expected, implying that the HDR strategy may continue to be effective at delaying the evolution of resistance to Bt maize in *S. nonagrioides* in the Ebro Valley. However, the frequency of resistance in 2016 (0.0036) is three times the recommended value for implementation of the HDR approach (< 0.001), which reduces the expected time to resistance failures (31 years from 2016). Now that a resistance allele has been detected, there is less time to react before homozygous resistant individuals emerge and start damaging Bt fields. This suggests additional measures should be considered to complement the HDR strategy, in order to further delay evolution of resistance and secure the long-term sustainability of Bt maize in northeast Spain.

The use of pyramided maize varieties that express several *B. thuringiensis* toxins would be a natural step towards delaying resistance evolution (Bates *et al.*, 2005; Carrière *et al.*, 2016). These varieties should be carefully designed to combine toxins with low probability of cross-resistance between them, so as to increase their effectiveness (Roush, 1998; Gressel *et al.*, 2017). However, the longer evolution proceeds against Cry1Ab Bt maize, the more likely a pyramid with Cry1Ab will be merely sequential use of two toxins, eliminating the advantages of a pyramid for resistance management (Sudo *et al.*, 2017). Pyramided Bt maize varieties are not available for cultivation in the EU. The approval of new GE crop varieties for cultivation in the EU is a lengthy and complex process that ultimately requires a majority of Member States to vote in favor of them (EC, 2016; Smart *et al.*, 2017). At this time, however, a growing number of European countries have stopped or even banned cultivation of GE crops in their territories (Rabesandratana, 2015) and there is a strong public opposition to genetically modified crops in the EU (Gaskell *et al.*, 2010). Hence despite the substantial advantages of pyramided varieties for resistance management, it seems unlikely that they will be available in the EU anytime soon.

Alternatively, increasing the percentage of the crop allotted to conventional maize, that is, increasing refuge size, could reduce the selection pressure exerted on the pest by promoting disassortative mating between susceptible individuals

coming from refuges and insects carrying resistance alleles. This would help to increase heterozygosity (Jiang *et al.*, 2013), thus lowering the frequency of homozygous resistant individuals (RR). This approach has been suggested to manage field-evolved resistance to Cry3Bb1 maize in Western Corn Rootworm in the US (Tabashnik and Gould, 2012).

## *4.5. Latest results: Selection of a S. nonagrioides strain resistant to Bt maize in the laboratory*

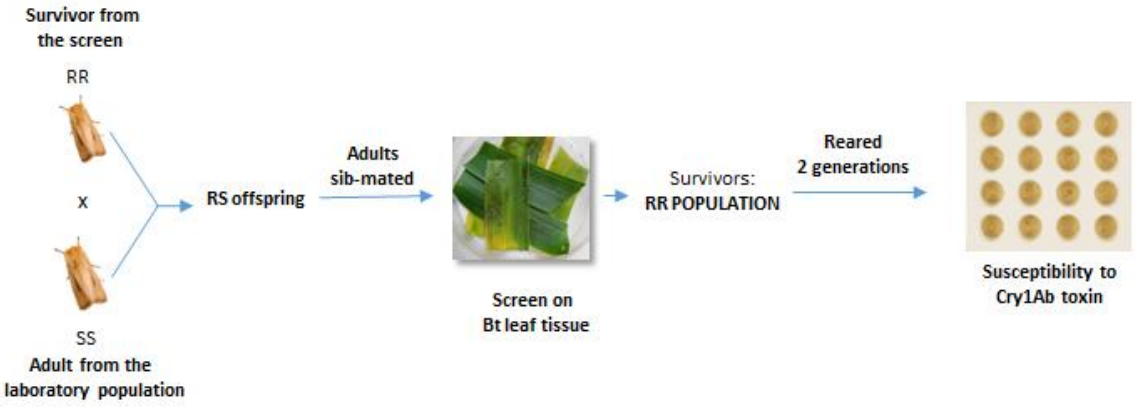
Owing to the detection of a resistance allele in the $F_2$ screen, selection of a line carrying the resistance allele on Bt maize was undertaken in a process that is still underway. The final aim of this task is to establish a stable population of *S. nonagrioides* resistant to Bt maize, in which the genetic basis of resistance, as well as the possible associated fitness costs, can be studied. This information would help improve the management of resistance of *S. nonagrioides* in the Ebro Valley. We are focusing on two objectives: i) to select a population of *S. nonagrioides* resistant to Bt maize and confirm that resistance is inherited recessively in this line, in compliance with the requirements of the HDR strategy; ii) to verify that the resistant strain is significantly less susceptible to Cry1Ab toxin by dose-response bioassays.

### I) Selection process and confirmation of recessive inheritance

Selection of a line resistant to Bt maize began with the recovery of the 26 larvae that survived the $F_2$ and $F_3$ screens in line P350, which proved to carry a resistance allele. These individuals were considered to be homozygous for resistance (RR), given that resistance is inherited recessively in cases where Bt plants meet the high-dose standard (Tabashnik and Carrière, 2017), as happens with MON 810 Bt maize that targets *S. nonagrioides*. Survivors of the screens were transferred to boxes (Ø 11.5 cm x 4.5 cm high first; 20.7 x 15.8 x 3.8 cm later) and reared on a combination of meridic diet and leaves of conventional maize. When larvae reached the last larval instar, vermiculite was added to the bottom of the box to facilitate pupation. Only 7 of the larvae that survived the screens (4 individuals from the $F_2$ screen and 3 from the $F_3$ screen) gave way to

healthy adults. Most of the remaining 19 larvae died before they could complete their larval development, probably due to the high toxin doses they had been exposed to during the screen on Bt maize. Each emerging adult was paired with 3-4 adults of the opposite sex of a laboratory population that was known to be susceptible to the protein Cry1Ab (SS). Oviposition took place in cages consisting of 8-10 maize seedlings confined by a ventilated methacrylate cylinder (Ø 11 cm x 29.5 cm height). Each cross made up a line, and the offspring of each line were considered to be heterozygous for resistance (RS) (Fig. 4.9). Egg masses of each line were collected seven days later and placed on moistened filter paper in plastic boxes (Ø 8.9 cm x 2.3 cm high), and a maximum of 200 larvae per line were reared on a meridic diet following the same procedure as the previous generation. Adults of each line were sib-mated in cages consisting of 25-30 maize seedlings confined by ventilated methacrylate cylinders (Ø 20 cm x 45 cm high). Seven days later, egg masses were collected and placed in plastic boxes (Ø 11.5 cm x 4.5 cm high), on top of moistened filter paper for egg hatching.

**Figure 4.9.** Selection process of a line resistant to Bt maize from line P350.




The offspring of each of the 7 lines ($F_{S+2}$) were screened on Bt maize leaf tissue to assess their susceptibility to the plant. For this purpose, between 150 and 1000 neonates (<24 h) were screened on leaves of Bt maize, according to the same method used in the $F_2$ and $F_3$ screens (see 4.2.4 in this chapter). A control consisting of 30-100 neonates fed on conventional maize leaves was also set up for offspring of each line and cross. Five days later, the number of surviving larvae and their larval instar was recorded, and the leaf tissue was inspected for

feeding damage. For comparison purposes, the same method was used to evaluate survival on Bt leaf tissue of 1000 neonates (<24 h) of the laboratory population.

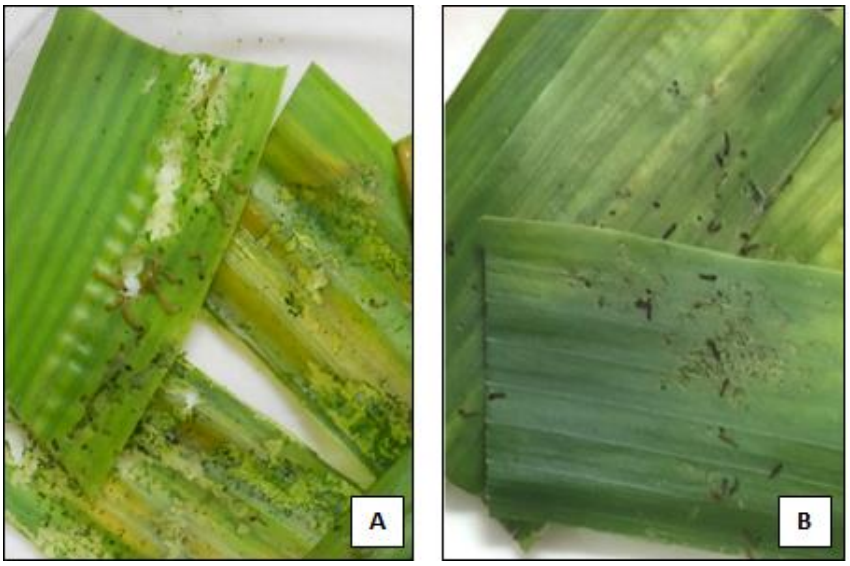
The results of the $F_{S+2}$ screens confirm that line P350 carries a resistance allele and suggest that inheritance of resistance is recessive, in compliance with the requirements of the HDR strategy (Gould, 1998). According to Mendel's principle of allele segregation, if resistance was recessive, individuals resulting from crosses between survivors of the $F_2$ and $F_3$ screens – supposedly homozygous resistant (RR) – and the laboratory population – supposedly homozygous susceptible (SS) – would be heterozygous for resistance (RS). In case this were true, the expected ratios of RR, RS and SS individuals in the offspring of sib-mating between RS individuals would be 25:50:25, so that approximately 25% of the individuals would be expected to survive the screen. Larval survival – corrected according to Abbott (1925) – around the expected values (19.4-31.4%), as well as molted larvae (1.0-18.9%) and extensive feeding damage (Fig. 4.10), were recorded in all 7 lines derived from line P350, suggesting recessive inheritance of the resistance allele. On the other hand, only 0.2% larvae of the laboratory population survived the 5-day bioassay, and no molted larvae or feeding damage were recorded in this assay (Table 4.2).

**Table 4.2.** Larval survival and molting to second instar after 5 days feeding on Bt leaf tissue in the lines carrying a resistance allele and in the laboratory population.

| Origin | Line | Generation screened | Larvae screened on Bt leaf tissue (N) | Corrected survival on Bt leaf tissue on day 5 (%) | L2 larvae in Bt leaf tissue on day 5 (%)[a] |
|---|---|---|---|---|---|
| Survivor of $F_2$ screen | **1** | $F_4$ | 500 | 31.4 | 13.4 |
| | **2** | $F_4$ | 1000 | 26.2 | 15.1 |
| | **3** | $F_4$ | 300 | 29.0 | 1.0 |
| | **4** | $F_4$ | 1000 | 29.2 | 3.5 |
| Survivor of $F_3$ screen | **5** | $F_5$ | 500 | 23.3 | 14.6 |
| | **6** | $F_5$ | 153 | 19.4 | 6.5 |
| | **7** | $F_5$ | 1000 | 24.6 | 18.9 |
| **Laboratory population** | | $F_{14}$ | 1000 | 0.2 | 0.0 |

[a] Percent of second instar larvae (%) with regards to the initial number of larvae tested on Bt leaf tissue.

**Figure 4.10.** Feeding damage on Bt leaves of larvae of line 3 carrying a resistance allele (A) and of neonates of the laboratory population (B).




Survivors of the $F_{S+2}$ screens were recovered to generate the resistant population (RR) and reared together for two generations on a combination of a meridic diet and leaves of conventional maize until pupation. Upon adult emergence, groups of 4-8 pairs of adults were sib-mated in oviposition cages consisting of 8-10 maize seedlings confined by a ventilated methacrylate cylinder (Ø 11 cm x 29.5 cm height). The whole selection process, depicted in Fig. 4.5, took place at a temperature of 25 ± 0.3 ºC and a 16:8 (L:D) photoperiod.

### II) Verification of lower susceptibility to Cry1Ab protein in the resistant line

The Cry1Ab protein used in the susceptibility assessment was provided by Dr. J. Ferré (Universitat de Valencia, Valencia, Spain), as described in section 2.2.2.

Susceptibility of the population RR to Cry1Ab protein was determined in the $F_{S+4}$ using a dose-response assay, as described in section 2.2.4. Eight Cry1Ab protein solutions at concentrations ranging from 2.00 to 120.99 ng Cry1Ab/cm$^2$ were prepared on carbonate-bicarbonate buffer (pH = 10.5), and 50 µl of toxin solution were dispensed over the surface of the diet (~ 0.5 ml) contained in each well of a 128-wells bioassay tray (Bio-Ba-128, C-D International, Pitman, NJ). Ten

replicates of the assay were performed, each of them involving neonates of different oviposition cages and testing eight larvae per toxin concentration – 16 in the control, in which carbonate-bicarbonate buffer was applied over the surface of the diet –. A week later, larval mortality and larval instar of the survivors were recorded. For comparison purposes, susceptibility to Cry1Ab protein was also estimated in the laboratory population using the same procedure. Six replicates of 16 larvae per toxin concentration – 32 in the controls – were evaluated in this population. Given the significant larval mortality observed at high Cry1Ab protein doses, larvae of this population were not exposed to the highest toxin concentration (120.99 ng Cry1Ab/$cm^2$) tested on neonates of the RR population.

Only seven out of the ten replicates that were evaluated in the resistant population had a mortality below 25% in the controls and thus were considered as valid to estimate the susceptibility of this population to the protein Cry1Ab. By contrast, control mortality was <10% in most replicates and all 6 of them were considered as valid.

Susceptibility to Cry1Ab of the resistant population was of 158 (87-601) ng Cry1Ab/$cm^2$ when measured by its $LC_{50}$, and 153 (86-647) ng Cry1Ab/$cm^2$ when measured by its $MIC_{50}$. These values were significantly higher than those estimated in the laboratory population, in which $LC_{50}$ was 12 (9-15) ng Cry1Ab/$cm^2$ and $MIC_{50}$ was 9 (5-12) ng Cry1Ab/$cm^2$. Additionally, the results of the concentration ratios indicate that the resistant population was nearly 13-fold less susceptible to the protein Cry1Ab than the laboratory population when lethal concentrations were considered, and 17-fold less susceptible to the Bt toxin when molt inhibition was considered (Table 4.3).

**Table 4.3.** Susceptibility of the resistant and the laboratory populations to Cry1Ab protein, measured by lethal (A) and molt inhibiting concentration (B).

**(A) Lethal concentration**

| Population | N[a] | Slope (SE) | $\chi^2$ | df | $LC_{50}$ (CI 95%)[b] | $LC_{90}$ (CI 95%)[b] | LCR ($LC_{50}$) (CI 95%)[c] |
|---|---|---|---|---|---|---|---|
| **Resistant** | 557 | 1.4 (0.2) | 134 | 54 | 158 (87-601) | 1261 (395-23507) | 12.9 (6.9-24.0)* |
| **Laboratory** | 903 | 3.4 (0.3) | 123 | 44 | 12 (9-15) | 29 (23-43) | 1 |

**(B) Molt inhibiting concentration**

| Population | N[a] | Slope (SE) | $\chi^2$ | df | $MIC_{50}$ (CI 95%)[b] | $MIC_{90}$ (CI 95%)[b] | MICR ($MIC_{50}$) (CI 95%)[c] |
|---|---|---|---|---|---|---|---|
| **Resistant** | 557 | 1.4 (0.2) | 111 | 54 | 153 (86-647) | 1345 (399-46957) | 17.4 (8.3-36.2)* |
| **Laboratory** | 903 | 2.7 (0.3) | 127 | 44 | 9 (5-12) | 26 (20-43) | 1 |

[a] Number of neonate larvae tested in diet-overlay assays, including controls.
[b] 50 % and 90 % lethal concentrations ($LC_{50}$ and $LC_{90}$) and molt inhibiting concentrations ($MIC_{50}$ and $MIC_{90}$) and their 95 % confidence intervals (CI 95 %) are expressed in ng Cry1Ab $cm^{-2}$
[c] $LC_{50}$ and $MC_{50}$ are significantly different ($p < 0.05$) if the 95 % confidence interval of the lethal concentration ratio (LCR) or the molt inhibiting concentration ratio (MICR) does not include 1. Asterisks indicate susceptibility to Cry1Ab protein of the resistant population is significantly lower from that of the laboratory population, indicated in that column by the number 1.

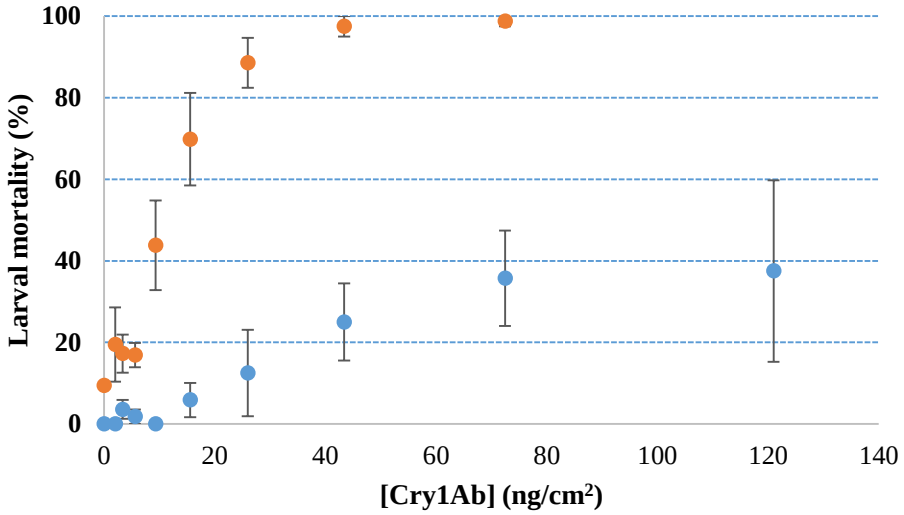
The remarkable decrease in susceptibility of the resistant population after just a few generations of selection on Bt maize was probably an underestimation, given that the estimated $LC_{50}$ in this population is above the highest Cry1Ab concentration tested and that the mean mortality at that dose was low (<40 %). A marked decrease in susceptibility (21-fold) to the toxin was also observed in a strain of *S. nonagrioides* selected in the laboratory for 8 generations on increasing concentrations of Cry1Ab protein over a meridic diet (Farinós *et al.*, 2004).

Additionally, both the $LC_{90}$ and the $MIC_{90}$ were significantly higher than the $LC_{50}$ and $MIC_{50}$, respectively, in the resistant strain (>8-fold) in comparison with the lower difference observed between the same parameters in the laboratory strain (<3-fold), suggesting that the population is indeed becoming resistant to the toxin Cry1Ab.

In both the resistant and the laboratory populations larval mortality increased as Cry1Ab toxin concentration rose. However, very low or null larval mortality was recorded in the resistant strain at toxin doses below 15 ng Cry1Ab/$cm^2$, a

concentration higher than the $LC_{50}$ estimated for the laboratory strain, whereas mortality at 120.99 ng Cry1Ab/cm$^2$, the highest toxin dose tested, did not get to 40% (37.5 ± 22.2 %). Higher values of mortality were recorded at all toxin concentrations in the laboratory population when compared with the resistant line, and nearly no survival was observed in this population from Cry1Ab protein concentrations 43.38 ng/cm$^2$ onwards (Fig. 4.11).

**Figure 4.11.** Larval mortality per Cry1Ab protein concentrations in diet-overlay bioassays in the resistant (blue dots) and the laboratory (orange dots) populations.



# V. Genetic basis of resistance to bt maize: the case of *Spodoptera frugiperda* and Cry1F maize

## *5.1. Introduction*

The fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is a highly polyphagous species and one of the most important pests of maize, cotton, and other crops of economic importance in both North and South America (Luginbill, 1928; Sparks, 1979). On maize, this species feeds extensively in the whorl and can also damage other parts of the plant, including the ear, which can lead to important yield reductions (Cruz and Turpin, 1983; Buntin, 2008; Lima *et al*., 2010). Recently, this pest has arrived and rapidly expanded throughout most of sub-Saharan Africa, causing severe damage in maize fields and harming other crops like millet and sorghum (Georgen *et al.*, 2016; Wild, 2017). This event, along with the highly migratory profile of this species and the increasing interception of *S. frugiperda* in the European Union (EU) in vegetable imports from South America and Africa, have risen concerns about the possible arrival of *S. frugiperda* to Europe (Johnson, 1987; EFSA Panel on Plant Health, 2017; EUROPHYT, 2018). The fall armyworm could be potentially harmful to maize and other crops in Europe, especially in the southernmost coastal areas of the continent, where the climate is mild and suitable for this pest (Abrahams, 2017; EFSA Panel on Plant Health, 2017).

Traditionally, infestations of *S. frugiperda* have been controlled by the intensive use of synthetic pesticides, which has resulted in the development of resistance to several groups of insecticides (Yu *et al*., 2003). A new approach based on the use of genetically modified maize expressing different toxins of the bacterium *Bacillus thuringiensis* (Bt) proteins that have proved to be highly active and effective controlling this pest (Cry1F, Cry2Ab2 and Vip3A) has been adopted recently (Siebert *et al.*, 2008; Storer *et al.*, 2012). In this context, maize hybrids which express the protein Cry1F are widely used in both single-toxin and pyramided varieties that target *S. frugiperda* (DiFonzo, 2018). On the other hand, the Bt toxin Cry1Ab is only moderately active against the fall armyworm (Luttrell *et al.*, 1999; Waquil *et al.*, 2013; Omoto *et al.*, 2016), and single-toxin events that express this crystal protein are not considered high-dose for the control of *S. frugiperda* (Hardke *et al.*, 2011).

One of the main threats to the effectiveness of Bt crops is the evolution of resistance in target species (Tabashnik, 1994). *Spodoptera frugiperda* is, so far, the only target pest species that has developed field resistance to Bt crops expressing different toxins and in multiple areas across different countries and continents (Dangal and Huang, 2015; Tabashnik and Carrière, 2017). Failure in field control of *S. frugiperda* due to resistance was first documented in Cry1F maize fields in Puerto Rico just a few seasons after these hybrids were first deployed (Storer *et al*., 2010). Recently, field resistance of *S. frugiperda* to Cry1F maize has been reported in several areas in Brazil (Farias *et al*., 2014a) and Argentina (Chandrasena *et al.*, 2017). A similar resistance problem has been documented in the southeast region of the US mainland, including populations in the states of Florida and North Carolina (Huang *et al.*, 2014; Li *et al*., 2016). Field-evolved resistance to Cry1Ab maize was recently reported in Brazil, probably associated to the low effectiveness of the single-toxin hybrid MON 810 against the fall armyworm and cross-resistance from widespread Cry1F-resistant populations (Omoto *et al.*, 2016). Cross-resistance between the two toxins has been proved to be high in *S. frugiperda*, owing to the high identity in their amino-acid sequences (76.7%) and their shared binding site in the midgut of the insect (Hernández-Rodríguez *et al.*, 2013; Bernardi *et al.*, 2015).

Inheritance of resistance is one of the factors that significantly affect the rate of resistance evolution, so that gaining knowledge on the dominance of resistance or the number of genes involved in this trait can improve insect resistance management (IRM) strategies and make them more suitable for each specific case (Gould, 1998; Tabashnik *et al.*, 2008). This work focuses on resistance to Cry1F maize, owing to the importance of this toxin in Bt varieties that target *S. frugiperda* and the number of cases of resistance to Cry1F maize detected in fall armyworm populations from different areas. A comparative analysis of resistance inheritance in Cry1F-resistant populations from different locations would help discern whether resistance shares a common origin in these colonies or it has evolved independently multiple times, which would suggest this pest has a high potential to develop resistance to Bt toxins. Inheritance of Cry1F resistance in *S. frugiperda* has been investigated in populations originated from Puerto Rico

(Blanco *et al.*, 2010; Storer *et al.*, 2010; Vélez *et al*., 2013), Brazil (Santos-Amaya *et al*., 2016) and Argentina (Chandrasena *et al.*, 2017). The aim of the present study was to perform a comparative analysis of the inheritance of Cry1F resistance in two colonies originated from Puerto Rico and Florida, to determine whether the genetic basis of resistance is similar in the two populations. The results could help understand the origin of Cry1F resistance in U.S. mainland, and they should be useful in the development of strategies to manage resistance of *S. frugiperda* to Bt plants.

## 5.2. Material and methods

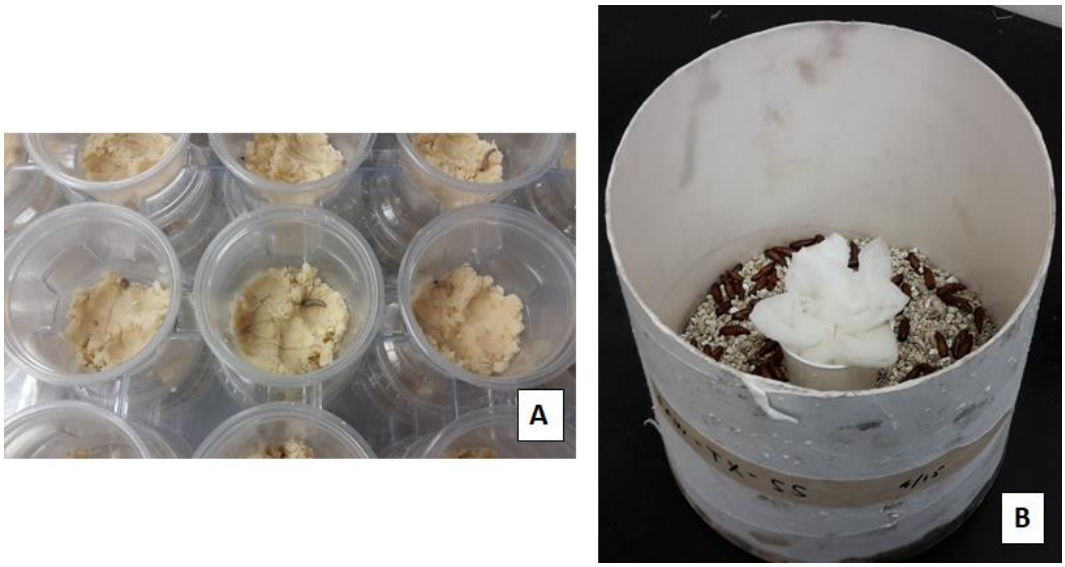
### 5.2.1. Sources of *Spodoptera frugiperda*

One Cry1F-susceptible (SS) and two Cry1F-resistant (PR and FL) colonies of *S. frugiperda* were used as the original insect sources for this study (Table 5.1). SS was collected from non-Bt maize fields near Weslaco in southwestern Texas in 2013, and it had been maintained in the laboratory of Dr. Huang (LSU Ag Center, Baton Rouge, LA, US) without exposure to any Bt toxins or insecticides for approximately 15 generations before it was used in this study. Previous studies showed that SS was susceptible to Cry1F, Cry2Ab2, and Cry1A.105 proteins, as well as to Bt maize plants expressing these Bt proteins (Huang *et al*., 2014; Niu *et al*., 2016a,b). PR was collected from maize fields in Puerto Rico in 2011, while FL was isolated using an $F_2$ screen from a field population collected in non-Bt maize fields in south Florida in 2011 (Huang *et al*., 2014). Both PR and FL have been documented to be highly resistant to both Cry1F-treated diet and Cry1F maize plants (Niu *et al*., 2013, 2014; Huang *et al*., 2014).

Larvae of these colonies were reared individually in 30 ml plastic cups containing meridic diet (Ward's Stonefly Heliothis diet, Rochester, NY), covered by ventilated plastic lids and arranged in 30-well trays kept at room conditions until pupation (Niu *et al*., 2013) (Fig. 5.1A). Pupae of each colony were placed on 3.8-L cylindrical cardboard containers (Huhtamaki Foodservice, De Soto, Kansas), containing approximately 100 g of vermiculite (Sun Gro, Pine Bluff, AR), a sugary solution for adult feeding and a mesh cloth on top as an appropriate

substrate for oviposition. Adult emergence, mating, and oviposition took place in growth chambers (PERCIVAL I-36VL, Iowa) at 28 ± 0.5 °C, >90% rh and a 14:10 (L:D) photoperiod (Fig, 5.1B). Eggs were collected daily and bioassays were performed with neonates (< 24 h). Before the two resistant colonies were used in this study, they had been backcrossed with SS three times and reselected for Cry1F resistance on Cry1F maize leaf tissue, so as to minimize the differences in the genetic background among the three colonies. The backcrossed and re-selected colonies were used as the sources of the resistant insects in this study.

**Figure 5.1.** Rearing of *S. frugiperda* larvae. Cups containing artificial diet and a single larva (A) and oviposition containers (B).




### 5.2.2. Genetic crosses

Besides the three original colonies mentioned above, 14 other colonies (Table 5.1) were established with four types of genetic crosses: (1) reciprocal crosses between SS and resistant colonies; (2) $F_1$ by $F_1$ crosses; (3) backcrosses of $F_1$ with resistant colonies; and (4) intercolony crosses between PR and FL. In the genetic crosses, pupae of each parental colony were first separated based on sex. For each cross, 30-50 female or male pupae from one colony and 30-50 pupae of the opposite sex from another colony were placed in oviposition containers, prepared as described above. Containers with pupae were placed in growth chambers and maintained at

28 ± 0.5 °C, >90% rh and a 14:10 (L:D) photoperiod for adult emergence, mating, and oviposition.

Among the 14 cross-colonies (1-14) (Table 5.1), four were produced by reciprocal crosses between SS and PR or FL. These colonies were:

(1) $F1_{PR♀xSS♂}$: $F_1$ generation of the cross between PR females and SS males.

(2) $F1_{PR♂xSS♀}$: $F_1$ generation of the cross between PR males and SS females;

(3) $F1_{FL♀xSS♂}$: $F_1$ generation of the cross between FL females and SS males

(4) $F1_{FL♂xSS♀}$: $F_1$ generation of the cross between FL males and SS females.

Four $F_2$ colonies were generated from sib-mating within each of the four $F_1$ colonies, respectively, and they were denoted as:

(5) $F2_{PR♀xSS♂}$: produced by sib-mating within $F1_{PR♀xSS♂}$;

(6) $F2_{PR♂xSS♀}$, produced by sib-mating within $F1_{PR♂xSS♀}$;

(7) $F2_{FL♀xSS♂}$, produced by sib-mating within $F1_{FL♀xSS♂}$

(8) $F2_{FL♂xSS♀}$, produced by sib-mating within $F1_{FL♂xSS♀}$.

Four colonies were developed by backcrossing each of the four $F_1$ colonies with their corresponding resistant colonies, and they were denoted as:

(9) $BC_{PR♀xF1♂}$, produced by backcrossing pooled $F1_{PRxSS}$ males to PR females;

(10) $BC_{PR♂xF1♀}$, produced by backcrossing pooled $F1_{PRxSS}$ females to PR males;

(11) $BC_{FL♀xF1♂}$, produced by backcrossing pooled $F1_{FLxSS}$ males to FL females;

(12) $BC_{FL♂xF1♀}$, produced by backcrossing pooled $F1_{FLxSS}$ females to FL males.

Two colonies were produced from the reciprocal intercolony crosses between PR and FL, they were:

(13) $F1_{PR♀xFL♂}$, produced from crosses between PR females and FL males;

(14) $F1_{PR♂xFL♀}$, produced from crosses between PR males and FL females.

**Table 5.1.** *Spodoptera frugiperda* colonies used in the assessment of the genetic basis of resistance.

| Colony ID | Source |
|---|---|
| **SS** | A Cry1F-susceptible colony collected from non-Bt maize field in Texas in 2013 |
| **PR** | A Cry1F-resistant colony collected from maize fields in Puerto Rico in 2011 |
| **FL** | A Cry1F-resistant colony collected from maize fields in Florida in 2011 |
| **$F1_{PR♀SS♂}$** | $F_1$ progeny of the cross between PR females and SS males |
| **$F1_{PR♂SS♀}$** | $F_1$ progeny of the cross between PR males and SS females |
| **$F1_{FL♀SS♂}$** | $F_1$ progeny of the cross between FL females and SS males |
| **$F1_{FL♂SS♀}$** | $F_1$ progeny of the cross between FL males and SS females |
| **$F2_{PR♀SS♂}$** | Progeny of the sib-mating within $F1_{PR♀SS♂}$ |
| **$F2_{PR♂SS♀}$** | Progeny of the sib-mating within $F1_{PR♂SS♀}$ |
| **$F2_{FL♀SS♂}$** | Progeny of the sib-mating within $F1_{FL♀SS♂}$ |
| **$F2_{FL♂SS♀}$** | Progeny of the sib-mating within $F1_{FL♂SS♀}$ |
| **$BC_{PR♀F1♂}$** | Progeny of the backcross of pooled $F1_{PRxSS}$ males to PR females |
| **$BC_{PR♂F1♀}$** | Progeny of the backcross of pooled $F1_{PRxSS}$ females to PR males |
| **$BC_{FL♀F1♂}$** | Progeny of the backcross of pooled $F1_{FLxSS}$ males to FL females |
| **$BC_{FL♂F1♀}$** | Progeny of the backcross of pooled $F1_{FLxSS}$ females to FL males |
| **$F1_{PR♀FL♂}$** | Progeny of the cross between PR females and FL males |
| **$F1_{PR♂FL♀}$** | Progeny of the cross between PR males and FL females |

### 5.2.3. Plant material

For leaf tissue bioassays, a Cry1F maize hybrid, Pioneer 31D59 (Herculex® I; Pioneer Hi-Bred, Johnston, Iowa) and a closely related non-Bt maize hybrid, Pioneer 31P40 (Pioneer Hi-Bred) were grown in the greenhouse at 25ºC and a 16:8 (L:D) photoperiod. For each hybrid, two seeds were sown in each 18.9-L pot containing standard potting soil mixture (Perfect Mix™, Expert Gardener products, St. Louis, US), and plants were grown to the V8 phenological stage.

### 5.2.4. Source of Cry1F toxin

The purified Cry1F protein used in diet-incorporated bioassays had a purity of 99.9% and it was obtained from Dr. Marianne Pusztai-Carey at the Case Western Reserve University, Cleveland, Ohio, US. A recombinant *Escherichia coli* culture was used to produce the Cry1F proteins, which were activated with trypsin and lyophilized prior to the bioassays.

### 5.2.5. Insect bioassays

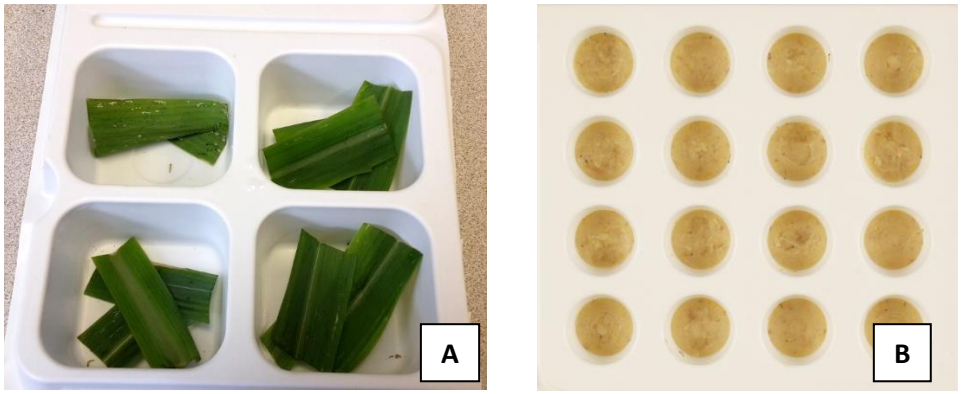
Susceptibility of the 17 insect colonies of *S. frugiperda* mentioned above was determined using two methods: leaf tissue and diet-incorporated bioassays.

Leaf tissue bioassays used leaf tissue that was excised from fully-expanded leaves of plants in the V5-V8 phenological stage. In the bioassays, three pieces of Bt or non-Bt leaf tissue that were approximately 3 cm long were placed in each well of a 32-well bioassay tray (C-D International, Pitman, NJ) and then, four newly-hatched larvae (<24 h) of an insect colony were released on the surface of the leaf tissue in each well and confined using vented covers (Bio-CV-4, C-D International, Pitman, NJ) (Figure 5.2A). Fresh leaf tissue was added to each well on days 3 and 5. Four replicates were performed for each combination of maize hybrid and insect colony, each replicate consisting of 32 larvae (8 wells), so that the total number of larvae tested per colony and maize hybrid was n=4×32=128.

In the case of diet-incorporated bioassays, for each colony three Cry1F concentrations (3.16, 10.00 and 31.60 µg/g) and a control solution (no Bt protein) were prepared by diluting the appropriate amount of Cry1F protein in distilled water, and then each solution was mixed with the appropriate amount of the same meridic diet used for insect rearing (Ward's Stonefly Heliothis diet, Rochester, NY). The use of these concentrations in the bioassays was based on the results of the past studies and the average Cry1F concentration expressed in the leaves of Herculex® I maize plants (event TC1507) in the V9 growth stage, which was estimated to be 12.1 ng/mg of dry weight (EPA, 2005). In the bioassays, approximately 1 g of treated or control diet was placed in each cell of a 128-cell bioassay tray (C-D International, Pitman, NJ), and then a neonate larva (<24 h)

was placed on the diet surface in each cell and confined using a cover that allowed air circulation (Bio-CV-16, C-D International, Pitman, NJ) (Fig. 5.2B). Four replicates of 25 larvae per replicate were run for each combination of insect colony and Cry1F concentration, so that a total of 100 larvae were tested per Cry1F concentration and colony (n= 4×25=100).

**Figure 5.2.** Susceptibility bioassays. Leaf tissue (A) and diet-incorporated (B) bioassays.




For both assay methods, bioassay trays were placed in growth chambers and maintained at 28 ± 0.5 °C, 50% rh and a 16:8 h (L:D) photoperiod for a week. The number of surviving larvae in each replicate was checked 7 days after larval release. A larva was considered dead if it did not move when prodded with a fine hair brush. Surviving larvae were weighed and the number of larvae that had a body weight of <6 mg/larva was also recorded for each replicate.

### 5.2.6. Statistical analysis

For each replicate, mortality was measured as 'practical mortality', which was calculated based on the total number of dead larvae plus the number of larvae weighing less than 6 mg (Huang *et al*., 2014). Practical mortality (thereafter called mortality in this chapter) was corrected according to the method described in Abbott (1925), and then transformed using the arcsine$\sqrt{x}$ to normalize the data. A one-way analysis of variance (ANOVA) was performed using the General Linear Model procedure with insect colony as the main factor. Treatment means based on

insect colonies were separated using Tukey's HSD tests at α=0.05 level (SAS Institute, 2010).

Sex linkage of Cry1F resistance was evaluated separately for PR and FL by comparing differences in the susceptibility of the two $F_1$ colonies obtained from the reciprocal crosses between SS and each resistant colony. If significant differences in the mortalities between the two $F_1$ reciprocal colonies were observed, resistance was considered to be sex-linked. Otherwise, if the mortalities were similar, resistance was considered to be autosomal.

Effective dominance level ($D_{ML}$), which evaluates the relative mortality level for an indicated insecticide concentration, was measured for each resistant colony according to the method described in Bourguet *et al*. (2000b), so that:

$$D_{ML} = (ML_{RS} - ML_{SS}) / (ML_{RR} - ML_{SS})$$

where $ML_{RS}$ is the mortality level at a given insecticide concentration for the heterozygotes; $ML_{SS}$ is the mortality level at a given insecticide concentration for the susceptible homozygotes; and $ML_{RR}$ is the mortality level at a given insecticide concentration for the resistant homozygotes. $D_{ML}$ was calculated for the leaf tissue and diet-incorporated bioassays at each of the three Cry1F concentrations tested. Values of $D_{ML}$ can range between 0 (completely recessive) and 1 (completely dominant).

Larval mortality data of $F_2$ and backcrossed colonies on the Cry1F-leaf tissue and Cry1F-treated diet were analyzed with chi-square tests to determine if the Cry1F resistance in PR and FL fitted the Mendelian single gene model (Tabashnik, 1991). For the diet-incorporated bioassays, the test for the monogenic model was performed for Cry1F concentrations at 10.00 and 31.60 μg/g only, because the concentration of 3.16 μg/g was not a good dose to discriminate heterozygotes from homozygotes. Given that leaf tissue bioassays were not conducted for the backcrossed colony related to FL, the corresponding chi-square test for fitting the monogenic model was not conducted for this colony.

Complementation tests for allelism were performed to find out whether the two resistant strains share a resistance locus (Tabashnik *et al*., 1997). This hypothesis was evaluated by comparing the mortalities of the two $F_1$ reciprocal colonies generated from the intercolony crosses of PR and FL ($F1_{PR♀FL♂}$ and $F1_{PR♂FL♀}$) with that of the parental colonies. A similar performance in the susceptibility assays between $F_1$ colonies and their parents (i.e. PR and FL) would indicate a similar genetic basis between PR and FL, since resistance alleles located at different loci would restore susceptibility to the toxin in $F_1$ offspring.

## *5.3. Results*

### 5.3.1. Overall larval mortalities of the insect colonies on Cry1F leaf tissue and Cry1F-treated diet

The effect of insect colony on larval mortality was significant for both leaf tissue bioassays ($F_{14,45} = 49.63$; $p < 0.0001$) and each of the three Cry1F concentrations in the diet-incorporated bioassays ($F_{16,51} \geq 40.29$; $p < 0.0001$) (Table 5.2). Mortality of SS was 100 % after 7 days on Cry1F leaf tissue and on Cry1F-treated diet at any of the three concentrations. On the other hand, the populations PR and FL were highly resistant to both Cry1F maize leaf tissue and Cry1F-treated diet, as indicated by the low values of larval mortality recorded in both populations on Cry1F maize leaf tissue and Cry1F-treated diet (0-11.8 %), values which were similar ($p > 0.05$) between the two resistant colonies.

**Table 5.2.** Corrected larval mortalities (%, mean ± SE) of different genetic colonies of *S. frugiperda* after 7 days on Cry1F maize leaf tissue or diet treated with three concentrations of purified Cry1F protein.

| Colony | Leaf tissue assay | Diet-incorporated assay | | |
|---|---|---|---|---|
| | | 3.16 µg/g | 10.00 µg/g | 31.60 µg/g |
| **SS** | 100 ± 0.0 f | 100 ± 0.0 e | 100 ± 0.0 e | 100 ± 0.0 f |
| **PR** | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 1.0 ± 1.0 a |
| **FL** | 0.0 ± 0.0 a | 11.8 ± 9.9 ab | 0.0 ± 0.0 a | 7.4 ± 3.7 ab |
| **F1$_{PR♀SS♂}$** | 98.2 ± 1.0 ef | 71.0 ± 8.1 d | 100 ± 0.0 e | 100 ± 0.0 f |
| **F1$_{PR♂SS♀}$** | 88.0 ± 4.3 cedf | 97.0 ± 3.0 e | 100 ± 0.0 e | 100 ± 0.0 f |
| **F1$_{FL♀SS♂}$** | 96.7 ± 1.9 def | 68.2 ± 3.5 cd | 85.8 ± 6.4 cde | 98.8 ± 1.2 f |
| **F1$_{FL♂SS♀}$** | 88.5 ± 5.3 cdef | 75.4 ± 2.9 d | 91.5 ± 3.6 de | 97.6 ± 2.4 ef |
| **F2$_{PR♀SS♂}$** | 78.2 ± 8.3 bcde | 54.6 ± 7.9 cd | 78.1 ± 4.4 bcd | 77.6 ± 5.6 de |
| **F2$_{PR♂SS♀}$** | 42.8 ± 12.0 b | 54.2 ± 8.5 cd | 63.9 ± 10.3 bcd | 61.9 ± 4.4 cd |
| **F2$_{FL♀SS♂}$** | 67.6 ± 5.8 bc | 66.7 ± 8.6 cd | 66.7 ± 13.9 bcd | 80.0 ± 8.6 def |
| **F2$_{FL♂SS♀}$** | 77.1 ± 8.0 bcde | 58.8 ± 4.9 cd | 61.4 ± 4.9 bcd | 71.4 ± 3.3 d |
| **BC$_{PR♀F1♂}$** | 44.0 ± 9.9 bc | 50.5 ± 6.7 cd | 55.8 ± 8.2 bc | 30.9 ± 10.4 bc |
| **BC$_{PR♂F1♀}$** | 75.0 ± 6.9 bcd | 76.4 ± 5.1 d | 62.6 ± 8.4 bcd | 73.3 ± 4.5 d |
| **BC$_{FL♀F1♂}$** | n/a | 31.9 ± 4.3 bc | 46.7 ± 6.5 b | 54.3 ± 8.4 cd |
| **BC$_{FL♂F1♀}$** | n/a | 2.6 ± 2.6 a | 47.6 ± 5.3 b | 55.2 ± 1.7 cd |
| **F1$_{PR♀FL♂}$** | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 0.0 ± 0.0 a | 0.0 ± 0.0 a |
| **F1$_{PR♂FL♀}$** | 3.8 ± 2.3 a | 3.1 ± 1.8 a | 9.4 ± 9.4 a | 6.3 ± 6.3 ab |
| **Analysis of variance** | $F_{14,45}$ = 49.63 $P$ < 0.001 | $F_{16,51}$ = 43.59 $P$ < 0.001 | $F_{16,51}$ = 40.29 $P$ < 0.001 | $F_{16,51}$ = 48.37 $P$ < 0.001 |

[a] Mean values followed by a common letter in a column were not significantly different at α = 0.05 (Tukey's HSD test).

### 5.3.2. Maternal effect of Cry1F resistance in PR and FL

Larval mortality on Bt maize leaf tissue and at any of the toxin concentrations tested in the diet-incorporated assays did not differ significantly ($p > 0.05$) between the two reciprocal $F_1$ colonies in either PR or FL, with the exception of mortality on 3.16 µg/g, which was significantly different between the two $F_1$

populations associated with PR (Table 5.2). Thus, the results did not provide evidence of sex-linkage of resistance in PR or FL. Mortalities of the four $F_1$ colonies ranged from 68.2 ± 3.5 % to 100 ± 0.0 %, and they were only lower ($p < 0.05$) than that of SS in three out of the four $F_1$ colonies tested on 3.16 µg/g, the lowest toxin concentration used in diet-incorporated assays.

### 5.3.3. Dominance level of Cry1F resistance in PR and FL

Given the absence of significant differences in larval mortalities between the $F_1$ reciprocal colonies in the different assays and toxin concentrations tested, the results of the two $F_1$ colonies were pooled for PR and FL. The effective dominance level, $D_{ML}$, of the pooled $F_1$ colonies based on the leaf tissue bioassays was 0.07 for both PR and FL, whereas the value of $D_{ML}$ based on the diet-incorporated bioassays for PR and FL was 0.16 and 0.32 at 3.16 µg/g, 0 and 0.11 at 10.00 µg/g, and 0 and 0.02 at 31.60 µg/g, respectively (Table 5.3). These results suggest that Cry1F resistance was functionally recessive or incompletely recessive in both PR and FL.

**Table 5.3.** Effective dominance values ($D_{ML}$) of Cry1F resistance in two colonies of *S. frugiperda* collected in Puerto Rico (PR) and Florida (FL).

| Colony | Genetic cross | Leaf tissue assay | Diet-incorporated assay | | |
|---|---|---|---|---|---|
| | | | 3.16 µg/g | 10.00 µg/g | 31.60 µg/g |
| PR | $F1_{PR♀SS♂}$ | 0.02 | 0.29 | 0 | 0 |
| | $F1_{PR♂SS♀}$ | 0.12 | 0.03 | 0 | 0 |
| | Pooled $F_1$ | 0.07 | 0.16 | 0 | 0 |
| FL | $F1_{FL♀SS♂}$ | 0.03 | 0.36 | 0.14 | 0.01 |
| | $F1_{FL♂SS♀}$ | 0.12 | 0.28 | 0.09 | 0.03 |
| | Pooled $F_1$ | 0.07 | 0.32 | 0.11 | 0.02 |

### 5.3.4. Testing for fitting the Mendelian monogenic model

Larval mortalities of the four $F_2$ colonies were significantly ($p < 0.05$) lower than the mortality of SS, but significantly greater ($p < 0.05$) than that of PR and FL (Table 5.2). There were no significant ($p > 0.05$) differences in larval mortality among the four $F_2$ colonies on the leaf tissue bioassays and at each of the three Cry1F concentrations in the diet-incorporated bioassays. Larval mortalities of the four $F_2$ colonies ranged from 42.8 to 78.2% on Cry1F leaf tissue, 54.2-66.7% at 3.16 µg Cry1F/g, 61.4-78.1% at 10.00 µg Cry1F/g, and 61.9-80.0% at 31.60 µg Cry1F/g. Because Cry1F resistance was not sex-linked in neither PR nor FL, as mentioned in section 5.3.2, mortality data recorded for the two $F_2$ colonies associated with each resistant colony were pooled to test for fitting the Mendelian monogenic model. The results of the chi-square test showed that the segregation of the $F_2$ colonies derived from FL fitted the monogenic model well ($p > 0.05$) on Cry1F leaf tissue and Cry1F-treated diet at 10.00 and 31.60 µg/g, whereas for PR, it fitted the single-gene model in the diet-incorporated bioassays, but not ($p < 0.05$) in the maize leaf tissue bioassays (Table 5.4).

Larval mortalities of the four backcrossed colonies were also significantly greater ($p < 0.05$) than those of PR and FL, and significantly lower ($p < 0.05$) than the mortality of SS, with the exception of the colony $BC_{FL♂F1♀}$ at 3.16 µg/g in the diet-incorporated bioassays, in which mortality was low (2.6 %) and not significantly different ($p > 0.05$) to the mortality of FL at that toxin dose (Table 5.2). Given that resistance was not sex-linked in either PR or FL, the data was pooled for the two backcrosses associated with PR and FL. As observed with the $F_2$ colonies, the results of the chi-square tests based on the pooled data of the backcrossed colonies showed that the segregation of the backcrossed colonies derived from FL fitted ($p > 0.05$) the single-gene model well, while, for PR, it only followed ($p > 0.05$) the model at the concentration of 31.60 µg/g (Table 5.4).

**Table 5.4.** Test for fitting the Mendelian monogenic model for Cry1F resistance in two colonies of *S. frugiperda* originated from Puerto Rico (PR) and Florida (FL). The results shown in this table correspond to pooled data of either $F_2$ or BC colonies.

| Assay method | Cry1F concentration (µg/g) | Segregation | Insects tested (N) | Number of dead | | $\chi^2$ | P-value |
|---|---|---|---|---|---|---|---|
| | | | | Observed | Expected | | |
| **PR** | | | | | | | |
| **Leaf tissue** | **n/a** | **$F2_{PRXSS}$** | 256 | 154.9 | 183.2 | 15.356 | <0.001* |
| | **n/a** | **$BC_{PRXF1}$** | 256 | 152.3 | 119.2 | 17.255 | <0.001* |
| **Diet-incorporated** | **10.00** | **$F2_{PRXSS}$** | 197 | 139.9 | 147.8 | 1.681 | 0.195 |
| | | **$BC_{PRXF1}$** | 197 | 116.6 | 98.5 | 6.670 | 0.01* |
| | **31.60** | **$F2_{PRXSS}$** | 193 | 134.6 | 145.2 | 3.138 | 0.077 |
| | | **$BC_{PRXF1}$** | 200 | 104.2 | 101.0 | 0.205 | 0.651 |
| **FL** | | | | | | | |
| **Leaf tissue** | **n/a** | **$F2_{FLXSS}$** | 256 | 185.2 | 182.5 | 0.01 | 0.920 |
| **Diet-incorporated** | **10.00** | **$F2_{FLXSS}$** | 200 | 128.1 | 138.7 | 2.617 | 0.106 |
| | | **$BC_{FLXF1}$** | 200 | 94.3 | 88.65 | 0.647 | 0.421 |
| | **31.60** | **$F2_{FLXSS}$** | 200 | 151.4 | 151.9 | 0.007 | 0.933 |
| | | **$BC_{FLXF1}$** | 200 | 112.9 | 105.6 | 1.069 | 0.301 |

n/a: not available
* Significantly different ($p < 0.05$) from the Mendelian monogenic model.

### 5.3.5. Complementation tests for allelism between PR and FL

Similar to their parental colonies (PR and FL), the two $F_1$ reciprocal colonies ($F1_{PR♀FL♂}$ and $F1_{PR♂FL♀}$) derived from the intercolony-crosses between PR and FL were also highly resistant to both Cry1F maize leaf tissue and Cry1F-treated diet (Table 5.2). Larval mortality of $F1_{PR♀FL♂}$ was zero across the two bioassay methods and the three Cry1F concentrations in the diet-incorporated bioassays. The corresponding mortality of $F1_{PR♂FL♀}$ was also low, ranging from 3.1 to 9.4 %. No significant differences ($p > 0.05$) were observed in larval mortality between the two parental colonies and the two $F_1$ colonies.

## *5.4. Discussion*

Since resistance of *S. frugiperda* to Cry1F was first detected in TC1507 maize fields in Puerto Rico in 2006 (Storer *et al*., 2010), inheritance of resistance has been investigated in several populations from the island (Blanco *et al*., 2010; Storer *et al*., 2010; Vélez *et al*., 2013). In all the populations evaluated, resistance was described to be inherited as a single recessive or incompletely recessive trait and, in general, resistance was autosomal, except for one population in which resistance was reported to be likely associated with males (Blanco *et al*., 2010). Additionally, Cry1F resistance was recently reported to be inherited as a single, autosomal, recessive or incompletely recessive gene in two Brazilian populations of *S. frugiperda* (Farias *et al*., 2016; Santos-Amaya *et al*., 2016), and it was reported to be a recessive trait in a fall armyworm population resistant to Cry1F maize from Argentina (Chandrasena *et al.*, 2017). On the other hand, resistance to Cry1F maize was proved to be conferred by a non-recessive allele in a population from North Carolina in the only other work that has studied resistance inheritance in mainland US (Li *et al.*, 2016).

The results of the present study suggest that resistance was likely recessive, autosomal and associated with a single gene in both the Puerto Rico and the Florida strains. These data, together with the data from the studies mentioned above, suggest that, generally, Cry1F resistance in *S. frugiperda* populations from different locations is inherited similarly. Nevertheless, the differences observed in resistance inheritance between the closely-located Florida and North Carolina populations deserve further study.

It has been hypothesized that the Cry1F resistance in *S. frugiperda* populations in Florida might have arisen from migration of resistant individuals from Puerto Rico (Huang *et al*., 2014). The low mortalities values presented here for the $F_1$ colonies resulting from the reciprocal crosses between PR and FL suggest that (major) alleles conferring resistance in the two colonies are located at the same locus, which could point to a common origin of the two colonies. Due to the recessive nature of resistance in both colonies, resistance alleles located in different loci would render higher mortality rates for the heterozygous $F_1$ progeny

than those registered in the parental colonies. In addition, the migration patterns of *S. frugiperda* are consistent with this hypothesis (Johnson, 1987; Nagoshi *et al*., 2012), and the weather systems occurring in the area could promote such kind of journey (Arias *et al*., 2011). However, the results of recent studies refute this hypothesis.

For a specific Bt protein, it is not uncommon that similar genetic bases are associated with resistance among populations of the same species (Yang *et al*., 2018). For example, the Cry1Ac resistance in two populations of *Pectinophora gossypiella* selected with Bt cotton bolls and Bt treated diet, were linked to recessive alleles at the same cadherin locus (Fabrick and Tabashnik, 2012). In the diamondback moth, *Plutella xylostella*, Cry1Ac resistance in two populations originated from Hawaii and South Carolina was associated to a same gene (Baxter *et al.*, 2005). Recently, Farias *et al*. (2014b) reported that eight Cry1F-resistant populations of *S. frugiperda* collected from different areas in Brazil shared a same locus of resistance. On the other hand, studies have also shown that multiple mutations can occur at the same locus that is responsible for Bt resistance. For example, in *P. gossypiella*, there are at least three mutations at the cadherin locus that is associated with Cry1Ac resistance, while the phenotype of these three recessive alleles is the same. Individuals with two resistance alleles in any combination of the mutations are resistant, whereas those with one or none are susceptible (Morin *et al*., 2003). Diverse mutations in a cadherin gene conferring Cry1Ac resistance were also found in the Chinese populations of the cotton bollworm, *Helicoverpa armigera* (Zhao *et al*., 2010).

Results of the current study suggest that the two Cry1F-resistant colonies of *S. frugiperda* originated from Puerto Rico and Florida likely shared a (major) locus for resistance. However, resistance in these populations could be due to a same mutation or multiple mutations at the locus, as observed in the Cry1Ac resistance in *P. gossypiella* and *H. armigera*. Up to the date, the molecular basis of resistance has only been studied in populations from Puerto Rico. Early studies indicated that resistance in these populations was associated with reduced Cry1F binding and midgut alkaline phosphatase expression (Jurat-Fuentes *et al*., 2011;

Jakka *et al*., 2016). However, recent studies indicate a mutation in the ATP Binding Cassette subfamily C2 (SfABCC2) is more likely the cause of resistance in populations from Puerto Rico, although reduced alkaline phosphatase levels could also play a role in resistance to Cry1F maize in these populations (Banerjee *et al.*, 2017; Flagel *et al.*, 2018). On the other hand, the absence of the mutation in the receptor SfABCC2 in the Cry1F-resistant populations from Florida suggests resistance would be conferred by different alleles in *S. frugiperda* populations from this state and Puerto Rico (Banerjee *et al.*, 2017). Furthermore, the absence of this mutation in resistant populations of *S. frugiperda* from other areas in North, Central and South America suggests that resistance to Cry1F likely evolved repeatedly in separate populations, and it is not the result of migration of resistant individuals originating in a single spot (Flagel *et al.*, 2018). Establishing whether resistance to Cry1F maize shares a common origin in fall armyworm populations from different locations or it has evolved independently in different regions has important implications for resistance management in this species. Much more efforts are needed to elucidate the molecular mechanisms of resistance in different populations of *S. frugiperda* that provide reliable evidence of the origin of resistant populations. This information would contribute to improve existing IRM programs of this species where it is already present and would help to establish adequate IRM strategies in Europe if the pest arrived to the continent.

# VI. General discussion

Genetically modified maize that expresses *Bacillus thuringiensis* toxin genes (Bt maize) is the most prevalent Bt crop in the world, and an efficient tool to control a wide range of noctuid pests. Bt maize technology was rapidly adopted in the Ebro Valley, where over 50% of the maize grown every year in the last decade expressed the toxin Cry1Ab. This poses a high selective pressure on the occurring pests, leading to an increasing risk of resistance development to the toxin, which would render Bt maize ineffective to control them. In this thesis, we have focused on three noctuid pests in the Mediterranean area and elsewhere. More specifically, we have considered a primary pest, *Sesamia nonagrioides*, and a secondary pest, *Mythimna unipuncta*, in Spain, as well as the potentially invasive pest *Spodoptera frugiperda*, which is expanding its distribution range from America to the Old World, and the insect pest in which the highest number of cases of field-evolved resistance to Bt crops has been reported.

There is a wide consensus that insect resistance management (IRM) plans are crucial to delay resistance evolution of the target pests and that resistance monitoring is a key feature of IRM programs (Head and Greenplate, 2012). The high-dose/refuge (HDR) strategy is the most commonly used IRM approach, and the one implemented in the Ebro Valley. IRM strategies should be dynamic and adapt to the new information available, so that management can change and control failures can be delayed (Andow and Ives, 2002; MacIntosh, 2010). Thus, the results of this thesis will contribute to optimize the implementation of adaptive IRM strategies for the aforementioned primary and secondary noctuid pests, so as to guarantee the long-term sustainability of Bt maize.

The ongoing IRM plan is carried out on a yearly basis, considering the susceptibility to the toxin Cry1Ab of *S. nonagrioides* populations from different zones within the Ebro Valley. Recently, EFSA recommendations suggested that monitoring efforts in the EU should focus in the Ebro Valley in order to maximize the probability of early detection of resistance evolution, considering small areas of about 10 x 10 km for a close tracking of variations in the susceptibility to the Bt toxin at this scale (EFSA, 2015). However, the low variation in susceptibility to the toxin Cry1Ab recorded between (<5-fold) and within (typically <10-fold)

populations from different locations suggests that, at this point, there are no areas where resistance evolution is more likely to happen because susceptibility is lower. Therefore, resistance monitoring should not focus in any particular location, but it should keep on considering populations from different zones in the Ebro Valley because, as far as we know, resistance could evolve in any part of the region. This information is essential to determine whether future fluctuations in susceptibility to the Bt toxin observed in the Ebro Valley are within the natural variation observed in this species or they can be a sign of resistance development (Siegfried *et al.*, 2007). Additionally, the low susceptibility to the toxin Cry1Ab recorded in populations of *M. unipuncta* from Galicia, where Bt maize has never been sown, and its similarity with the values obtained in populations from the Ebro Valley, where Bt maize cultivation has been intense for over a decade, suggest that this pest has been little exposed to the toxin in the latter area. Nevertheless, given the known potential of *M. unipuncta* to develop resistance to this Bt toxin rapidly (González-Cabrera *et al.*, 2013), our data support the recommendations of previous studies that suggested *M. unipuncta* should not be disregarded in Bt maize IRM plans in Spain. Additionally, the susceptibility value estimated in the population from Galicia can be considered the baseline susceptibility of *M. unipuncta* to the protein Cry1Ab in this area in forthcoming IRM programs.

Broadening the knowledge on the range of plant hosts that can support larval development and oviposition of *S. nonagrioides* is key for understanding the biology and ecology of this pest and for an adequate management of resistance. The rationale behind this is that the presence of unstructured refuges composed of natural hosts near Bt maize fields can help delay resistance evolution (MacIntosh, 2010). We have found that, apart from maize, *S. nonagrioides* could complete their life cycle in two cultivated plant species (sorghum and rice) and a weed (johnsongrass), but individuals fed solely on these hosts experienced a higher mortality and were developmentally delayed and growth-restricted in comparison with those reared on maize. Thus, the *S. nonagrioides* populations hosted by these plants would be composed of a low number of low quality individuals that would likely emerge later than potentially resistant adults from Bt fields, thus

compromising the requirement of random mating between susceptible and resistant individuals of the HDR strategy. These results indicate that neither of the four wild alternative hosts tested is suitable as a refuge, since they do not meet the criteria that natural hosts must comply with to delay resistance development (Van den Berg, 2017). Sorghum was the only cultivated host that could be considered as a potential refuge for *S. nonagrioides* in areas where this crop is cultivated close to Bt maize fields, but at present it is not a common agronomic practice in Spain. Therefore, our data do not provide any evidence that can lead to reduce the requirements of a structured refuge in the Ebro Valley, in agreement with the results obtained for several stem-boring maize pests (Bourguet *et al.*, 2000a; Losey *et al.*, 2001; Van den Berg, 2017). Refuges for susceptible individuals should continue to be composed of non-Bt maize plants, and it would be desirable that compliance with refuge requirements increased from the 89% reported in 2016 (EFSA Panel on GMO, 2018) to the maximum possible extent in maize based agro-ecosystems in Spain.

The nutritional and morphological quality of plants varies with time, so that each generation of multivoltine pest species like *S. nonagrioides* uses host plants in different phenological stages. This could have an effect on oviposition of this noctuid, and could ultimately influence the size of the pest population, a parameter that affects the rate of resistance evolution to Bt crops (Sisterson *et al.*, 2004). Therefore, obtaining a reliable estimate of the size of each generation of *S. nonagrioides* exposed to Bt maize is necessary to improve the resistance evolution model of this species. The lower fecundity recorded during the maize reproductive stage kernel dough with regards to plants during anthesis (VT) agrees with the decreasing fecundity reported in other lepidopteran species in older plants compared to younger plants (Navasero and Ramaswamy, 1993; Ndemah *et al.*, 2001; Campos *et al.*, 2003), and it could be associated with the decreasing quality proved in plants as they age (Cockfield, 1988; Francis *et al.*, 1993; Zalucki *et al.*, 2002). Interestingly, fecundity in VT and R4 maize plants is virtually the same as that recorded in females of the same species reared under the photoperiods experienced by the second and third generations, respectively, of this pest in the Ebro Valley (Fantinou *et al.*, 2004), indicating that both photoperiod and maize

plant phenology are important variables for the oviposition rates of the 2nd and 3rd generations of *S. nonagrioides*. Moreover, this finding supports the validity of this parameter in the published model (Castañera *et al.*, 2016).

The HDR strategy has been successfully implemented in the only EU hotspot (Ebro Valley) where resistance evolution is more likely and the main target pest, *S. nonagrioides*, has been exposed to Cry1Ab maize continuously since 1998 (Castañera *et al*., 2016). One of the important objectives of this thesis was to update in 2016 the previous estimate of the frequency of resistance alleles to Cry1Ab toxin in the area, which dated back from 2004-2005, by using the same methodology ($F_2$ screen). This method is the most efficient one of the several ones that can be used to detect rare and recessive resistance alleles, since it preserves genetic variation among isofemale lines and concentrates resistance alleles into homozygous genotypes in the $F_2$ generation so that they can be detected (Andow and Alstad, 1998). Our data indicate that resistance increased slightly between 2004-2005 and 2016, probably associated to the increasing adoption rate in the decade elapsed between the two estimates and the continuous selection pressure exerted on *S. nonagrioides* populations in the area during that period. However, this variation was not significant and it did not occur faster than estimated by the resistance evolution model previously developed by our group. These results validate the predictions of the model and suggest that the HDR strategy has worked in the Ebro Valley. This success is likely associated with the high refuge compliance by farmers in the area (on average > 80% in the last decade) and also to the fact that MON 810 maize represents a high-dose scenario for *S. nonagrioides* (EFSA Panel on GMO, 2018; Farinós *et al.*, 2018). However, the frequency of resistance alleles estimated in 2016 is more than threefold that recommended for this approach to be effective. For this reason, additional efforts should be made to maximize refuge compliance, to guarantee Bt maize continues to be effective in controlling *S. nonagrioides* in the Ebro Valley. The fact that the updated model foresees resistance will occur in this area in 2047, three years earlier than predicted by the previous version of the model, emphasizes the importance of providing robust updated data on the variables included in this resistance evolution model.

The detection for the first time of a resistance allele to a Bt toxin in the EU over the course of this study, more specifically in *S. nonagrioides* from the Ebro Valley to MON 810 maize, supports the need of maintaining regular and proactive resistance monitoring in this area. The resistance allele is currently being selected in the laboratory, and up to now, there has been a remarkable decrease in susceptibility of the resistant population to the toxin Cry1Ab in comparison with that recorded in the laboratory population. Preliminary data suggest that resistance is inherited recessively. This population will enable to obtain information on inheritance of resistance, as well as on whether it entails any fitness costs, which could affect resistance evolution in the studied area.

Inheritance of resistance is one of the factors that significantly affect the rate of resistance evolution (Tabashnik *et al.*, 2013), so that gaining knowledge on the dominance of resistance or the number of genes involved in this trait can improve IRM strategies and make them more suitable for each specific case (Gould, 1998; Tabashnik *et al.*, 2008). We have studied the genetic basis of resistance to Cry1F maize in two populations of S. *frugiperda*, the pest species in which the highest number of cases of field-evolved resistance has been reported (Tabashnik and Carrière, 2017). Both populations have a similar genetic basis of resistance, so that in the two colonies resistance was recessive, autosomal and associated with a single gene, as previously observed in other Cry1F-resistant populations of this pest from Puerto Rico (PR) and Brazil (Vélez *et al.*, 2013; Farias *et al.*, 2016; Santos-Amaya *et al.*, 2016). However, the results of recent studies on the molecular basis of resistance in *S. frugiperda* populations from Florida and PR (Banerjee *et al.*, 2017; Flagel *et al.*, 2018), together with the high number of cases of field-evolved resistance to Bt maize expressing different toxins reported in different areas of North, Central and South America (Tabashnik and Carrière, 2017), suggest *S. frugiperda* has a high potential to become resistant to Bt maize. Additionally, the findings of a recent study suggest African populations of *S. frugiperda* could be the result of the entrance of individuals originating from the Caribbean and Florida areas (Nagoshi *et al.*, 2018), where field-evolved resistance to Cry1F maize had been already reported and it was well established and widespread in some regions like PR (Storer *et al.*, 2012). Therefore, it is likely

that the *S. frugiperda* populations detected in Africa were already resistant to the Bt protein when they arrived to the continent. Moreover, high resistance levels were observed in PR several years after Cry1F maize was removed from there (Storer *et al.*, 2012), probably owing to the lack of strong fitness costs associated with resistance generally observed in populations from the island (Jakka *et al.*, 2014; Vélez *et al.*, 2014). Likewise, potentially resistant African populations of *S. frugiperda* could have remained resistant to Cry1F maize even though they are not under selective pressure in the continent. This could have important implications for IRM of Bt maize in the EU. If Cry1F-resistant fall armyworm populations were successful in arriving to Europe from Africa, it is likely that the only Bt maize hybrids grown in the EU (MON 810) would be ineffective in controlling the pest if used on their own, due to both the low activity of the toxin Cry1Ab against *S. frugiperda* and the high cross-resistance that has been proved between this protein and Cry1F (Hernández-Rodríguez *et al.*, 2013; Bernardi *et al.*, 2015).

In summary, this thesis provides novel information on several aspects that could help to optimize the ongoing resistance management plan and at the same time might contribute to support the European competent authority on defining more precise monitoring protocols. Additionally, the new data have also allowed to validate and update the resistance evolution model of *S. nonagrioides* in the Ebro Valley (Castañera *et al*., 2016), improving the accuracy of its predictions.

# VII. Conclusions

1. The low variation in susceptibility of *Sesamia nonagrioides* to the toxin Cry1Ab between and within closely sampled populations of the Ebro Valley reveals that there is no advantage in shifting the current sampling strategy, which covers large areas in the Ebro Valley, to smaller ones.

2. The similar low values of susceptibility to the toxin Cry1Ab recorded in a population of *Mythimna unipuncta* from a non-Bt region (Galicia) and a hotspot Bt-maize area (Ebro Valley) suggest low exposure of this pest to the toxin in the latter, given its known great potential to develop resistance to Bt maize.

3. To our knowledge, this is the first report of the susceptibility of *M. unipuncta* populations from Galicia to the toxin Cry1Ab. The $LC_{50}$ value estimated in this population could be considered a baseline susceptibility of *M. unipuncta* to Cry1Ab protein in this area in forthcoming IRM programs.

4. Apart from maize, *S. nonagrioides* was able to complete its development on the two cultivated hosts, sorghum and rice, and on the weed johnsongrass. Nevertheless, none of them meet the criteria established for alternative hosts to serve as unstructured refuges for Bt maize within the HDR strategy.

5. Maize phenology affected significantly the oviposition performance of *S. nonagrioides*, so that fecundity on plants at the phenological stage met by the third generation (dough, R4) was nearly cut in half in comparison with the phenological stage met by the second generation (anthesis, VT). These values, which are virtually identical to the theoretical ones used in the current simulation model, corroborate the projections of resistance evolution.

6. The frequency of alleles conferring resistance to the toxin Cry1Ab has increased slightly in *S. nonagrioides* populations from the Ebro Valley,

from 0.0015 in 2004-2005 to 0.0036 in 2016. According to the updated version of the resistance evolution model, control failure is predicted to happen in 2047, three years earlier than previously forecasted.

7. The HDR strategy has been efficient in delaying resistance evolution in the Ebro Valley. However, the current frequency of resistance alleles, which triples the value recommended for an effective implementation of this strategy, heightens the importance of full compliance with refuge requirements in this hotspot area.

8. This is the first report in Europe of a *S. nonagrioides* resistance allele to Bt maize, arising from a field population of the Ebro Valley. Preliminary results of the selection process suggest that the resistance allele is recessive.

9. Resistance to Cry1F maize in two resistant populations of *Spodoptera frugiperda* was recessive, autosomal and associated with a single gene, suggesting that both likely shared a major locus for resistance. The high capacity of this pest to develop resistance to Bt maize supports the raised concerns on the potential incidence of this pest in the EU.

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