Use and application of Rna interference in plant protection: Case study on Western Corn Rootworm

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USE AND APPLICATION OF RNA INTERFERENCE IN PLANT PROTECTION: CASE STUDY ON WESTERN CORN ROOTWORM

MASTER THESIS

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STUDENT'S STATEMENT

ON ACADEMIC RECTITUDE

I, **Mihaela Trčak**, JMBAG 0058205079, born on 24th of September 1994 in Zagreb, declare that I have independently written the thesis under the title:

USE AND APPLICATION OF RNA INTERFERENCE IN PLANT PROTECTION: CASE STUDY ON WESTERN CORN ROOTWORM

With my signature, I guarantee:

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REPORT

ON EVALUATION AND MASTER'S THESIS DEFENSE

Master thesis written by Mihaela Trčak, JMBAG 0058205079, titled:

USE AND APPLICATION OF RNA INTERFERENCE IN PLANT PROTECTION: CASE STUDY ON WESTERN CORN ROOTWORM

Is defended and evaluated with the grade_____, on _____.

Committee for thesis evaluation and defense: Signatures:

1. prof. dr. sc. Renata Bažok, mentor _____
2. prof. dr. sc. Snježana Kereša, member _____
3. doc. dr. sc. Darija Lemić, member _____

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SUMMARY

Of the master thesis written by **Mihaele Trčak**, titled:

USE AND APPLICATION OF RNA INTERFERENCE IN PLANT PROTECTION: CASE STUDY ON WESTERN CORN ROOTWORM

Despite the remarkable increase in pesticide use and advances in plant protection, the global crop loss due to pest damage has remained high. Some pests like western corn rootworm, *Diabrotica virgifera virgifera* LeConte have shown considerable ability to adapt to different management practices such as crop rotation, chemical pesticides, and biotechnological control. Therefore, there is a need for the development of new technologies which can be efficiently and sustainably used for managing economically important pests. One of the new technologies which show promising results in the control and management of western corn rootworm is RNA interference. It is a novel biotechnological tool which is based on a sequence-specific repression of gene expression by using small double-stranded RNAs. It can induce cessation of feeding and ultimately morbidity of pests. Since western corn rootworm management is a challenging task due to substantial adaptability and resistance to various management strategies, RNAi technology presents a valuable tool for WCR control. It is highly efficacious and considered as ecologically safe, but before the wider use of this technology, its weak points shall be considered.

Keywords: RNAi, western corn rootworm, RNAi for western corn rootworm control, RNAi risk assessment, RNAi product registration

1. Introduction

Plant pest damage poses a great threat to agricultural production since they often cause significant yield losses in agriculture. Despite substantial advances in plant protection strategies, agricultural production is still threatened by the pests which show great ability to adapt and adjust to different management strategies. Therefore, there is a need for the development of new methods, technologies, and products which can be used to combat and eradicate resilient pests. In the last two decades, scientists have been exploring the possibility of using RNA interference (RNAi) as a method to control pests and plant diseases. RNAi is a novel technology which is used as silencing machinery in posttranscriptional regulation of genes. It is used in genomics for determination of gene function, in biotechnology for pest and plant disease control and medicine as a therapeutic agent. The principle of RNAi is the sequence-specific degradation of mRNA induced by dsRNA homologous to the target sequence (Taxman, 2009). The uptake of dsRNA by feeding on transgenic plants producing specific dsRNA against pests causes pest lethality due to disruption of translation and cellular processes. It is the most promising method for pest control on a large scale.

RNAi shows a great possibility for combating Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) (Baum et al. 2007, Bolognesi et al., 2012, Fishilevich et al., 2016, Niu et al., 2017) which is the most important economic pest of continuous maize cultivation in the US and Europe (Levine et al., 2002, Ivezić et al., 2009). Remarkably efficient RNAi response in both WCR larvae and adults upon ingestion of dsRNA caused increased interest in the feasibility of RNAi-based technology for WCR management. For now, there are four registered RNAi-based plant incorporated protectant (PIP) products approved by the US EPA called “SmartStax PRO” which combine *Bt* traits and DvSnf7 dsRNA, designed to manage western corn rootworm in maize fields (US EPA, 2017).

Although RNAi technology opened up new approaches towards modern pest management, there are still some uncertainties related to environmental safety, registration process and regulatory framework of RNAi-based products. The main uncertainties arising with the use of

RNAi-biopesticides are linked to the effects of non-target exposure, environmental exposure, and management of resistant pest populations. Nevertheless, environmental risk assessment done by the US EPA for registration of the first RNAi-based PIP, “SmartStax PRO” has shown that it is safe for widespread usage and has no adverse effects on environmental and human health (US EPA, 2016). Therefore, it is to be expected that the number of registered RNAi-based PIP will increase in future due to their efficacy, selectivity for specific target species, and the possibility to control resistant populations.

1.1. Aims and objectives

The aims of this thesis are:

1. To give an overview of RNAi mechanism and possibilities of application in pest control
2. To review existing achievements in the use of RNAi technology for WCR control
3. To discuss the strong and weak points of RNAi technology
4. To examine the possibility of using RNAi as a technology for WCR control on a global scale

2. RNAi technology

RNA interference (RNAi) is a novel technology first discovered in plants, animals, and fungi and it is commonly used as silencing machinery in posttranscriptional regulation of genes. Since its discovery by Fire at al. (1998), it has been thoroughly studied and its potentials have been recognized in several fields: in genomics for determination of gene function, in biotechnology for pest control, and in medicine as a therapeutic agent. The RNAi pathway has been implicated as a mechanism that evolved for defense against viruses or integration of mobile genetic elements; RNAi is also effective in regulating gene expression in virtually all eukaryotic organisms, including plants and insects (Fishilevich et al., 2016). The phenomenon of RNAi as a method for gene silencing has allowed unique advancements in the understanding of gene function in many organisms and thus accelerated the use of reverse genetics to new levels (Joga et al., 2016).

The RNAi mechanism works at the messenger RNA (mRNA) level, exploiting a sequence-dependent mode of action, which makes it unique in potency and selectivity compared with conventional agrochemicals (Zotti et al., 2018). The application of RNAi in agriculture, specifically in crop protection was first investigated by Baum et al. (2007), Mao et al. (2007), Price and Gatehouse (2008), and Zotti and Smagghe, (2015). They concluded that dsRNA-mediated silencing of essential genes in insects can induce cessation of feeding and ultimately morbidity, however, need for the efficient uptake of dsRNA by feeding or topical application is essential for the development of cost-effective RNAi biopesticides. The main problem of pest control nowadays is resistance acquired to chemical products, therefore RNAi is an interesting method which can be applied in crop protection since it allows a wide range of target genes which can be silenced. The importance of novel techniques used in pest control is emphasized by the fact that the total market for agrochemicals increased by 2,6% in 2017, and reached $61,530 million (Zubok, 2019). The possibility of using RNAi as a method for pest control has created an interest in the market and it is expected that the number of registered RNAi-based plant protectants will increase in the future.

2.1. Molecular mechanisms of RNAi

Three major RNAi pathways have been characterized so far: the microRNA (miRNA), piwiRNA (piRNA), and small interfering RNA (siRNA) pathways (Joga et al., 2016). The siRNA pathway is activated upon introduction of dsRNA in insects, therefore it is the key pathway for pest control. The principle of RNAi is sequence-specific degradation of mRNA induced by dsRNA homologous to the target sequence (Taxman, 2009). Several events take place during siRNA interference process in the cell. First, RNase III like adenosine triphosphate (ATP) dependent enzyme Dicer-2 processes dsRNA into smaller 21- to 25-nucleotide (nt) interfering sequences (siRNAs) with dinucleotide 3' overhangs. Dicer encodes a multidomain protein containing an ATP-dependent RNA helicase, PAZ domain, two tandem RNase III domains, and a dsRNA-binding domain (Bernstein et al., 2001). Insects have two Dicers and their activities have been postulated based on their counterparts in *Drosophila* (Camargo et al., 2018). In insects, Dicer-1 exclusively recognizes miRNA and Dicer-2 recognizes dsRNA.

The siRNA duplexes serve as guides for specific mRNA degradation. The 'guide strand' or antisense strand of the siRNA is preferentially associated with RNA-induced silencing complex (RISC) while the 'passenger strand' or sense strand which has the same nt sequence as target mRNA is degraded. The RISC complex binds with the homologous mRNA which is cleaved by the Argonaute 2 protein (Ago2). Ago2 protein contains two distinctive domains: a PAZ domain and a PIWI domain (Parker and Barford, 2006). The PAZ domain is responsible for RNA binding whereas the PIWI domain guides the cleavage of the target mRNA. Ago2 and Dicer-2 are two of the core protein families involved in dsRNA-mediated RNAi response.

The complementary Watson-Crick base pairing of the antisense strand and target mRNA in RICS induces endonucleotic cleavage of the target mRNA by Ago2 protein. Ago 2 cleaves the target mRNA in the middle of the strand so that one fragment is missing polyA tail, and other 5' 7-methylguanosine cap which makes them susceptible for degradation. Such degradation of mRNA obstructs translation; hence this process is often referred to as posttranscriptional gene silencing. The process is closely related to post-transcriptional gene regulation by microRNAs (miRNAs) where the end-result is inhibition of translation initiation, and shares many of the

same components (Price and Gatehouse, 2008). Therefore, RISC can mediate the degradation of mRNA or inhibit translation.

The silencing ability of antisense RNA was originally observed in nematode *Caenorhabditis elegans* Maupas. It is used as a modal organism due to the known DNA sequence, short life span and simple management in the laboratory. The main advantages of using RNAi in nematodes are: (1) easy application (dsRNA delivery) by soaking or microinjection and (2) systemic gene suppression, (3) dsRNA amplification. Fire et al. (1998) noticed that silencing of genes in *C. elegans* isn't limited only to cells at the site of injection, but can be transported to other cells which proved the systemic response of RNAi. The basis of this effect is thought to lie in the presence of an RNA-dependent RNA polymerase (RdRP) that can interact with the RISC complex and generate new dsRNA based on the partially degraded target template by using the hybridized siRNA strands as primers (Price and Gatehouse, 2008). New dsRNA is then cleaved by Dicer and new small interfering RNAs (siRNAs) are generated which can be transported to other cells. This provides the long-lasting interference effect and the spreading of the gene knockout effect in organism. Furthermore, the systemic properties of RNAi, that is, the spread of RNAi signaling/effects from cell to cell and from tissue to tissue, can lead to a heritable transfer of the RNAi effect (parental RNAi) (Zotti et al., 2018). In other words, RNAi can occur in embryos if the silencing signal is transmitted to embryos of the next generation. However, nematodes and insects do not share the same mechanism of systemic response since RdRP activity isn't present in insects but it doesn't exclude the possibility of systemic response in insect via different mechanism.

The dsRNA can be taken up by insects using two different pathways (Figure 2.1.). Endocytosis and Sid-1-like transmembrane channel protein-mediated pathway are described for insect uptake, while Sid-1 and Sid-2 transmembrane channel proteins are needed for environmental dsRNA uptake in nematodes. *Sid-1* and *Sid-2* genes are essential for dsRNA uptake in nematodes and are not present in insects. SID-1 is a multi-span transmembrane protein essential for systemic RNAi (Huvenne and Smagghe, 2010). The other protein, SID-2m is mainly found in the intestine tissue of the worm and facilitates environmental RNAi (Winston et al., 2007). A phylogenic analysis suggested that *Sid-1* like genes in *Tribolium* may not be

orthologous to *Sid-1*, but rather to the *C. elegans Tag-130* gene which is not associated in systemic RNAi in nematodes (Tomoyasu et al., 2008). Therefore, systemic RNAi system differs in nematodes and insects. The Sid-1-like channel proteins have been shown to be involved in dsRNA uptake in some insect species, such as the brown planthopper (BPH, *Nilaparvata lugens* Stal) (Xu et al., 2013), the Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) (Cappelle et al., 2016), and the red flour beetle (*Tribolium castaneum* Herbst) (Tomoyasu et al., 2008). Also, the presence of *sid-1* homologs does not necessarily mean that dsRNA uptake is performed by the resulting Sid-1-like proteins (Cappelle et al., 2016). However, some insect genomes lack *Sid-1*-like genes completely and uptake of dsRNA depends on receptor-mediated endocytosis. For example, *Drosophila melanogaster* Meigen lacks *Sid-1*-like genes so the dsRNA transport is facilitated via endocytic pathway. Cappelle et al. (2016) have confirmed that in coleopteran CPB both the Sid-1-like channel proteins as well as the receptor-mediated endocytosis are involved in dsRNA uptake. Notably, the full disclosure of dsRNA uptake mechanism in insect tissue is of key importance for better understanding of systemic RNAi in insects and the development of more potent delivery and application strategies of RNAi.

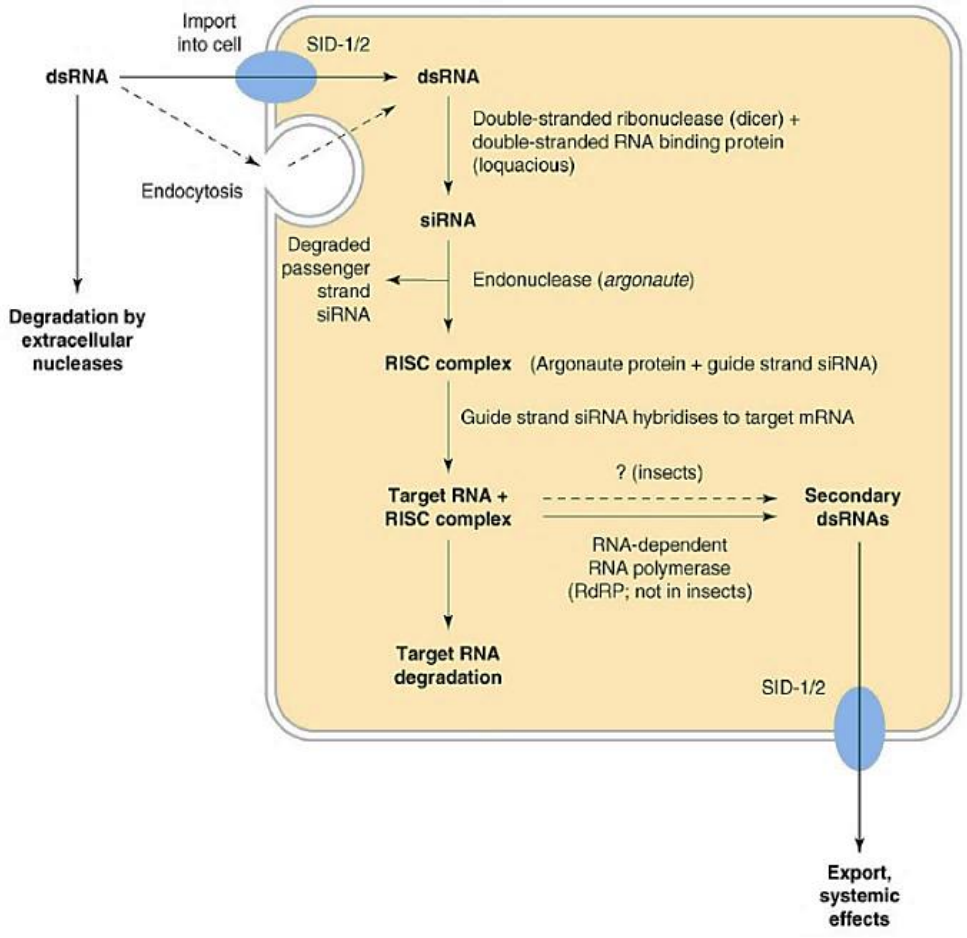

Figure 2.1. Posttranscriptional gene silencing – dsRNA is imported in cells via endocytosis in *D. melanogaster* or systemic RNA interference deficient-1 channel proteins (Sid-1 or Sid-2 detected only in nematodes closely related to *C. elegans*). Afterwards, dsRNA is processed by Dicer into small interfering (siRNA) and incorporated in RISC complex while passenger strand is degraded by endonuclease. RISC complex binds to target mRNA and degrades it based on specific siRNA sequence. Systemic RNAi can take place by producing secondary dsRNAs which can then be exported from the cell and cause PTGS in other cells.

Source: (Price and Gatehouse, 2008)

The possibility of the absence of dsRNA amplification mechanism in some insects is the main problem in achieving the long-lasting effect of gene knockdown since effects of RNAi are visible only in cells exposed to dsRNA. Due to lack of *Sid-1* and *Sid-2* genes and their homologs in some

insects, the transport mechanism of dsRNA between cells in insects is unknown. This does not exclude the possibility of amplification of dsRNA in insects since the true mechanism is still unknown and may be based on the entirely different enzyme. Indeed, in some species, for example coleopterans, the RNAi effect is so strong and can last so long that it would be likely that such a system is present in these insects (Joga et al., 2016). However, the absence of amplification mechanism in some insects presents the problem when it comes to the application of RNAi since insects have to continuously be exposed to large amounts of dsRNA in order to have efficient gene knockout effect. Degradation of dsRNA in the gut would require continuous administration of high levels of dsRNA; production of sufficient dsRNA in transgenic plant and its delivery in a sufficiently undegraded state to the insect would provide another significant technical problem, if a role in defense against insect pests was required (Price and Gatehouse, 2008). However, results obtained in 2007 by Baum et al., and Mao et al., have shown that there is a possibility to resolve this problem by producing dsRNAs in plants (Baum et al., 2007; Mao et al., 2007).

Baum et al. (2007) published breakthrough research done on Western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte) in which they used transgenic plants producing hairpin dsRNA directed against a gene encoding V-type ATP-ase A against WCR and showed a significant reduction in WCR-caused root damage. There are different methods in which pests can intake dsRNA, but intake via transgenic plants producing specific dsRNA against pests is the most promising one for pest control on large scale.

2.2. dsRNA delivery method and application for insect control

Development of a robust dsRNA feeding methodology in insects that mimics the results obtainable with *C. elegans* (where efficient suppression of gene expression by orally delivered dsRNA is routine) is a prerequisite for utilization of RNAi for crop protection against insect pests (Price and Gatehouse, 2008). The best delivery dsRNA methods for insects are: (1) autonomous oral delivery (feeding) and (2) topical application of dsRNA on soil and leaves. Nevertheless, the

one that holds the promise for widespread usage is by feeding on transgenic plant material expressing specific dsRNA since it is the most potent method for pest control.

Autonomous uptake of dsRNA in insects and digestion in the gut is the most applicable method of inducing interference in insects for pest control. The insect midgut plays a critical role in the regulation of important physiological functions such as digestion, metabolism, immune response, electrolyte homeostasis, osmotic pressure, and circulation (Hu et al., 2016) which makes it suitable target for RNAi application. The midgut columnar cells with microvilli and endocytosis apparati are responsible for absorbing nutrients and present the target for dsRNA uptake. The insect gut consists of foregut, midgut, and hindgut. The midgut region is the most appropriate site for dsRNA uptake since it contains exposed cells and is the main part of the exchange between the circulatory system and the gut. RNAi effects occurring in insects as a result of oral delivery of dsRNA are presumably mediated by the midgut and the Malpighian tubules to dsRNA in the gut contents (Price and Gatehouse, 2008). Uptake of dsRNA by the epithelial cells of the insect midgut is critical to the effectiveness of RNAi response (Joga et al., 2016). Feeding physiology and persistence of dsRNA in gut is an important factor for its efficiency. The stability of dsRNA is influenced by gut conditions, especially Ph which varies among insects from acidic (Coleopteran larvae) to alkaline (Lepidoptera species), along the gut and with distance from the epithelium. Also, it is unclear to what degree the peritrophic matrix in the midgut of Coleoptera and Lepidoptera species can obstruct dsRNA transmission.

Many researches have shown the great possibility of RNAi in a wide variety of insects applied through feeding (Araujo et al., 2006, Turner et al., 2006, Baum et al., 2007, Zhao et al., 2008, Zhou et al., 2008, Walshe et al., 2009). An appealing aspect of gene silencing for pest control is its potential for selectivity based entirely on nucleotide-sequence identity (Baum et al., 2007). The key to the success of this approach is: (1) identification of a suitable insect target and (2) dsRNA delivery of sufficient amounts of intact dsRNA for uptake by the insect (Price and Gatehouse, 2008) (Figure 2.2.). The breakthrough research done by Baum et al. (2007) showed that ingestion of dsRNA supplied in transgenic corn plants engineered to express WCR dsRNA triggers RNAi in WCR which results in reduced feeding damage, larval stunting and mortality. They tested possible target genes from WCR cDNA library and selected V-ATPase A which

showed significant larval mortality and rapid knockdown of endogenous mRNA within 24 hours of ingestion. Three other coleopteran species, Colorado potato beetle, Southern corn rootworm (SCR; *Diabrotica undecimpunctata howardii* Barber), and cotton boll weevil (*Anthonomus grandis* Boheman), were also tested for sensitivity to dsRNA targeting β-tubulin, V-ATPase A and V-ATPase E orthologues of WCR. The dsRNA showed significant larval mortality in CPB and SCR but larvae of cotton boll weevil showed no effects on mortality upon ingestion of dsRNA orthologues compared to control. This might suggest that not all coleopteran larvae are sensitive to orally delivered dsRNAs. Also, synthesis of gene-specific dsRNAs for CPB V-ATPase A and V-ATPase E showed higher activity than the orthologous WCR dsRNA since the V-ATPase A target sequence of CPB and WCR share 83% nucleotide-sequence identity. Transgenic corn plants were assembled by using putative V-ATPase A coding region from WCR into a corn transformation expression cassette designed to express dsRNA targeting 245 bp of the WCR gene. Transformed corn plants were infested with WCR eggs in the root zone and assessed for feeding damage after three weeks. The results showed obvious reductions in root damage compared to the control. RNA extracted from individual transgenic plants showed that dsRNA constructs were expressed as full-size transcript and small 21-bp siRNAs processed from the larger dsRNA, while plants which had the highest nodal injuries didn't express dsRNA. This research demonstrated that autonomous feeding with plant material expressing specific dsRNA can cause high mortality in WCR and presents a feasible method for crop protection and has obvious commercial implications.

Another study done by Mao et al. (2007) on cotton bollworm (*Helicoverpa armigera* Hubner) showed that when larvae are fed plant material expressing specific dsRNA, levels of cytochrome P450 gene (CYP6AE14) transcript in the midgut decreased and obstructed larval growth. P450 monooxygenase CYPAE14 plays an important role in detoxification of the cotton metabolite, gossypol, in cotton bollworm. Production and storing of gossypol is important cotton defense mechanism against pests, but insect P450 monooxygenase serves as an adaptation mechanism to plant defense compounds and in developing resistance. Induction of CYP6AE14 expression by gossypol on the inner surface of cotton bollworm's midgut showed a link between CYP6AE14 expression and tolerance to gossypol, therefore CYP6AE14 was targeted for downregulation.

Feeding on transgenic plants expressing dsCYP6AE14, an inverted repeat driven from the CYP6AE14 coding sequence, resulted in a reduction of transcript levels in cotton bollworm which lead to reduced larval tolerance to gossypol and retarded larval growth. The study demonstrated that hairpin dsRNA expressed in plant tissue directed against CYP6AE14 provide sufficient amounts of dsRNA needed for RNAi in the insect midgut and that this method of delivery has great potential for pest control and immense agricultural importance.

Both cotton bollworm and western corn rootworm are economically important pests. RNAi provides a unique mode of action for the control of insect pests that could complement the current strategy of expressing *B. thuringiensis* (*Bt*) insecticidal proteins in crops such as corn, cotton and soybeans (Baum et al., 2007). As a consequence of its highly specific mode of action compared with other pest control strategies such as conventional neurotoxic insecticides, RNAi technology comprises a suite of tools with a vast range of potential applications for generic studies and agriculture, including protection of beneficial insects against viruses and parasites (Hunter et al., 2010, Zotti and Smagghe, 2015), resistance management (Zhu et al., 2014), and pest control against insect species (Huvenne and Smagghe, 2010), plant pathogens (Fu et al., 2005, Duan et al., 2012, Koch et al., 2013, Koch et al., 2016, Wang et al., 2016), mites (Wu et al., 2014, Kwon et al., 2016, Li et al., 2017), ticks (de la Fuente et al., 2007), and nematodes (Youssef et al., 2013, Walawage et al., 2013) in a wide range of crops (Zotti et al., 2018). This technology enables the control of a single insect species while minimizing the negative impact on non-target organisms. RNAi-based products are expected to reach the market in the form of transformative products (plant-incorporated protectants (PIPs)) and non-transformative/non-PIP products (sprayable, stem injection, root drenching, seed treatment or powder/granule products) (Joga et al., 2016, San Miguel and Scott, 2016, Andrade and Hunter, 2016, Andrade and Hunter, 2017).

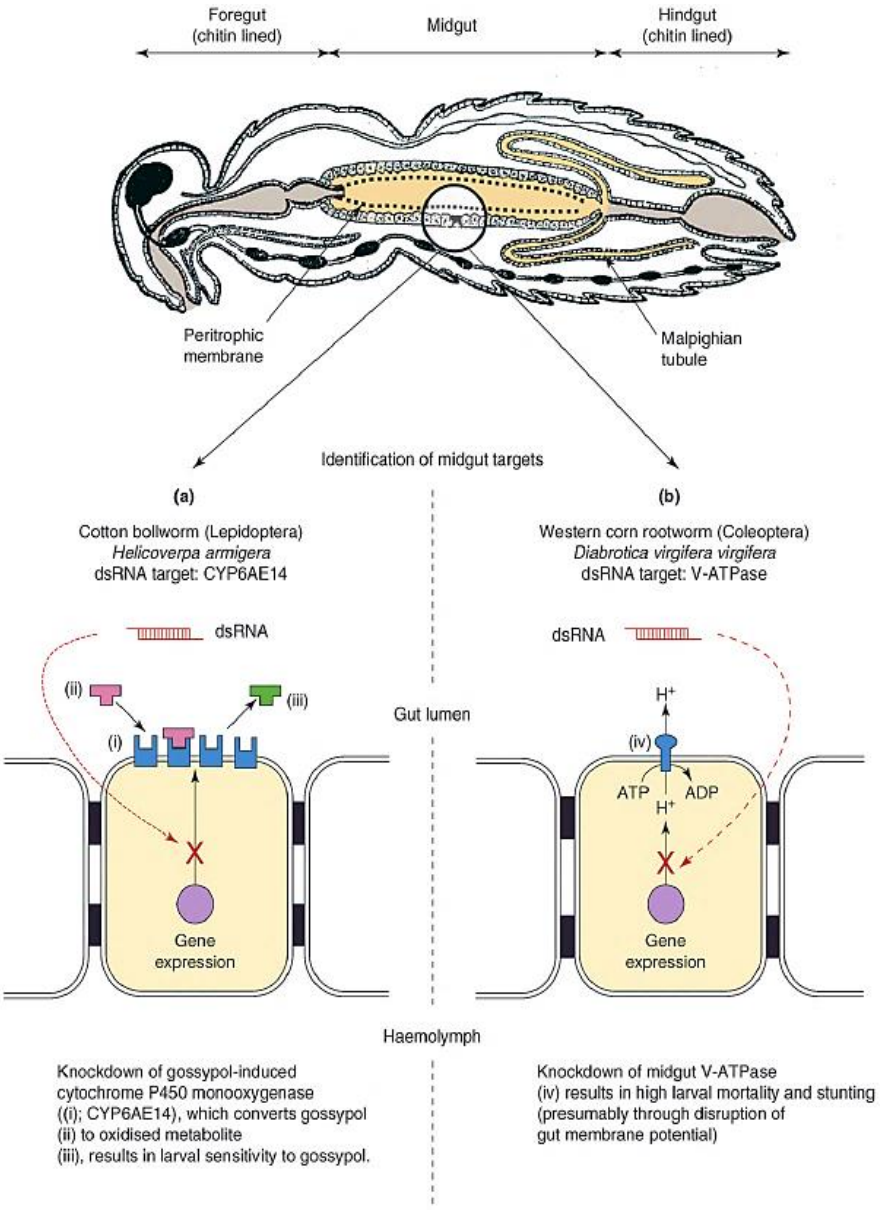

Figure 2.2.: Oral delivery of dsRNA. Specific dsRNAs encoding for target genes expressed in transgenic plants are cleaved by Dicer-2 into siRNAs. The dsRNA and siRNA are expressed through plant tissues and orally delivered to pests feeding on plant material. In sufficient quantities, dsRNA and siRNA are delivered in gut and taken up by the gut cells. In case of Lepidoptera, a gut specific cytochrome monooxygenase (CYP6AE14), presumably involved in detoxification of gossypol, is knocked down by dsRNA products delivered by feeding method. In case of Coleoptera, midgut V-ATPase A is knocked down by dsRNA delivered by feeding on transgenic plant material which results in larval mortality and stunting presumably through disruption of gut membrane potential.

Source: (Price and Gatehouse, 2008)

There is also a possibility of topical application of dsRNA on leaves or soil since dsRNA can be translocated through the plant vascular system. This is especially important for sap-feeding insects and root-feeding insects since plant roots can take up dsRNA and enable delivery through phloem and xylem. Focus on the development of topically applied RNAi-based approaches to manage insect pest and pathogens may provide environmentally sound products for use across all agriculture production systems (Andrade and Hunter, 2017). Further development of such products will be required in order to determine the stability of topically applied dsRNA and efficiency of dsRNA taken up by the insects due to susceptibility to sunlight degradation (Li et al., 2015), degradation by environmental microbes (Dubelman et al., 2014) and in the cells by natural dsRNA processing mechanism (Palli, 2014). The applicability of sprayable dsRNA relies on the development of cost-effective methods for the mass production and formulation of the dsRNA (Zotti et al., 2018). Mitter et al. (2017) provided evidence that using designer, non-toxic, degradable, layered double hydroxide (LDH) clay nanosheets can substantially increase naked dsRNA stability sprayed on plants. They concluded that once loaded on LDH (BioClay nanocarrier system), the dsRNA does not wash off, shows sustained release and can be detected on sprayed leaves even 30 days after application. This proves the versatility of RNAi technology application and reinforces the high potential of RNAi products for their utilization in crop protection.

The US EPA recently registered four products containing a new and innovative RNAi-based PIP called 'SmartStax PRO' that will help US farmer to control CRW (Zotti et al., 2018). Despite the growing market interest in RNAi-based biopesticides, there are still unresolved questions on environmental safety and lack of field experimentation. Therefore, as all new technologies, RNAi biopesticides and transgenic plants have to be more carefully examined for their potential hazards.

2.3. RNAi risk assessment

RNAi, as a novel technology, opened up new approaches towards modern pest management. There are similarities and differences in the risk associated with insecticidal RNAi relative to

those posed by chemical and microbial pesticides and *Bt* crops, which have pesticidal effects derived from the bacteria *Bacillus thuringiensis* (Heinemann et al., 2013). Although most regulatory agencies for biotech products such as US EPA and the European Food Safety Authority (EFSA) have extensive experience in the ecological risk assessment of newly introduced PIPs, based on several years of *Bt*-based crop assessments (US EPA, 2016, EFSA, 2014), the hazards or ecological risks of dsRNA may not be assessed using the same framework because of the unique mode of action of dsRNA (Zotti et al., 2018). Lundgren and Duan (2013) argue that some of the potential hazards posed by RNAi-based pesticides and transgenic crops to non-target organisms include off-target gene silencing, silencing the target gene in unintended organisms, immune simulation, and saturation of the RNAi machinery.

US-Environmental Protection Agency stated in the RNAi risk assessment (2013) that the growing potential of RNAi based biopesticides has lead Scientific Advisory Panel (SAP) to consider aspects of risk assessment to determine whether and RNAi based PIP can meet the environmental safety standard. The US EPA's consideration for the environmental fate of dsRNA and non-target effects are the same for all RNAi biopesticides (PIP, foliar spray, seed treatment, granule/powder). Two main uncertainties related to RNAi-biopesticides are determination of effects of non-target exposure and environmental exposure. Non-target organisms include: birds, mammals, freshwater and marine fish, aquatic invertebrates, non-target insects, honey bees, non-target plants and soil invertebrates. The degree of non-target exposure depends on the distribution and fate of the dsRNA PIP within the environment, as well as the potential routes of exposure, exposure duration, and potential for uptake (US EPA, 2013). Significant considerations of environmental risk assessment are potential paths of dsRNA distribution in the environment. Factors that affect the distribution of dsRNA in the environment and the potential for exposure to non-target organisms include physical movement of the PIP crop plant tissue, persistence and physical movement of dsRNA released from the plant, either while plant is living or following the breakdown of plant tissue (US EPA, 2013). It is also possible that dsRNA could be present in root exudates, guttation droplets, and nectar which emphasizes the possibility of on-field non-target exposure. The US EPA (2013) also considers off-site movement of plant pollen expressing dsRNA relation to non-target exposure

which depends on the characteristics of the pollen (morphology and weight) and the mechanism relied upon for pollination (i.e. wind, pollinators). Another point is release of dsRNA in environment upon transgenic plant tissue degradation. Degradation of the plant tissue will affect the potential for exposure, since RNA is degraded within the plant during senescence (Pietramellara et al., 2009). Nevertheless, some dsRNA may not be degraded by the time plant material reaches the ground which means that it could easily end up in the soil where it may persist for a certain time or be transported to other environments. Studies show that DNA can persist in soil for a few days or several years by binding to humic substances or minerals, or it may be degraded by microbe and nucleases (Levy-Booth et al., 2007, Pietramellara et al., 2009). The information on the potential of dsRNA PIPs vertical and lateral movement in soil with water and the effects of binding to soil particles have to be more closely examined. Another possibility of non-target exposure is secondary exposure where organisms which have ingested dsRNA are consumed by non-target organisms. Garbian et al. (2012) showed that dsRNA could be transferred from bees to Varroa mites showing that secondary exposure to dsRNA is possible.

In order to assess risk, the US EPA (2013) proposes measuring the exposure levels which are assumed to be equal to the highest amount expressed in various plant tissues. The environmental risks for chemical and microbial pesticides, as well as *Bt* crops, are usually assessed using a tiered approach (Zotti et al., 2018) which is based on maximum-hazard dose for predicting toxic events and off-target events. The US EPA (2013) also states that optimum concentrations of dsRNA for adequate RNAi effect should be used which then leads to the possibility of diminishing environmental exposure levels. Finally, the ecological risk assessment should rely on environmental fate and non-target toxicity and consider the unintended effects of dsRNA PIPs due to its unique mode of action.

There should be a balance between benefits for crop protection and environmental risks, and therefore potential hazards posed by RNAi-based pesticides and RNAi-based engineered crops should be carefully assessed using methods that take into consideration the fact that RNAi has a unique mode of action that can also silence unintended genes (Zotti et al., 2018).

3. Western corn rootworm

Western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) (Figure 3.1.) was first discovered in Kansas, USA and described by LeConte in 1867. Shortly after its discovery, Gillette (1912) recognized it as maize (*Zea mays* L.) pest in sweetcorn in the central USA. From 1909 to 1948 the insect spread eastward at an average rate of about 19 km per year (Metcalf, 1986). From 1950s onwards, the species invaded new areas at a rate of 64 to 80 km per year, and the practice of continuous maize planting without crop rotation allowed *D. v. virgifera* to become a major pest (Youngman et al., 1999) (Figure 3.2.). Fifty years after, *D. v. virgifera* was detected in Europe in Serbia and spread to other Central and Southern European countries within 10 years. Total area infested in Europe by WCR reached approximately 311 000 km^2 by the end of the 2003 maize growing season, with about 70 000 km^2 of land area with economic adult activity and approximately 97 km^2 of larval damage (Kiss et al., 2005). Nowadays, WCR is the most important economic pest of continuous maize cultivation in US and Europe, difficult to manage and to eradicate.

Figure 3.1.: Adult western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Photo: R. Schmidt)

Source (Cullen et al., 2013)

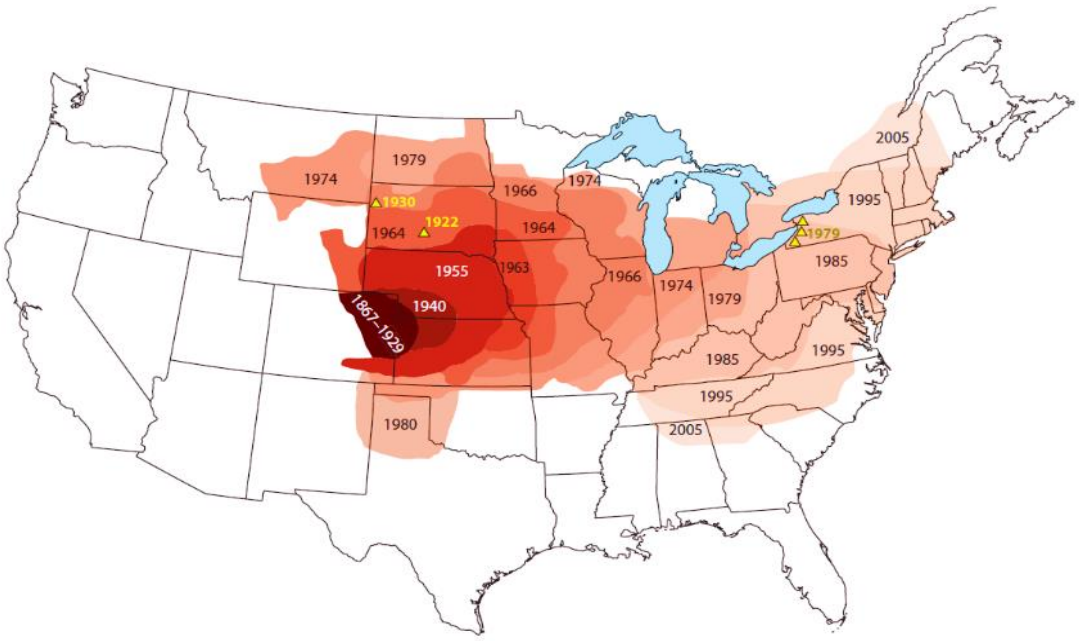

Figure 3.2.: Reconstruction of WCR expansion in US from 1867 to 2005 based on data reports in the literature (Tate and Bare, 1946, Foott and Timmins, 1977, Metcalf, 1983, Smith, 1983, Krysan, 1986, Youngman and Day, 1993, McPherson et al., 1996., Sutter, 1999, Meloche and Hermans, 2004, Meloche et al., 2005). Distribution boundaries are approximate and triangles mark reports of WCR before their distribution was established.

Source (Gray et al., 2009)

The significance of this pest is illustrated by the cost of the damage caused by WCR corn infestation in maize. Metcalf (1986) reported the cost greater than 1 billion $/year due to yield losses and control costs and Dun et al. (2010) suggested that a 17.9% of yield loss can be expected for each node of roots injured by WCR larvae and that current management costs associated with WCR could be significantly greater than 1 billion $/year. Recent development of new WCR variants, resistant to various management practices such as chemical insecticides, crop rotation, and *Bt* maize, resulted in maize yield decrease and increase of control costs. Therefore, the magnitude of WCR infestation in US became substantial, especially in major maize growing areas (Figure 3.3.). The main areas of economic damage in Europe are southeastern countries like Croatia, Serbia, Hungary, Slovakia, and Italy (Hummel et al., 2008) (Figure 3.4.).

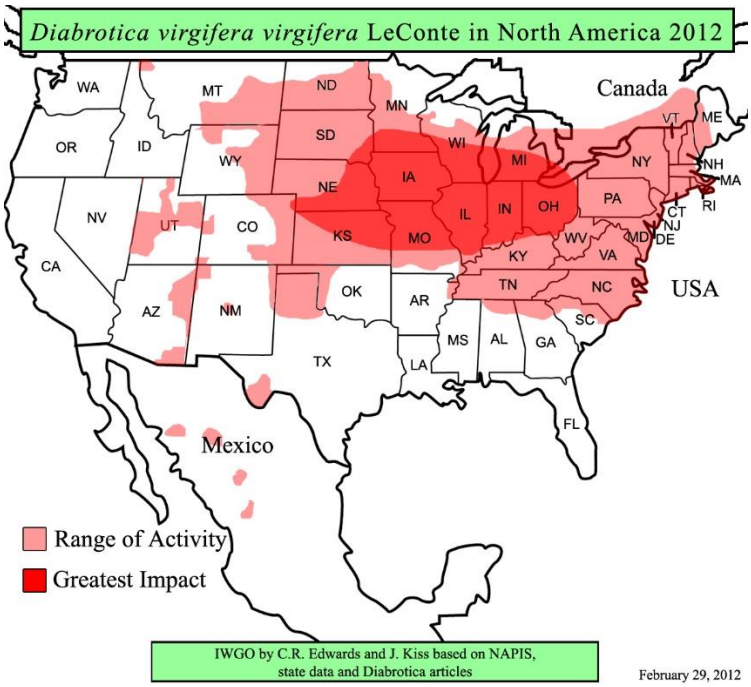

Figure 3.3.: Area of maize acres infested by the WCR in North America in 2012
Source: (https://extension.entm.purdue.edu/wcr/)

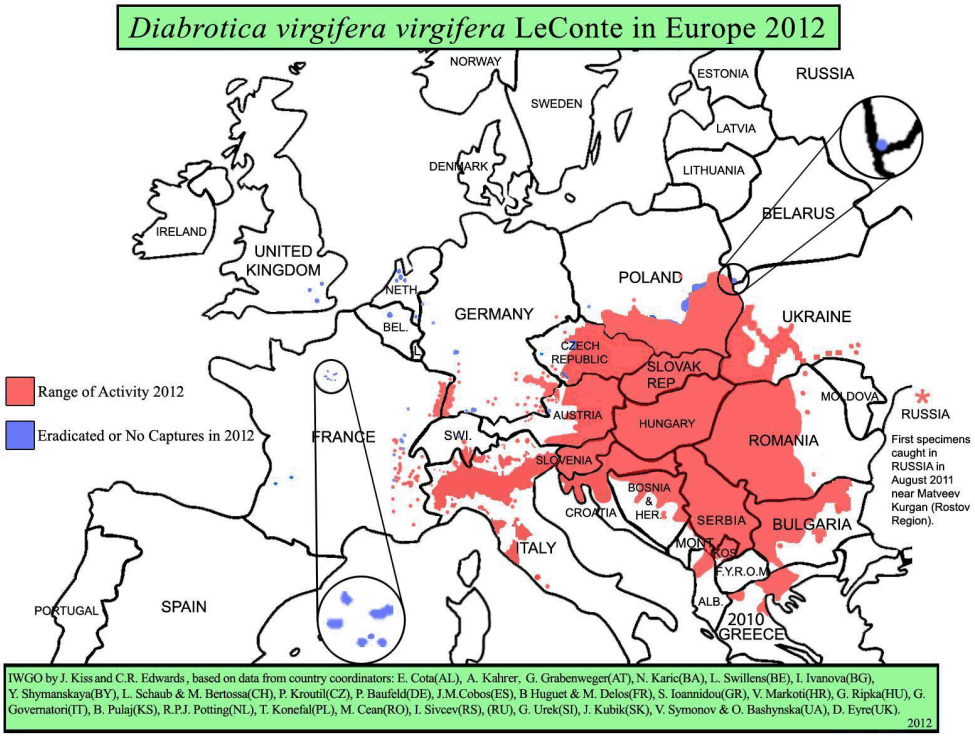

Figure 3.4.: Area of maize acres infested by the WCR in Europe in 2012
Source: (https://extension.entm.purdue.edu/wcr/)

3.1. Pest characteristics and maize damage

Knowing pest biology and ecology is essential for its successful management. The amount of injury caused by an insect pest to a crop plant depends on the feeding habit of the pest species, the size of its population, and the capacity of the plant to withstand the type and amount of injury that results from the species feeding habit and size of population (Kogan, 1994). The population management of WCR depends on a multitude of connected factors such as emergence, phenology, oviposition, adult movement, and dispersal.

The WCR pests have one generation per year (univoltine), overwinter in the egg stage and are present in maize fields from July until the first frost. WCR beetles feed on the leaves, silk, pollen and young kernels of maize, although pollen is the preferred food (Kiss et al., 2005). After the silking period WCR are able to feed on a variety of alternate host plants (Clark and Hibbard, 2004, Musická et al., 2004) and they can survive to adulthood on several grass species, including foxtail, wheatgrasses, Johnsongrass, rye, rice, millet, fescue, ryegrass (Branson and Ortman, 1970), alfalfa (Ball, 1957), soybean (Shaw et al., 1978), red clover, velvetleaf, giant rageweed, and Jerusalem artichoke (Levine et al., 2002), although the fitness of WCR females is reduced when feeding on plants other than maize.

The ovipositional habit of WCR females is critical for larval survival, as larvae can develop on the roots of maize, but high mortality occurs on the roots of grassy-type weeds (Branson and Ortman, 1970). Soil moisture is an important factor for oviposition, and if the soil is sufficiently moist during August females will lay a larger number of eggs (Weiss et al., 1983, Spike and Tollefson, 1988, Toepfer et al., 2007, Meinke et al., 2009). Diapausing eggs are laid in the soil in late autumn and overwinter in the soil until late May and early June when they hatch. Because larvae are essentially monophagous and immobile, ovipositional activity of adults in the late summer and fall plays an important role in linking the insects temporally and spatially with the host plant (Naranjo and Sawyer, 1988). After larvae hatch, they are established on their host plants' roots. If egg hatch timing doesn't overlap with host plant root development, larval establishment and survival are reduced. Therefore, delayed planting results in reduced root damage and adult emergence, and peak emergence time (Musick et al., 1980). WCR larvae feed

on maize roots which causes the reduction of water and nutrient availability to developing plants, disruption of root system function, reduction of grain yield (Spencer et al., 2005), and facilitate infection by root and stalk fungi resulting in further damage (Levine et al., 2002) such as lodging (Figure 3.5.). High temperatures in summer can increase larval feeding damage since larvae predominantly feed on maize roots due to lack of soil water. Ivezić et al. (2009) reported high maize root injury in 2003 due to warm summer and drought stress caused by a low amount of precipitation during the vegetation period. Finally, adverse environmental conditions (i.e. drought) in conjunction with rootworm feeding injury can greatly diminish plant health and subsequently productivity (Boetel et al., 2003) (Figure 3.6.).

WCR adults are mainly pollen feeders that also use above-ground plant organs of maize (Chiang, 1973; Ludwig and Hill, 1975). Corn pollen and silk were shown to be the best for egg production in female *D. virgifera virgifera* (Elliot et al., 1990). Increased number of WCR adults in maize fields may hamper maize pollination due to silk feeding and cause reduced ear filling and seed shape deformation which results in economic damage, although lower than that caused by WCR larvae.

Figure 3.5.: WCR damage to maize root; the resistant GM line is symptom-free (left) and susceptible control is severely injured (right)

Source: (Ball et al., 2008)

Figure 3.6.: WCR injury to a commercial maize field in Illinois, 1995. Maize injury resulted from larvae feeding on the plants' root system with the added stress of low precipitation.

Source: (Levine et al., 2002)

3.2. Chemical measures

Largescale applications of soil insecticides were first made for *D. v. virgifera* control in 1949 and more than 700 000 ha were being treated with insecticides by 1954 (Ball and Weekman, 1962). Chemical treatments included BHC, aldrin, chlordane, heptachlor, and cyclodiene. First outbreak insecticide-resistant strains occurred shortly after the start of extensive use of soil insecticides and resistant strains rapidly spread over the US Corn Belt. Metcalf (1983) indicated that insecticide resistance results from an intensive selection of a large insect population having a substantial gene pool that incorporates, often at a very low frequency, mutant alleles conferring fitness for survival under the modified environment contaminated by the insecticide. He concluded that WCR cross-resistance to the soil insecticides aldrin, heptachlor and chlordane sharply decreased the benefit/cost ratio of the soil insecticide treatments in corn. Also, he noted that the rise of widespread cyclodiene resistant WCR occurred simultaneously with the acceleration in the rate of WCR expansion, probably due to increased fitness of resistant beetles and behavioral changes associated with the resistance gene.

The failure of chemical measures is emphasized by many researches showing the inefficiency of chemical insecticides application on WCR population. Gray et al. (1992) conducted a 3-year evaluation of WCR emergence as affected by insecticide and tillage system and suggested that planting-time insecticide applications, although intended to protect maize roots, do not result in actual rootworm population management. Sutter and Gustin (1989) established artificial infestations of WCR and observed more adults emerging from insecticide-treated soil than from untreated control plots. Woodson et al. (1999) observed avoidance behavior in the third stage WCR in response to the organophosphate insecticides terbufos, chlorethoxyfos, and fonofos. This results in negative chemotaxis and movement to untreated plots avoiding toxic chemicals. Furthermore, certain chemicals can act as repellants to WCR causing movement of adults to neighboring fields where females can lay eggs. Such example was recorded by Levine et al. (2002) who concluded that application of permethrin to seed-production cornfields may have led to WCR oviposition in the nearest untreated fields, specifically adjacent seed-production soybean fields. Larvae that emerge from neighboring fields could then cause injury to rotated maize crop.

The greatest problem of chemical insecticides is routine application despite the actual WCR population present in the cornfield or the economic injury level, and low efficacy of chemical treatments on WCR pest management. Rapid development of resistance to pesticides in some populations of corn rootworms, human safety and ecological concerns have motivated the development and adoption of environmentally rational management practices (Kiss et al., 2005).

3.3. Cultural measures

Annual rotation of maize and soybean has successfully managed WCR pest population in the US Corn Belt and Canada from the introduction of practice until 1990s. A corn and soybean rotation disrupts the rootworm life cycle because eggs are not normally laid outside of cornfields and larvae cannot survive on soybean roots (Levine et al., 2002). Behavioral change in WCR population caused the failure of crop rotation as cultural measure of maize protection.

In 1995, crop rotation failed as a pest management tactic in a large number of first-year commercial cornfields across east Illinois and northwestern Indiana (Levine and Gray, 1996, Edwards, 1996, Gray et al., 1998a, 1998b). Delayed planting, a later than usual egg hatch (mid-June) and prolonged hot and dry conditions in July worsened the impact of rootworm larval feeding (Levine and Gray, 1996, Gray et al., 1998). Injury to first-year corn was caused by ovipositional changes of WCR females. The main obstacle for successful corn protection using cultural measures is reduced preference of WCR females to lay eggs on corn fields due to reproductive advantage.

Although crop rotation proved itself as unsuccessful measure against WCR in some areas, it is still cost-effective and environmentally friendly tool for many countries in Europe. It is used as integrated pest management (IPM) control option for both management and eradication. In general, its application has been highly recommended to control established alien invasive pest populations (Wittenberg and Cock, 2001). European crop rotation systems are more diverse than the corn/soybean rotation in the US Corn Belt and incorporate more monocot crops (Moeser and Hibbard, 2005). Crop rotation as a method of control is still effective in Europe because farmers use more diverse array of cropping systems (Kiss et al, 2005), rotate more often with a greater variety of crops, and frequently utilize relatively smaller fields compared with farmers in the USA who operate on much larger scale (Gray et al., 2009). On the other hand, monocot weed flora in Europe may present a problem in WCR management if WCR adapts to changing environments and alternative host plants. Moeser and Hibbard (2005) argue that monocot crops grown in close spatial range to maize fields are capable of acting as a reservoir, even in the absence of maize, and that WCR females could use monocot weeds or crops as a place for oviposition. This could provide new areas of infestation and expansion of WCR's ecological niche due to the high adaptability of WCR regarding host plants.

Igrc Barčić et al. (2007) conducted a study on WCR adult movement and egg-laying in fields bordering corn in Croatia. WCR egg-laying reached approximately 20 m into field neighboring maize fields and significant root damage caused by WCR larvae in first-year corn following soybean and wheat can happen up to a distance of 20 m into those fields due to edge effect.

Their findings indicated possible WCR larval damage in rotated fields in Croatia, since most of them are approximately 50 m wide, without WCR being the variant form resistant to crop rotation. The main goal for successful cultural measures against WCR pest is to assess adult population level in the donor field at which economic damage in neighboring fields could occur or to assess the number of WCR adults in pre-maize fields.

The oviposition by WCR females in soybean and other neighboring fields is an immense threat to the efficacy of cultural measures in WCR management. Due to the failure of chemical and cultural measures in corn protection against WCR, *Bt* maize was commercialized in the US as a solution for management and eradication of WCR.

3.4. Biotechnical control

Genetically engineered *Bt* crops produce toxins (*Bt* δ-endotoxin) derived from the bacterium *Bacillus thuringiensis* Berliner (*Bt*) which kill pests and reduce the use of chemical insecticides. Transgenic *Bt* maize hybrids that produce insecticidal proteins from the bacterium *Bacillus thuringiensis* (*Bt*) have become the standard management tactic against insects across the US Corn Belt (Cullen et al., 2013). In 2018, 80% of total 89 129 000 acres of maize planted in the US contained at least one *Bt* trait – stacked gene (USDA-NASS, 2012) (Figure 3.7.). Stacked maize hybrids contain traits targeting WCR and one or more additional traits targeting stalk-boring insect pests and herbicide tolerance (HT). *Bt* maize provides effective control of several key insect pests, with additional benefits of reduced reliance on conventional insecticides and, in some cases, regional suppression of pests (Romeis et al., 2008, Hutchison et al., 2010) as well as reduced harm to non-target organisms and increased profit to farmers (Cattanero et al., 2006, Marvier et al., 2007, Carpenter, 2010, Hutchison et al., 2010). It was first commercialized in 1996 in the US and in 1998 in the EU, although Cry3Bb1 maize containing toxin for management of WCR wasn't registered until 2003. Three additional *Bt* proteins registered in following years: Cry34/35Ab1 in 2005 – a binary toxin, mCry3A in 2006, and eCry3.1Ab (commercialized in maize containing mCry3A, available as "pyramid" trait) in 2012.

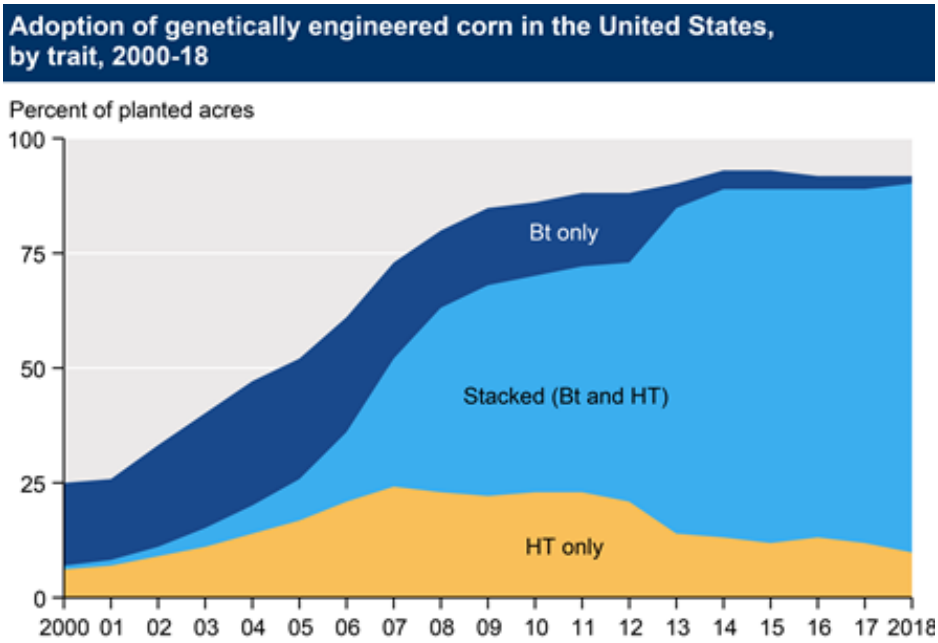

Figure 3.7.: Increase of adoption rate for stacked corn varieties which have *Bt* and HT traits.

Source: (USDA-ERS, 2019)

Rapid increase of stacked *Bt* maize acres in the US is caused by the emergence of WCR varieties resistant to crop rotation while in the EU, due to the effectiveness of crop rotation against WCR, *Bt* maize is grown continuously at a large scale only in Spain. Although intended to be a long-lasting solution for WCR management, first *Bt* maize resistant rootworms appeared in 2009. Widespread planting of *Bt* corn imposes selection on target insects to develop resistance (Cullen et al., 2013).

Gassmann et al. (2014) reported that during 2011, injury to *Bt* maize in the field expanded to include mCry3A corn in addition to Cry3Bb1 corn and laboratory analysis from these fields found resistance to CryBb1 and mCry3A and cross-resistance between these toxins. They noted the increase of resistant WCR between 2009 and 2011 in Iowa with severe root injury and the ability of WCR to survive on Cry3Bb1 maize. As they concluded, the resistant WCR appeared as a result of rapid development of resistance to *Bt* crops that are not high dose which highlights the potential of insects to develop resistance when *Bt* crops do not achieve a high dose of *Bt* toxin. For example, the refuge and high-dose of *Bt* toxin have worked in tandem to prevent resistance development in the European corn borer population (Tabashnik, 1994, Gould, 1998,

Huang et al., 2011). Respectively, planting *Bt* crops which are not high-dose allows some WCR survivors in every field. When heterozygotes (individuals with a mixture of alleles for resistance and susceptibility) can survive on a *Bt* crop, the frequency of *Bt* resistance alleles within a population can increase rapidly (Cullen et al., 2013).

Current approaches to resistance management for *Bt* crops, enacted by the US EPA, promote the use of refuges and planting of pyramided *Bt* crops to delay the evolution of resistance (Tabashnik and Gould, 2012). Ideally, pyramids contain two or more efficacious *Bt* toxins that kill the same pest insect but have different modes of action, making it difficult for a pest population to develop resistance to both toxins (Carriere et al., 2015, Roush, 1998). Additionally, the US EPA (2017a) recommended IPM approach to delay further resistance which includes crop rotation, use of maize rootworm soil insecticides at-planting with a non *Bt*-hybrid, using *Bt* hybrid with a different corn rootworm Cry protein than one that may have performed poorly in the past, and long-term IPM implementation that uses multiple tactics for WCR suppression. Also, recognizing the threat of resistance, the US EPA requires registrants (seed companies) to include an insect resistance management (IRM) plan when applying to register a *Bt* trait (Cullen et al., 2013) in order to detain field-evolved resistance of target insects.

In the last two decades, *Bt* maize has been the main management strategy for WCR control. Emerging field resistance to mCry3A and Cry3Bb1 decreased the efficiency of *Bt* maize against WCR. To improve future prospects for managing resistance to *Bt* crops, resistance monitoring data should be analyzed retrospectively in conjunction with data on the spatial and temporal distribution of *Bt* corn and refuges (Carriére et al., 2012). Resistance monitoring is essential for the detection of the occurrence of resistant insects in the field while IPM management strategies can contribute to the long-term effectiveness of *Bt* maize against WCR.

4. RNAi technology in WCR management

Western corn rootworm management is a challenging task since WCR have shown significant adaptability and resistance to various management strategies. In instances where resistance has been documented, it has always been associated with uniform adoption of a given technology over large geographic areas (Fishilevich et al., 2016). The most recent technology, genetically engineered corn expressing *Bt* insecticidal proteins, failed to circumvent WCR's ability to adapt to novel management technologies. In addition, using only one WCR management technique isn't sufficient for adequate control and combining multiple techniques and modes of action is essential for efficient control of this resilient pest. Therefore, there is a need for the development of a more robust control option with a special emphasis on resistance management, which can be used along with existing techniques, is environmentally safe and efficient.

RNAi is an effective novel biotechnological tool which can be used for WCR control. Baum et al. (2007) first reported the potential of in planta RNAi as efficient WCR management tool. More recent reports (Rangasamy and Siegfried 2011, Bolognesi et al. 2012, Ramaseshadri et al. 2013, Khajuria et al. 2015, Hu et al. 2016, Niu et al. 2017) have confirmed a great capability of oral exposure of WCR larvae and adults to dsRNA in corn protection and WCR management. Due to its efficiency, reliability and pest insect specificity it is expected to reach the market as novel PIP pest control in combination with *Bt* technology in the near future. For the moment, there are four RNAi PIP product approved by the US EPA called SMARTSTAX PRO which combine *Bt* traits and DvSnf7 dsRNA.

4.1. Molecular mechanisms of RNAi in WCR

Remarkably efficient RNAi response in both WCR larvae and adults upon ingestion of dsRNA caused increased interest in the feasibility of RNAi-based technology for WCR management. In order to assess the potential of RNAi as WCR management technique, it is important to understand the RNAi pathway on a molecular level.

The RNAi pathway in WCR starts with the initial uptake of dsRNA in the midgut cells and continues with systemic spread of RNAi silencing signal. Although WCR exhibits strong systemic response to RNAi, transitive RNAi mechanism which is present in nematodes (Ketting et al., 2001, Alder et al., 2003, Sijen et al, 2007), plants (Vaistij et al., 2002, Chen et al., 2010) and fungi (Fernandez et al., 2012) and responsible for secondary siRNA amplification and magnification of RNAi response isn't present in WCR. In organisms which have amplification mechanisms, secondary siRNA is produced by RdRP. The resulting secondary siRNAs trigger a secondary gene silencing that is termed transitive RNAi (Ketting et al., 2001). Fishilevich et al. (2016) suggested three possible explanations of strong systemic RNAi response in WCR: (1) the potent RNAi response in WCR might not involve transitive RNAi, (2) transitive RNAi in WCR is dependent on an enzyme other than RdRP, and (3) the secondary siRNAs generated by WCR are modified in such way that they are not detectable by the standard sequencing methods. All in all, the presence of strong systemic response to orally delivered dsRNA is only adding to the efficiency in protecting maize from WCR damage.

Another interesting remark is the susceptibility of WCR adults to dsRNA which can produce transgenerational control of WCR. This effect, also called parental RNAi (pRNAi) has been observed in multiple insects (Bucher et al., 2002, Paim et al., 2013). Therefore, pRNAi in WCR may provide an additional population management strategy for this important insect pest (Fishilevich et al., 2016). Khajuria et al. (2015) investigated pRNAi of genes involved in embryonic development of WCR. They showed that targeting genes essential for progeny of adult WCR could prevent crop injury by impacting the population of larval progeny of exposed adults. Aside from the great potential of pRNAi to target progeny of WCR adults and achieve maize protection in the subsequent generation, pRNAi is can also be used to reduce resistance by preventing oviposition or loss of egg viability and manage WCR population by preventing resistance alleles to be passed on to subsequent generation.

Resistance management is of the essence for RNAi-based insecticides. Past experience showed great adaptability and resistance of WCR to biotechnological control techniques which rapidly diminished the efficiency and increased the economic damage caused by WCR corn infestation. Transgenic crops that produce substances that provide protection from insect feeding are

vulnerable to the evolution of resistance in the target insect pest population, resulting in a reduction in the durability of the insect resistance substance(s) and the associated loss of benefits (Fishilevich et al., 2016). Therefore, changes in RNAi pathway genes and the subsequent effect on resistance development to RNAi in WCR has been examined (Vélez et al., 2016, Camargo et al., 2018, Davis-Vogel et al., 2018). Although there is no reported WCR resistance to RNAi technology, various adaptations to RNAi pathway may occur and it is important to understand the potential pathways in which WCR can develop resistance to RNAi technology. Preliminary evidence suggests that western corn rootworm could develop broad resistance to all insecticidal RNAs through changes in RNAi pathway genes; however, the likelihood of field-evolved resistance occurring through this mechanism remains unclear (Davis-Vogel et al., 2018). Recent evidences suggest evolution of RNAi pathway gene functionality in insects is a slow and complex process (Meister, 2013, Jun Tong et al., 2015, Dowling et al., 2017). While certain regions of the sequences themselves may show rapid change, conserved regions that preserve protein function – and indeed the miRNA pathway genes themselves which participate in certain aspects of siRNA-mediated RNAi – show little or no evidence of positive selective pressure (Obbard et al., 2006). Therefore, changes in expression of these genes were considered to be a more viable route to resistance than outright loss or functional mutation (Davis-Vogel et al., 2018). Some studies (Velez et al., 2016, Wu et al., 2017) suggested that changes in key RNAi processing components could also be a route for WCR resistance development. Camargo et al. (2018) reported that knockdown of RNAi pathway genes Argonaute 2, Dicer-1, and Dicer-2 showed no fitness costs in WCR larvae which suggests that they do not have essential function in WCR larvae, but knockdown of Agronaute 1 reduced larval survivorship and delayed development which confirmed the important role of AGO 1 in the regulation of processes such as embryonic development. They concluded that the potential for resistance may depend on whether RNAi pathway genes have essential function such that the loss of their function will reflect in fitness cost of WCR and if not, the resistance could occur through downregulation or loss-of-function of RNAi pathway genes which do not have fitness costs.

Since RNAi-technology is most likely to appear along with *Bt* traits in transgenic plants, the potential interactions between *Bt* and dsRNA have to be evaluated. Theoretically, stacking *Bt* and RNAi traits in maize could delay resistance development of WCR. If the insect-protected GE crop simultaneously produces two or more insecticidal substances with different modes of action such that cross-resistance is less likely to occur, insects that carry resistance alleles to one of the substances will continue to be controlled by the other substance(s) and fail to pass resistance alleles on to the next generation (Fishilevich et al., 2016). However, the competition between these two traits isn't thoroughly examined. Zukoff and Zukoff (2017) evaluated the host recognition response of WCR larvae to RNAi and Bt vector stack in maize. They concluded that all examined corn lines (Bt, RNAi, stacked RNAi and Bt, and isoline of these) were recognized as suitable hosts for WCR and that WCR could be attracted to hybrids, fed, and exposed to the Bt toxins or RNA in roots the same as the isoline plants, therefore equal larval pressure can be expected to all maize lines which is positive for resistance management. Additionally, stacking two or more RNAi traits in maize has to be researched since there is a possibility of competition between dsRNAs, along with competition with plant-derived siRNAs. Miyata et al. (2014) reported that mixture of dsRNA can result in competition between dsRNAs for RNAi machinery and cellular uptake/transport components and that competitions usually depends on the dose and length of dsRNA. RNAi treatment with more dsRNA molecules and longer dsRNA molecules wins out other RNAi treatment.

Ivashuta et al. (2015) investigated RNA movement between host plants and WCR and demonstrated that only long dsRNAs were selectively accumulated in insects respective to environmental RNAi (eRNAi) while abundant plant small RNAs and smaller RNA fragments were not taken up or were unstable in the gut lumen environment. Furthermore, 12% of siRNAs found in the WCR third instar larval body originated from the maize corn roots but had little or no effect on WCR transcriptome. This indicates that competition between plant-derived siRNAs and transgenically produced dsRNA is unlikely due to high identity on-target effects of transgenes producing dsRNAs and insignificant gene regulation by plant-derived siRNAs at the level of transcript accumulation.

4.2. Recent advances

In order for RNAi to be successfully adapted into plant protection, various factors have to be considered: (1) screening potential target sequences, (2) choosing efficient dsRNAs which produce fast response in target pest, (3) having stable expression and accumulation of dsRNA in plant tissue, (4) reducing development of resistance, and (5) environmental safety. The first step of efficient RNAi-based pesticide development is screening and choosing efficient dsRNAs against WCR which result in mortality, larval growth inhibition, reduction of WCR's fertility, and/or inhibition of embryonic development. Important aspects of RNAi bioassays are (1) length of dsRNA fragments, (2) dsRNA dose – LC_{50}, GI_{50}, and (3) sensitivity of target genes – LT_{50}.

Measurements other than the overall lethality, such as LC50 (concentration that leads to 50% lethality), LT_{50} (time to reach 50% mortality in the tested population) or GI_{50} (concentration that leads to 50% growth inhibition) can be useful to identify potential target sequences and discriminate among multiple efficacious dsRNA targets (Fishilevich et al., 2016). Rangasamy and Siegfried (2011) concluded that there is an upper limit of exposure to dsRNA that produces a response, and that lower exposure has to be determined to establish reliable LC_{50}.

Bolognesi et al. (2012) noted that dsRNAs greater or equal to approximately 60 base-pairs (bp) are required for biological activity in artificial diet bioassays. Moreover, they observed that 240 bp dsRNAs containing a single 21 bp match to the target sequence are efficacious while 21 bp siRNAs matching the sequence were not which suggests that the dsRNA length plays an essential role in the effectiveness of the RNAi response in WCR larvae. They hypothesized that the lack of activity of 21 bp siRNAs could be due to the fact that the processing of long dsRNAs with 100% match with the target mRNA results in multiple target-specific siRNAs which provides a greater number of siRNAs available to target mRNA and cause mortality. Recent studies have shown that various genes can be targeted in WCR for efficient RNAi-mediated knockdown which can be successfully used in plant protection (table 4).

Table 4: List of WCR genes for RNA interference control:

TARGET GENE	*GENE FUNCTION*	*RESULT OF GENE KNOCKDOWN*	*SOURCE*
Vacuolar ATPase (V-ATPase)	Conserved ATP-dependent proton pumps present in intracellular organelles such as endosomes, lysosomes and secretory vesicles and in plasma membrane of animal cells where they control cytoplasmic pH or energization of membrane	Loss of cell membrane integrity, cell-cell junctions, larval stunting, larval mortality, adult mortality	Baum et al. (2007) Rangasamy and Siegfried (2011) Koči et al. (2014)
DvSnf7	Part of the ESCRT (Endosomal Sorting Complex Required for Transport) pathway which has crucial role in cellular housekeeping by internalization, transport, sorting and lysosomal degradation of transmembrane proteins	Malfunctioning of cellular processes in midgut and fat body tissues, larval stunting, larval mortality	Bolognesi et al. (2012) Ramaseshadri et al. (2013)
hunchback (hb) ***brahma (brm)***	*Hunchback* (*hb*) gene is a gap gene which encodes a zinc-finger-containing transcription factor important for axial patterning in a number of insects; *brahma* (*brm*) gene obtains maternal and zygotic functions, Brm is an ATP-dependent remodeling enzyme of the SWI2/SNF2 family associated with nucleosome remodeling that is essential for regulated gene expression	Impacts the population of larval progeny of exposed WCR adults; total oviposition was not significantly affected but complete absence of hatching in the eggs was reported – potential pRNAi effect	Khajuria et al. (2015)

dvssj1 ***dvssj2***	Encode membrane proteins associated with smooth separate junctions (SSJ) which are required for intestinal barrier function	Loss of the midgut epithelium, larval growth inhibition, larval mortality	Hu et al. (2016)
dvvgr ***dvbol***	Essential reproductive genes; expressed in ovarian tissue and involved in sperm maturation division respectively	Exposure of adult WCR and larvae caused reduction of fecundity	Niu et al. (2017)

4.3. Registration process

Monsanto Company was first to develop and register transgenic maize, MON 87411, which targets *Snf7* gene stacked on *cry3Bb1* gene under the name SmartStax PRO. The DvSnf7 RNA expressed in MON 87411 is composed of 968 nucleotide sequence containing 240 base pair dsRNA component plus the addition of a poly A tail (Urquhart et al., 2015). Upon consumption, the plant-produced dsRNA in MON 87411 is specifically recognized by the RNA interference machinery of WCR and other closely related CRW species, resulting in downregulation of the targeted DvSnf7 gene and leading to mortality (Bolognesi et al., 2012, Koči et al., 2014). Studies demonstrated that cross-resistance between DvSnf7 and Cry3Bb1 is unlikely (Moar et al., 2017). In addition, incorporation of multiple modes of action against CRW by pyramiding *Bt* and RNA-based traits will offer increased efficacy and durability of a product while maintaining a high degree of specificity for the target pest and environmental safety (Baum and Roberts, 2014).

Ecological risk assessment (ERA) is the main step in the registration of transgenic plants and PIPs. ERA of transgenic plants is designed to determine potential risks of introducing genetically engineered plants into the environment and include problem formulation, analysis, and risk characterization (US EPA, 1998). Assessment of potential ecological impacts, associated with the introduction of a PIP, is based on the characteristics of the crop and the introduced trait (Bachman et al., 2016). Risk assessment continuum based on tiered approach starts with laboratory research of worst-case scenario, continues with extended lab/semi-field research and finishes with field research (Figure 4.1.). Ecological complexity, realistic exposure and comprehensiveness of ERA increase in each step of risk assessment continuum.

In 2017, US EPA (US EPA, 2017b) published ERA for a registration of MON 89034 x TC1507 x MON 87411 x DAS-59122-7 combined trait corn (SmartStax PRO) expressing Bt insecticidal proteins, and DvSnf7 dsRNA submitted by Monsanto Company based on previously developed problem formulation. The goal of risk assessment was to determine (1) human risks and toxicology, (2) exposure and environmental fate of DvSnf7 dsRNA, (3) non-target toxicity, (3) possibility of synergism between DvSnf7 dsRNA and Cry proteins. They concluded that (1) No

Observed Adverse Effect Level (NOAEL) and Lowest Observed Adverse Effect Level (LOAEL) showed no significant toxicity and that, based on literature, exogenous dsRNAi will not be taken up as intact molecules in mammals (US EPA, 2017b) (2) exposure in terrestrial environment is limited to organisms which directly feed on dsRNA-producing plant material and can be extended to organisms which feed on herbivorous arthropods (secondary exposure), (3) exposure do DvSnf7 dsRNA in aquatic environments is expected to be minimal due to low persistence, (4) synergism between DvSnf7 dsRNA and the Cry proteins expressed in SmartStax PRO does not occur. Other studies have also confirmed that DvSnf7 dsRNA has no impact on human and mammalian health and that adverse impact on non-target organisms is relatively low.

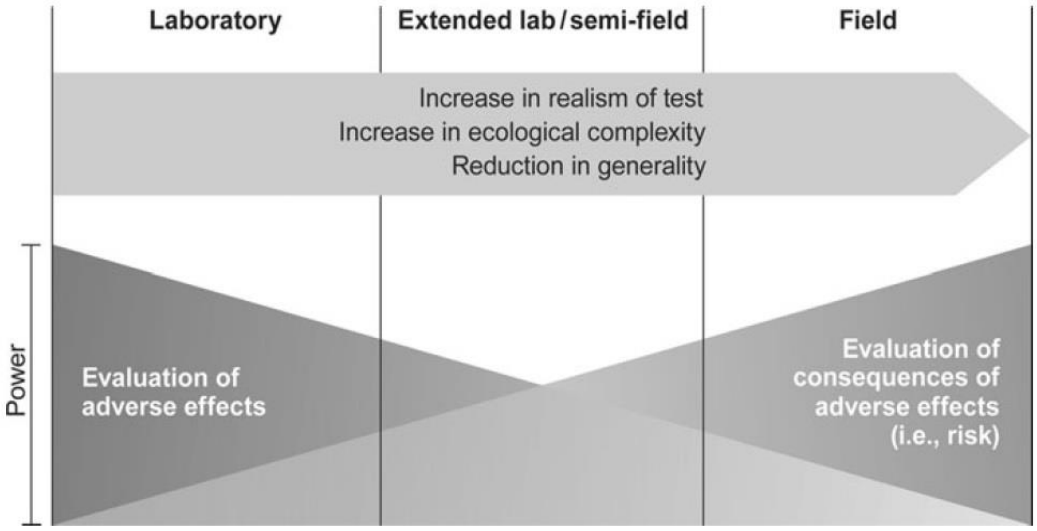

Figure 4.1.: Risk assessment continuum. Power indicates the ability to evaluate negative effects.

Source (Romeis et al., 2011)

Petrick et al. (2016) claim that RNAi and dsRNA molecules, naturally occurring in eukaryotes, are present in plants and animals and have a substantial history of safe consumption. This safe consumption is remarkable since commonly consumed foods such as maize, soybean, rice,

lettuce, and tomatoes contain both short (e.g. small interfering RNAs (siRNAs) and microRNAs) and long dsRNAs encoding short RNAs with perfect sequence identity to human and mammalian genes (Ivashuta et al., 2009, Jensen et al., 2013). The safety of ingested nucleic acids, including RNA, is well understood from several perspectives, including the simple fact that humans consume significant amounts of RNA with every meal of plant and animal-derived ingredients (Jonas et al., 2001). This is due to effective biological barriers against exogenous RNAs which cause degradation of dsRNA. The efficacy of biological barriers against ingested dietary RNAs in vivo is demonstrated by feeding studies with plant-derived materials/foods and/or dsRNAs (Dickinson et al., 2013, Petrick et al., 2015, Snow et al., 2013, Witwer and Hirschi, 2014, Witwer et al., 2013). Petrick et al. (2016) evaluated the potential toxicity to mammals of DvSnf7 RNA using toxicology study in mice at doses of 1, 10 and 100 mg/kg body weight. The results demonstrated the safety of DvSnf7 RNA expressed in MON 87411 since there was no toxicity observed and subsequently the No Observed Adverse Effect Level (NOAEL) was determined to be 100 mg/kg.

Additionally, Bachman et al. (2016) developed and evaluated ecological risk assessment for DvSnf7 RNA against WCR. The ERA problem formulation for MON 87411 was based on the biology and familiarity with the crop and the trait, the mode of action (MOA), the spectrum of activity, the tissue-specific expression profile, routes of exposure for ecological receptors and an assessment of potential persistence in the environment. Laboratory tests evaluated ecologically relevant endpoints such as survival, growth, development, and reproduction of non-target organisms (NTO's) since the protection goal is to maintain the ecological functions, namely, pollination, predation and parasitism, decomposition of soil organic material, and soil nutrient cycling. Testing required laboratory toxicity testing on individual species and placing the results into the context of ERA. All margins of exposure for NTOs were > 10-fold the maximum expected environmental concentration. They concluded that unintended effects in non-target organisms from immune stimulation and RNA machinery saturation are extremely unlikely to result from relatively low exposure to dsRNA resulting from cultivation of MON 87411.

Although no RNAi-based GM crops are registered in Europe to this date, the European Food Safety Authority (EFSA) reviewed risk assessment of RNAi. EFSA determined whether existing approaches for risk assessment are appropriate (EFSA, 2014), commissioned separate external scientific reports in which relevant scientific literature has been reviewed to inform human and environmental risks of RNAi-based GM plants (Paces et al., 2017, Christiaens et al., 2018), and developed a strategy for identification and assessment of plant non-target effects in RNAi-based GM plants (EFSA GMO Panel, 2018). Even though EFSA has not yet developed ERA plant for in-planta expressed dsRNA-based pesticides, the US EPA framework for ERA of dsRNA will largely help in RNAi-based pesticides registration in EU.

4.4. Future aspects

The successful demonstration of transgenic dsRNA to provide corn root protection against WCR feeding damage has catalyzed industry-wide interest in RNAi as a novel mode of action to combine with Bt technology to reduce the probability of field-evolved resistance to currently marketed traits (Fishilevich et al., 2016). Products that utilize the RNAi mode of action have the potential to offer new and complementary insect, bacteria, fungal, viral, and weed control solutions that have a degree of potency and selectivity beyond what has been possible to date using conventional pesticides (Sherman et al., 2015). The main advantages of RNAi-based pesticides are their selectivity for specific target species, and a possibility to control resistant populations. Head et al. (2017) evaluated the performance of SmartStax (which contains only *Bt* traits) and SmartStaxPRO in preventing root injury, estimated the reduction in adult beetle emergence, and compared the durability of SmartStax and SmartStaxPRO with 5% seed blend refuge, based on the trials conducted by Monsanto and Dow AgroSciences on naturally infested grower maize fields selected to challenge CRW products. They provided evidence that the addition of DvSnf7 in SmartStax PRO can reduce maize root damage compared to SmartStax (which contains only Bt traits) and prolong the durability of CryBb1 and Cry34Ab1/Cry35Ab1.

Nevertheless, several key uncertainties remain that represent potential hurdles to realizing the widespread commercial application of RNAi as a mode of action to pyramid with traits based on

Bt proteins: (1) RNAi trait performance over multiple field seasons and commercial hybrid yield potential has to be reported (Fishilevich et al., 2016), (2) the regulatory framework to assess safety of dsRNA insecticidal traits may differ in certain regards from that established for Bt-based insecticidal traits (Fishilevich et al., 2016) and (3) dsRNA environmental fate in soil; concentrations in soil during the growing season and after the harvest.

All in all, first field results of dsRNA transgenic maize have proved RNAi technology to be efficacious and reliable. Therefore, it is to be expected that more RNAi-based pesticides will be developed, registered and marketed in the near future. The delivery of products that utilize the RNAi mode of action as a biocontrol may occur as a spray, drench or granular application (Sherman et al., 2015), although they are more likely to be marketed as plant-incorporated protectants (PIP's). Also, the registration process for RNAi products is very similar as for GM crops which will make it easier to register RNAi-based pesticides because of the preexisting framework.

5. Conclusions

1. RNA interference is a novel technology which has emerged as the most promising tool for control of economically important pests such as Western corn rootworm.

2. RNA interference can be efficiently applied in pest control by autonomous uptake (feeding) of dsRNA supplied in transgenic plants engineered to express specific nucleotide sequence which results in knockdown of genes in target pest, or by topical application of dsRNA on soil and leaves. The application results in the mortality of both larvae and adult pests and reduction of pest damage.

3. Western corn rootworm is the most important economic pest of continuous maize cultivation which causes great damage to maize roots, pollination, and grain yield. It is very difficult to manage since it shows great ability to adjust to adverse conditions such as crop rotation, chemical pesticide application, and biotechnological control (*Bt* maize).

4. Many of the recent reports have confirmed a great efficiency of RNA interference in plant protection against Western corn rootworm adults and larvae with a range of genes which can be effectively targeted and result in larval stunting, larval and adult mortality or reduction of fecundity. As for now, there are four RNAi PIP product approved by the US EPA called SMARTSTAX PRO which combine *Bt* traits and DvSnf7 dsRNA, designed to manage Western corn rootworm in maize fields.

5. Advantages of using RNA interference are its specificity based on precise nucleotide-sequence identity which is targeted, high potency of RNA interference based plant-incorporated protectant products, and the possibility to control resistant populations or impede their development.

6. There are still some environmental concerns regarding the use of RNA interference products due to lack of field experiments, non-target exposure information, uncertainties of dsRNA distribution in the environment and need for appropriate environmental risk assessment design for registration of RNA interference plant-incorporated products.

7. In order to achieve adequate crop protection without compromising environmental safety, it is necessary to carefully assess risks of extensive application of RNA interference-based products during the multiple seasons and establish a regulatory framework for evaluating the safety of dsRNA insecticidal traits in transgenic RNAi crops.

6. Literature

Alder, M.N., Dames, S., Gaudet, J., Mango, S.E. (2003). Gene silencing in *Caenorhabditis elegans* by transitive RNA interference. RNA. 9: 25-32.

Andrade, E.C., Hunter, W.B. (2016). RNA interference – natural gene-based technology for highly specific pest control (HiSPeC). In: RNA interference (Abdurakhmonov, I.Y.). InTech, Rijeka, 391–409.

Andrade, E.C., Hunter, W.B. (2017). RNAi feeding bioassay: development of a non-transgenic approach to control Asian citrus psyllid and other hemipterans. Entomol Exp Appl. 162: 389-396.

Araujo, R.N., Santos, A., Pinto, F.S., Gontijo, N.F., Lehane, M.J., Pereira, M.H. (2006). RNA interference of the salivary gland nitrophorin 2 in the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) by dsRNA ingestion or injection. Insect Biochemistry and Molecular Biology. 36: 683–693.

Bachman, P.M., Huizinga, K.M., Jensen, P.D., Mueller, G., Tan, J., Uffman, J.P., Levine, S.L. (2016). Ecological risk assessment for DvSnf7 RNA: A plant incorporated protectant with targeted activity against western corn rootworm. Reg. Toxic. And Pharm. 81: 77-88.

Ball, H.J. (1957). On the biology and egg-laying habits of the western corn rootworm. Journal of Economic Entomology. 50: 126-128.

Ball, H.J., Weekman, G.T. (1962). Insecticide resistance in the adult western corn rootworm in Nebraska. Journal of Economic Entomology. 55: 439-441.

Baum, J.A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T., Roberts, J. (2007). Control of coleopteran insect pests through RNA interference. Nature Biotechnology. 25(11): 1322-1326.

Baum, J.A., Roberts, J.K. (2014). Progress towards RNAi-mediated insect pest management. Adv. Insect Physiol. 47: 249-295.

Bernstein, E., Caudy, A. A., Hammond, S. M., Hannon, G. J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 409: 363–366.

Bolognesi, R., Ramaseshadri, P., Anderson, J., Bachman, P., Clinton, W., Flannagan, R., Ilagan, O., Lawrence, C., Levine, S., Moar, W., Mueller, G., Tan, J., Uffman, J., Wiggins, E., Heck, G., Segers, G. (2012). Characterizing the Mechanism of Action of Double-Stranded RNA Activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). PLoS ONE. 7(10): e47534.

Branson, T.F., Ortman, E.E. (1970). The host range of larvae of the western corn root-worm: further studies. Journal of Economic Entomology. 63: 800-803.

Bucher, G., Scholten, J., Klingler, M., Parental RNAi in *Tribolium* (Coleoptera). Current Biol. 12: 85-86.

Camargo, C., Wu, K., Fishilevich, E., Narva, K.E., Siegfried, B.D. (2018). Knockdown of RNA interference pathway genes in western corn rootworm, *Diabrotica virgifera virgifera*, identifies no fitness costs associated with Argonaute 2 or Dicer-2. Pesticide Biochemistry and Physiology. 148: 103-110.

Cappelle, K., de Oliviera, C.F., Van Eynde, B., Christiaens, O., Smagghe, G. (2016). The involvement of clathrin-mediated endocytosis and two Sid-1 like transmembrane proteins in double-stranded RNA uptake in Colorado potato beetle midgut. Insect Mol. Bio. 25: 315-323.

Carpenter, J.E. (2007) Peer-reviewed surveys indicate positive impact of commercialized GM crops. Nat. Biotechnol. 28: 319-321.

Carriére, Y., Ellers-Kirk, C., Harthfield, K., Larocque, G., Degain, B., Dutilleul, P., Dennehy, T.J., Marsh, S.E., Crowder, D.W., Li, X., Ellsworth, P.C., Naranjo, S.E., Palumbo, J.C., Fournier, A., Antilla, L., Tabashnik, B.E. (2012). Large-scale, spatially explicit test of the refuge strategy for delaying insecticide resistance. Proc. Natl. Acad. Sci. U.S.A. 109: 775-780.

Carriére, Y., Crickmore, N, Tabashnik, B.E. (2015). Optimizing pyramid transgenic Bt crops for sustainable pest management. Nat. Biotechnol. 33: 161-168.

Castañera, P., Farinós, G.P., Ortego, F., Andow, D.A. (2016). Sixteen Years of Bt Maize in the EU Hotspot: Why Has Resistance Not Evolved? PLoS ONE. 11(5): e0154200.

Cattanero, M. G., Yafuso, C., Schmidt, C., Huang, C.Y., Rahman, M., Olson, C., Ellers-Kirk, C., Orr, B.J., Marsh, S.E., Antilla, L., Dutilleul, P., Carriére, Y. (2006). Farm-scale evaluation of the impacts of transgenic cotton on biodiversity, pesticide use, and yield. Proc. Natl. Acad. Sci. USA. 103: 7571-7575.

Chen, H.M., Chen, L.T., Patel, K., Li, Y.H., Baulcombe, D.C., Wu, S.H. (2010). Nucleotide RNAs trigger secondary siRNA biogenesis in plants. Proc. Natl. Acad. Sci. USA. 107:15 269-15.

Chiang, H.C. (1973). Bionomics of the northern and western corn rootworm. Annual Review of Entomology. 18: 47-72.

Christiaens, O., Dzhambazova, T., Kostov, K., Arpaia, S., Joga, M.R., Urru, I., Sweet, J., Smagghe, G. (2018). Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi-based GM plants. EFSA supporting publication. 2017: EN-1246, 314 p.

Clark, T.L., Hibbard, B.E. (2004). A comparison of non-maize hosts to support western corn rootworm (Coleoptera: Chrysomelidae) larval biology. Environ. Entomol. 33: 722-727.

Cullen, E.M., Gray, M.E., Gassmann, A.J., Hibbard, B. (2013). Resistance to Bt Corn by Western Corn Rootworm (Coleoptera: Chrysomelidae) in the U.S. Corn Belt. J. Integ. Pest Management. 4(3), doi: http://dx.doi.org/10.1603/IPM13012

de la Fuente, J., Kocan, K.M., Almazán, C. and Blouin, E.F. (2007). RNA interference for the study and genetic manipulation of ticks. Trends Parasitol. 23:427–433.

Davis-Vogel., C., Ortiz, A., Procyk, L., Robeson, J., Kassa, A., Wang, Y., Huang, E., Walker, C., Sethi, A., Nelson, M.E., Sashital, D.G. (2018). Knockdown of RNA interference pathway genes impacts the fitness of western corn rootworm. Scientific reports. 8: 7858.

Dickinson, B., Zhang, Y., Petrick, J.S., Heck. G., Ivashuta, S., Marshall, W.S. (2013). Lack of detectable oral bioavailability of plant micro RNAs after feeding in mice. Nature Biotech. 31: 965-967.

Dowling, D., Pauli, T., Donath, A., Meusemann, K., Podsiadlowski, L., Petersen, M., Peters, R.S., Mayer, C., Liu, S., Zhou, X., Misof, B., Niehuis, O. (2016). Phylogenetic Origin and Diversification of RNAi Pathway Genes in Insects. Genome Biol. Evol. 8(12): 3784-3793.

Duan, C-G., Wang, C-H., Guo, H-S. (2012). Application of RNA silencing to plant disease resistance. Silence. 3: 5.

Dubelman, S., Fischer, J., Zapata, F., Huizinga, K., Jiang, C., Uffman, J., Levine, S., Carson, D. (2014). Environmental fate of double-stranded RNA in agricultural soils. PLoS One. 9: 1–7.

Dun, Z., Mitchell, P.D., Agosti, M. (2010). Estimating *Diabrotica virgifera virgifea* damage functions with field trial data: applying an unbalanced nested error component model. J. Appl. Entomol. 134: 409-419.

Edwards, C.R. (1996). The dramatic shift of the western corn rootworm to first-year corn. In: 1996 Proceedings Illinois Agricultural Pesticides Conference, Cooperative Extension Service, University of Illinois at Urbana-Champaign, pp. 14-15.

EFSA – European Food Safety Authority (2012). Scientific Opinion on the annual Post-Market Environmental Monitoring (PMEM) report from Monsanto Europe S.A. on the cultivation of genetically modified maize MON 819 in 2010. EFSA Journal. 10(4): 2610.

EFSA – European Food Safety Authority (2014). International scientific workshop 'Risk assessment considerations for RNAi-based GM plants <https://www.efsa.europa.eu/en/events/event/140604> Accessed on April 10th, 2019.

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms) (2018). Scientific Opinion on the assessment of genetically modified maize MON 87411 for food and feed uses, import and processing, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2015-124). EFSA Journal. 16(6): 5310. <https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5310> Accessed on 4th of May 2019.

Fernandez, E.Q., Moyer, D.L., Maiyuran, S., Labaro, A., Brody, H. (2012). Vector-initiated transitive RNA interference in the filamentous fungus *Aspergillus oryzae*. Fungal Genet. Biol. 49: 294-301.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 391: 806–811.

Fishilevich, E., Vélez, A.M., Storer, N.P., Li, H., Bowling, A.J., Rangasamy, M., Worden, S.E., Narva, K.E., Siegfried, B.D. (2016). RNAi as a management tool for the western corn rootworm, *Diabrotica virgifera virgifera*. Faculty Publications: Department of Entomology, University of Nebraska - Lincoln. 504.

Foott, W.H., Timmins, P.R. (1977). Observations on new insect pests of grain corn in Essex County, Ontarion. Proc. Entomol. Soc. Ont. 108: 49-52.

Fu, D.Q., Zhu, B.Z., Zhu, H.L., Jiang, W.B., Luo, Y.B. (2005). Virus-induced gene silencing in tomato fruit. Plant J. 43: 299–308.

Garbian, Y., Maori, E., Kalev, H., Shafir, S., Sela, I. (2012). Bidirectional transfer of RNAi between honey bee and *Varroa destructor*: *Varroa* gene silencing reduces *Varroa* population. PLoS Pathogens. 8: 1-9.

Gassmann, A.J., Petzold-Maxwell, J.L., Clifton, E.H., Dunbar, M.W., Hoffmann, A.M., Ingber, D.A., Keweshan, R.S. (2014). Field-evolved resistance by western corn rootworm to multiple *Bacillus thuringiensis* toxins in transgenic maize. Proceedings of the National Academy of Sciences. 111(14).

Gillette, C.P. (1912). *Diabrotica virgifera* LeC. as a corn root-worm. Journal of Economic Entomology. 5: 364-366.

Gould, F. (1998). Sustainability of transgenic insecticidal cultivars: Integrating pest genetics and ecology. Annu Rev Entomol. 43: 701-726.

Gray, M.E., Felsot, A.S., Steffey, K.L., Levine, E. (1992). Planting time application of soil insecticides and western corn rootworm (Coleoptera: Chrysomelidae) emergence: implications for long-term management programs. J. Econ. Entomol. 85: 544-553.

Gray, M.E., Levine, E., O'Neal, M.E., Spencer, J.L., Isard, S.A., Steffey, K.L. (1998a). Crop rotation and western corn rootworm, *Diabrotica virgifera virgifera* LeConte, management: have we lost a pest management tool in the eastern corn belt? In: Proceedings of the Sixth Australasian Applied Entomological Research Conference, Brisbane, Australia, 29 September-2 October, 155-163.

Gray, M.E., Levine, E., Oloumi-Sadeghi, H. (1998b). Adaptation to crop rotation: western and northern corn rootworms respond uniquely to a cultural practice. Rec. Res. Dev. Entomol. 2: 19-31.

Gray, M.E., Sappington, T.W., Miller, N.J., Moeser, J., Bohn, M.O. (2009). Adaptation and invasiveness of western corn rootworm: intensifying research on a worsening pest. Annu. Rev. Entomol. 54: 303-321.

Head, G.P., Carroll, M., Evans, S., Rule, D.M., Willse, A., Clark, T., Storer, N., Flanagan, R., Samuel, L., Meinke, L.J. (2017). Evaluation of SmartStax and SmartStax PRO Maize against Western Corn Rootworm and Northern Corn Rootworm: Efficacy and Resistance Management Evaluation of SmartStax and SmartStax PRO against WCR and NCR. Pest Manag. Sci. 73(9): 1883-1899.

Heinemann, J.A, Agapito-Tenfen, S.Z., Carman, J.A. (2013). A comparative evaluation of the regulation of GM crops or products containing dsRNA and suggested improvements to risk assessments. Environment International. 55: 43-55.

Hu, X., Richtman, N.M., Zhao, J-Z., Duncan, K.E., Niu, X., Procyk, L.A., Oneal, M.A., Kernodle, B.M., Steimel, J.P., Crane, V.C., Sandahl, G., Ritland, J.L., Howard, R.J., Presnail, J.K., Lu, A.L., Wu, G. (2016). Discovery of midgut genes for the RNA interference control of corn rootworm. Scientific Reports. 6: 30542.

Huang, F., Andow, D.A, Buschman, L.L. (2011). Success of the high dose/refuge resistance management strategy after fifteen years of Bt crop use in North America. Entomologia Experimentalis et Applicata. 140: 1-16.

Hummel, H.E., Dinnesen, S., Nedelev, T., Modic, S., Urek, G., Ulrichs, C. (2008). *Diabrotica virgifera virgifea* LeConte in confrontation mood: simultaneous geographical and host spectrum expansion in southeastern Slovenia. Mitt. Dtch. Ges. allg. angew. Ent. 16: 127-130.

Hunter, W., Ellis, J., Vanengelsdorp, D., Hayes, J., Westervelt, D., Glick, E., Williams, M., Sela, I., Maori, E., Pettis, J., Cox-Foster, D., Paldi, N. (2010). Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (*Apis mellifera*, Hymenoptera: Apidae). PLoS Pathog. 6: 1–10.

Hutchison, W., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S.J., Abrahamson, M., Hamilton, K.L., Steffey, K.L., Gray, M.E., Hellmich, R.L., Kaster, L.V., Hunt, T.E., Wright, R.J., Pecinovsky, K., Rabaey, T.L., Flood, B.R., Raun, E.S. (2010). Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science. 330: 222-225.

Huvenne, H., Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. J Insect Physiol. 56: 227–235.

Igrc Barčić, J., Bažok, R., Edwards, C.R., Kos, T. (2007). Western corn rootworm adult movement and possible egg laying in fields bordering maize. J. Appl. Entomol. 131(6): 400-405.

Ivashuta, S.I., Petrick, S., Heisel, S.E., Zhang, Y., Guo, L., Reynolds, T.L., Rice, J.F., Allen, E., Roberts, J.K. (2009). Endogenous small RNAs in grain: Semi-quantification and sequence homology to human and animal genes. Food and Chemistry Toxicology. 47(2): 353-360.

Ivashuta, S., Zhang, Y., Wiggings, B.E., Ramaseshadri, P., Segers, G.C., Johnson, S., Meyer, S.E., Kerstetter, R.A., McNulty, B.C., Bolognesi, R., Heck, G.R. (2015). Environmental RNAi in herbivorous insects. RNA. 21(5): 840-50. doi: 10.1261/rna.048116.114 Epub 2015 Mar 23.

Ivezić, M., Raspudić, E., Brmež, M., Majić, I., Džoić, D., Brkić, A. (2009). Maize tolerance to western corn rootworm larval feeding: screening through five years of investigation. Agric Conspec Sci. 74: 291-295.

Jensen, P.D., Zhang, Y., Wiggins, B.E., Petrick, J.S., Zhu, J., Kerstetter, R.A., Ivashuta, S.I. (2013). Computational sequence analysis of predicted long dsRNA transcriptomes of major crops reveals sequence complementarity with human genes. GM Crops Food. 4: 90-97.

Joga, M.R., Zotti, M.J., Smagghe, G., Christiaens, O. (2016). RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: What we know so far. Front. Physiol. 7: 553.

Jonas, D.A., Elmadfa, I., Engel, K.-H., Heller, K.J., Kozianowski, G., König, A., Müller, D., Narbonne, J.F., Wackernagel, W., Kleiner, J. (2001). Safety Considerations of DNA in Food. Ann. Nutr. Metab. 45: 235-254.

Jun Tong, K., Duchêne, S., Ho, S.Y., Lo, N. (2015). INSECT PHYLOGENOMICS. Comment on “Phylogenomics resolves the timing and pattern of insect evolution”. Science. 349: 487-489.

Kantack, B.H. (1965). Western corn rootworm in South Dakota. Proc. N. Centr. Br. Entomol. Soc. Am. 20: 62-63.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, I., Hannon, G.J., Plasterk, R.H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. Genes Dev. 15: 2654-2659.

Khajuria, C., Vélez, A.M., Rangasamy, M., Wang, H., Fishilevich, E., Frey, M.L.F., Carneiro, N.P., Gandra, P., Narva, K.E., Siegfried, B.D. (2015). Parental RNA interference of genes involved in embryonic development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Insect Biochemistry and Molecular Biology. 63: 54-62.

Kiss, J., Edwards, R., Berger, H.K., Cate, P., Cean, M., Cheek, S., Derron, J., Festić, H., Furlan, L., Igrc Barčić, J., Ivanova, I., Lammers, W., Omelyuta, V., Princzinger, G., Reynaud, P., Sivcev, I., Sivicek, P., Urek, G., Vahala, O. (2005). Monitoring of Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte) in Europe 1992-2003. In: Western Corn Rootworm: ecology and management (Vidal, S., Kuhlmann, U., Edwards, C.R) CAB International, Wallingford, UK, 29-41.

Kiss, J., Komaromi, J., Bayar, K., Edwards, C.R., Hatala-Zseller, I. (2005). Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) and the crop rotation systems in Europe. In: Western corn rootworm: ecology and management (Vidal, S., Kuhlman, U., Edwards, C.R.). CAB International, Wallingford, UK, 189-220.

Koch, A., Kumar, N., Weber, L., Keller, H., Imani, J., Kogel, K-H. (2013). Host-induced gene silencing of cytochrome P450 lanosterol C14-demethylase-encoding genes confers strong resistance to Fusarium species. Proc. Natl. Acad. Sci. 110: 19324–19329.

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., Linicus, L., Jelonek, L., Goesmann, A., Cardoza, V., McMillan, J., Mentzel, T., Kogel, K.H. (2016). An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. PLOS Pathog. 12: e1005901.

Koči, J., Ramaseshadri, P., Bolognesi, R., Segers, G., Flannagan, R., Park, Y. (2014). Ultrastructural Changes Caused by Snf7 RNAi in Larval Enterocytes of Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). PLoS ONE. 9(1): e83985.

Kogan, M. (1994). Plant resistance in pest management. In: Introduction to Insect Pest Management (Metcalf, R.L., Luckmann, W.H.) A Wiley-Interscience publication, US.

Krysan, J.L. (1986). Introduction: biology, distribution, and identification of pest *Diabrotica*. In: Methods for the Study of Pest *Diabrotica* (Krysan, J.L., Miller, T.A.) New York, Springer 25-47.

Kwon, D.H., Park, J.H., Ashok, P.A., Lee, U., Lee, S.H. (2016). Screening of target genes for RNAi in *Tetranychus urticae* and RNAi toxicity enhancement by chimeric genes. Pestic. Biochem. Physiol. 130: 1–7.

Levine, E., Gray, M. (1996). First year corn rootworm injury: east-central Illinois reaearch progress to date and recommendations for 1996. In: 1996 Proceedings Illinois Agricultural Pesticides Conference, Cooperative Extension Service, University of Illinois at Urbana-Champaign, 3-13.

Levine, E., Spencer, J.L., Isard, S.A., Onstad, D.W., Gray, M.E. (2002). Adaptation of the Western Corn Rootworm to Crop Rotation: Evolution of a New Strain in Response to a Management Practice. American Entomologist. 48(2): 94-107.

Levy-Booth, D.J., Campbell, R.G., Gulden, R.H., Hart, M.M., Powell, J.R., Klironomos, J.N., Pauls, K.P., Swanton, C.J., Trevors, J.T., Dunfield, K.E. (2007). Cycling of extracellular DNA in the soil. Soil Biology & Biochemistry. 39: 2977-2991.

Li, H., Guan, R., Guo, H., Miao, X. (2015). New insights into an RNAi approach for plant defense against piercing-sucking and stem-borer insect pests. Plant Cell Environ. 38: 2277–2285.

Li, G., Niu, J., Zotti, M., Sun, Q., Zhu, L., Zhang, J., Liao, C.Y., Dou, W., Wei, D.D., Wang, J.J., Smagghe, G. (2017). Characterization and expression patterns of key ecdysteroid biosynthesis and signaling genes in a spider mite (*Panonychus citri*). Insect Biochem. Mol. Biol. 87: 136–146.

Ludwig, K.A., Hill, R.E. (1975). Comparison of gut content of adult western and northern corn rootworm in northeast Nebraska. Environmental Entomology. 4: 435-438.

Lundgren, J.G., Duan, J.J. (2013). RNAi-Based Insecticidal Crops: Potential effects on Nontarget Species. BioScience. 63(8): 657-665.

Mao, Y.B., Cai, W.J., Wang, J.W., Hong, G.J., Tao, X.Y., Wang, L.J., Huang, Y.P., Chen, X.Y. (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat. Biotechnol. 25: 1307–1313.

Marra, M., Piggott, N., Goodwin, B.K. (2012). The Impact of Corn Rootworm Protected Biotechnology Traits in the United States. AgBioForum. 15(2): 217-230.

Marvier, M., McCreedy, C., Regetz, J., Kareiva, P. (2007) A meta-analysis of effects of Bt cotton and maize on non-target invertebrates. Science. 316: 1474-1477.

Matthew, L. (2009). Hairpin RNAi in plants. In: RNA interference: methods for plants and animals, (Doran, T., Helliwell, C.) Cambridge University Press, Cambridge.

McPherson, R.M., Douce, G.K., Riley, D.G. (1996). Summary of Losses from Insect Damage and Costs of Control in Georgia 1995. Univ. Ga. Spec. Publ. 90. 54 pp.

Meinke, J.L., Sappington, T.W., Onstad, D.W., Guillemaud, T., Miller, N.J., Komaromi, J., Levay, N., Furlan, L., Kiss, J., Toth, F. (2009). Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) population dynamics. Agric For. Entomol. 11: 29-46.

Meister, G. (2013). Argonaute proteins: functional insights and emerging roles. Nature Reviews. Genetics. 14: 447-459.

Meloche F., Hermans, P. (2004). Eastward expansion and discovery of the soybean biotype of western corn rootworm (*Diabrotica virgifera virgifera* LeConte) in Canada. Can. J. Plant Sci. 84: 305–9.

Meloche, F., Rhainds, M., Roy, M., Brodeur, J. (2005). Distribution of western and northern corn rootworms (Coleoptera: Chrysomelidae) in Quebec, Canada. Can. Entomol. 137: 226–29.

Metcalf, R.L. (1983). Implications and prognosis of resistance to insecticides. In: Pest resistance to Pesticides (Georghiou, G.P., Saito, T.). Plenum Press, New York, 703-733.

Metcalf, R.L. (1986). Foreword. In: Methods for the Study of Pest *Diabrotica* (Krysan, J.L., Miller, T.A.). Springer-Verlag, New York, 7-15.

Mitter, N., Worrall, E.A., Robinson, K.E., Li, P., Jain, R.G., Taochy, C., Fletcher, S.J., Carroll, B.J., Lu, G.Q., Xu, Z.P. (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. Nature Plants. 3: 16207.

Miyata, K., Ramaseshadri, P., Zhang, Y., Segers, G., Bolognesi, R., Tomoyasu, Y. (2014). Establishing an *In Vivo* Assay System to Identify Components Involved in Environmental RNA Interference in the Western Corn Rootworm. PLoS ONE. 9(7): e101661.

Moar, W., Khajuria, C., Pleau, M., Ilagan, O., Chen, M., Jiang, C., Price, P., McNulty, B., Clark, T., Head, G. (2017). Cry3Bb1-Resistant Western Corn Rootworm, *Diabrotica virgifera virgifera* (LeConte) Does Not Exhibit Cross-Resistance to DvSnf7 dsRNA. PLoS ONE 12(1): e0169175.

Moeser, J., Hibbard, E. (2005). A Synopsis of the Nutritional Ecology of Larvae and Adults of *Diabrotica virgifera virgifera* (LeConte) in the New and Old World – Nouvelle Cuisine for the Invasive Maize Pest *Diabrotica virgifera virgifera* in Europe? In: Western Corn Rootworm: ecology and management (Vidal, S., Kuhlmann, U., Edwards, C.R.) CAB International, Wallingford, UK, 41-67.

Musick, G.J., Chiang, H.C., Luckmann, W.H., Mayo, Z.B., Turpin, F.T. (1980). Impact of planting dates of field corn on beetle emergence and damage by the western and the northern corn rootworms in the Corn Belt. Annals of the Entomological Society of America. 73: 207-215.

Naranjo, S.E., Sawyer, A.J. (1988). Impact of Host Plant Phenology on the Population Dynamics and Oviposition of Northern Corn Rootworms, *Diabrotica barberi* (Coleoptera: Chrysomelidae), in Field Corn. Environ. Entomol. 17(3): 508-521.

Niu, X., Kassa, A., Hu, X., Robeson, J., McMahon, M., Richtman, N.M., Steimel., J.P., Kernodle, B.M., Crane, V.C., Sandahl, G., Ritland, J.L., Presnail, J.K., Lu, A.L., Wu, G. (2017). Control of Western Corn Rootworm (*Diabrotica virgifera virgifera*) Reproduction through Plant-Mediated RNA Interference. Scientific reports. 7: 12591.

Obbard, D.J., Jiggins, F.M., Halligan, D.L., Little, T.J. (2006). Natural selection drives extremely rapid evolution in antiviral RNAi genes. Current biology. 16: 580-585.

Oyediran, I.O., Hibbard, B.E., Clark, T.L. (2004). Prairie grasses as hosts of the western corn rootworm (Coleoptera: Chrysomelidae). Environ. Entomol. 33: 740-747.

Paces, J., Nic, M., Novotny, T., Svoboda, P. (2017). Literature review of baseline information to support the risk assessment of RNAi-based GM plants. EFSA supporting publication. 2017: EN-1246, 314 pp.

Paim, R.M., Araujo, R.N., Lehane, M.J., Gontijo, N.F., Pereira, M.H. (2013). Long term effects and parental RNAi in the blood feeder *Rhodnius prolixus* (Hemiptera; Reduviidae). Insect Biochem, Mol. Biol. 43: 1015-1020.

Palli, S.R. (2014). RNA interference in Colorado potato beetle: Steps toward development of dsRNA as a commercial insecticide. Curr. Opin. Insect Sci. 6: 1–8.

Parker, J.S., Barford, D. (2006). Argonaute: A scaffold for the function of short regulatory RNAs. Trends in Biochemical Sciences. 31(11): 622-630.

Petrick, J.S., Moore, W.M., Heydens, W.F., Koch, M.S., Sherman, J.H., Lemke, S.L. (2015). A 28-day oral toxicity evaluation of small interfering RNAs and a long double-stranded RNA targeting vacuolar ATPase in mice. Reg. Toxic. and Pharm. 71(1): 8-23.

Petrick, J.S., Frierdich, G.E., Carleton, S.M., Kessenich, C.R., Silvanovich, A., Zhang, Y., Koch, M.S. (2016). Corn rootworm-active RNA DvSnf7: Repeat dose oral toxicity assessment in support of human and mammalian safety. Regulatory Toxicology and Pharmacology. 81: 57-68.

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M.T., Guerri, G., Nannipieri, P. (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. Biology and Fertility of Soils. 45: 219-235.

Price, R.G.D., Gatehouse J.A. (2008). RNAi-mediated crop protection against insects. Trends in Biotechnology. 26: 393-400.

Ramaseshadri, P., Segers, G., Flannagan, R., Wiggins, E., Clinton, W., Illagan, O., McNulty, B., Clark, T., Bolognesi, R. (2013). Physiological and Cellular Responses Caused by RNAi-Mediated Suppression of Snf7 Orthologue in Western Corn Rootworm (*Diabrotica virgifera virgifera*) Larvae. PLoS ONE. 8(1): e54270.

Rangasamy, M., Siegfried, B.D. (2011). Validation of RNA interference in western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) adults. Pest Manag Sci. 68: 587-591.

Romeis, J., Hellmich, R.L., Candolfi, M.P., Carstens, K., De Schrijver, A.D., Gatehouse, A.M.R., Herman, R.A., Huesing, J.E., McLean, M., Raybould, A., Shelton, A.M., Waggoner, A. (2011). Recommendations for the design of laboratory studies on non-target arthropods for risk assessment of genetically engineered plants. Transgenic Res. 20: 1-22.

Roush, R. (1998). Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? Philos. Trans. R. Soc. Lond. B Biol. Sci. 353: 1777-1786.

Sági, L., Rakszegi, M., Spitkó, T., Mészáros, K., Németh-Kisgyörgy, B., Soltész, A., Szira, F., Ambrus, H., Mészáros, A., Galiba, G., Vágújfalvi, A., Barnabás, B., Marton, L.C. (2008). Genetic modification of cereals in the Agricultural Research Institute of the Hungarian Academy of Sciences. Acta Agronomica Hungarica. 56(4): 443-448.

San Miguel, K., Scott, J.G. (2016). The next generation of insecticides: DsRNA is stable as a foliar-applied insecticide. Pest Manag Sci. 4:801–809.

Shaw, J.T., Paullus, J.H., Luckmann, W.H. (1978). Corn rootworm oviposition in soybean. Journal of Economic Entomology. 71: 189-191.

Sherman, J.H., Munyikwa, T., Chan, S.Y., Petrick, J.S., Witwer, K.W., Choudhuri, S. (2015). RNAi technologies in agricultural biotechnology: The Toxicology Forum 40th Annual Summer Meeting. Regul. Toxicol. Pharmacol. 73(2): 671-80. doi: 10.1016/j.yrtph.2015.09.001.

Sijen, T., Steiner, F.A., Thijssen, K.L., Plasterk, R.K. (2007). Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. Science. 315: 244-247.

Smith, M.V. (1983). Northern (*Diabrotica longicornis*) and western (*Diabrotica virgifera*) corn rootworm beetles as competitors of foraging honey bees, *Apis mellifera*. Can. Beekeeping. 10: 173–74.

Snow, J.W., Hale, A.E., Isaacs, S.K., Baggish, A.L., Chan, S.Y. (2013). Inffective delivery of diet-derived microRNAs to recipient animal organisms. RNA Biol. 10: 1107-1116.

Spencer, J.L., Levine, E., Isard, S.A., Mabry, T.R. (2005). Movement, Dispersal and Behaviour of Western Corn Rootworm Adults in Rotated Maize and Soybean Fields. In: Western Corn Rootworm: ecology and management (Vidal, S., Kuhlmann, U., Edwards, C.R.) CAB International, Wallingford, UK, 121-145.

Spike, B.P., Tollefson, J.J. (1988). Western corn rootworm (Coleoptera: Chrysomelidae) larval survival and damage potential to corn subjected to nitrogen and plant density treatments. J. Econ. Entomol. 81: 1450-1455.

Steffey, K.L., Rice, M.E., All, J., Andow, D.A., Gray, M.E., Van Duyn, J.W. (1999). Handbook of Corn Insects. Entomological Society of America, Lanham, Maryland, 164 pp.

Sutter, G.R., Gustin, R.D. (1989). Environmental factors influencing corn rootworm biology and control. In: Proceeding Illinois agricultural pesticides conf. 1989, University of Illinois, Cooperative Extension Service, Urbana-Campaign, IL. 43-48.

Sutter, G.R. (1999). Western corn rootworm. In Handbook of Corn Insects (Steffey, K.L., Rice, M.E., All, J., Andow, D.A., Gray, M.E., Van Duyn, J.W.) Lanham, MD: Entomol. Soc. Am. 64–65.

Tabashnik, B.E. (1994). Evolution of resistance to Bacillus thuringiensis. Annual Review of Entomology. 39: 47-49.

Tabashnik, B.E., Gould, F. (2012). Delaying corn rootworm resistance to Bt corn. J Econ. Entomol. 105: 767-776.

Tate, H.D., Bare, O.S. (1946). Corn rootworms. Neb. Agric. Expt. Stn. Bull. 381: 3–12.

Taxman, D.J. (2009). siRNA and shRNA design. In: RNA interference: methods for plants and animals, (Doran, T. and Helliwell, C.) Cambridge University Press, Cambridge

Toepfer, S., Ellsbury, M.M., Eschen, R., Kuhlmann, U. (2007). Spatial clustering of *Diabrotica virgifera virgifera* and *Agriotes ustulatus* in small-scale maize fields without topographic relief drift. Entomol Exp Applic. 124: 61-75.

Tomoyasu, Y., Miller, S. C., Tomita, S., Schoppmeier, M., Grossmann, D., Bucher, G. (2008). Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*. Genome Biol. 9:1.

Turner, C.T., Davy, M.W., MacDiarmid, R.M., Plummer, K.M., Birch, N.P., Newcomb, R.D. (2006). RNA interference in the light brown applemoth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. Insect Molecular Biology. 15: 383–391.

Urquhart, W., Mueller, G.M., Carleton, S., Song, Z., Perez, T. Uffman, J.P., Jensen, P.D., Levine, S.L., Ward, J. (2015). A novel method of demonstrating the molecular and functional

equivalence between *in vitro* and plant-produced double-stranded RNA. Regul. Toxicol. Pharmacol. 73: 607-612.

US EPA – United States Environment Protection Agency (1998). Guidelines for ecological risk assessment. EPA/630/R-95/002F, April 1998, Washington.

US EPA – United States Environment Protection Agency (2013). White paper on RNAi technology as a pesticide: Problem Formulation for Human Health and Ecological Risk Assessment. <http://www.thecre.com/premium/wp-content/uploads/2012/04/RNAi-White-Paper.pdf>. Accessed on April 10th, 2019.

US EPA – United States Environment Protection Agency (2016). RNAi Technology: Human Health and Ecological Risk Assessments for SmartStax PRO. <https://www.epa.gov/sites/production/files/2016-12/documents/rnai_sap_sept_2016_final_minutes.pdf> Accessed on 10th of April 2019.

US EPA – United States Environment Protection Agency (2017). EPA Registers Innovative Tool to Control Corn Rootworm. <https://www.epa.gov/newsreleases/epa-registers-innovative-tool-control-corn-rootworm> Accessed on 3rd of May 2019.

US EPA – United States Environment Protection Agency (2017a). Framework to Delay Corn Rootworm Resistance. <https://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra/framework-delay-corn-rootworm-resistance#q1>. Accessed on 19th of April 2019.

US EPA – United States Environmental Protection Agency (2017b). Revised Addendum to Environmental Risk Assessment for a FIFRA Section 3 Registration of MON 89034 x TC1507 x MON 87411 x DAS-59122-1 Combined Trait Maize Expressing Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, Cry34/35Ab1, Bacillus thuringiensis Derived Insecticidal Protein and DvSnf7 Double Stranded RNA (dsRNA); Submitted by Monsanto Company <https://cfpub.epa.gov/si/si_public_file_download.cfm?p_download_id=532897&Lab=OPP>. Accessed on 3rd of May 2019.

USDA-ERS (2019). Recent Trends in GE Adoption. <https://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us/recent-trends-in-ge-adoption.aspx>. Accessed on 12th of August 2019.

Vaistij, F.E., Jones, L., Baulcombe, D.C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell. 14: 857-867.

Velez, A.M., Khajuria, C., Wang, H., Narva, K.E., Siegfried, B.D. (2016). Knockdown of RNA Interference Pathway Genes in Western Corn Rootworms (*Diabrotica virgifera virgifera*

LeConte) Demonstrates a Possible Mechanism of Resistance to Lethal dsRNA. PLos ONE. 11: e0157520.

Walawage, S.L., Britton, M.T., Leslie, C.A., Uratsu, S.L., Li, Y., Dandekar, A.M. (2013). Stacking resistance to crown gall and nematodes in walnut rootstocks. BMC Genomics. 14: 668.

Walshe, D.P., Lehane, S.M., Lehane, M.J., Haines, L.R. (2009). Prolonged gene knockdown in the tsetse fly *Glossina* by feeding double stranded RNA. Insect Molecular Biology. 18: 11–19.

Wang, M., Weiberg, A., Lin, F-M., Thomma, BPHJ., Huang, H-D., Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. Nat Plants. 2: 16151.

Weiss, M.J., Mayo, Z.B., Newton, J.P. (1983). Influence of irrigation practices on the spatial distribution of corn rootworm (Coleoptera: Chrysomelidae) eggs in the soil. Environ. Entomol. 12: 1293-1295.

Winston, W.M., Sutherlin, M., Wright, A.J., Feinberg, E.H., Hunter, C.P., (2007). *Caenorhabditis elegans* SID-2 is required for environmental RNA interference. Proceedings of the National Academy of Sciences of the United States of America. 104: 10565–10570.

Wittenberg, R., Cock, M.J.W. (2001). Invasive Alien Species: a Toolkit of Best Prevention and Management Practices. CAB International, Wallingford, UK, 225 pp.

Witwer, K.W., McAlexander, M.A., Queen, S.E., Adams, R.J. (2013). Real-time quantitative PCR and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of dietary miRNAs. RNA Biol. 10: 1080-1086.

Witwer, K.W., Hirschi, K.D. (2014). Transfer and functional consequences of dietary microRNAs in vertebrates. Bioessays, 36: 394-406.

Woodson, W.D., Smith, M.P., Fuller, B.W. (1999). Effect of insecticide treated soil on movement of third instar western corn rootworms (Coleoptera: Chrysomelidae). J. Kans. Entomol. Soc. 72: 99-103.

Wu, K. and Hoy, M.A. (2014) Clathrin heavy chain is important for viability, oviposition, embryogenesis and, possibly, systemic RNAi response in the predatorymite *Metaseiulus occidentalis*. PLoSOne. 9: e110874

Wu, K. Camargo, C., Fishilevich, E., Narva, K.E., Chen, X., Taylor, C.E., Siegfried, B.D. (2017). Distinct fitness costs associated with the knockdown of RNAi pathway genes in western corn rootworm adults. PLoS ONE. 12: e0190208.

Xu, H., Chen, T., Ma, X.F., Xue, J., Pan, P.L., Zhang, X.C., Cheng, J.A., Zhang, C.X. (2013). Genome-wide screening for components of small interfering RNA (siRNA) and micro-RNA (miRNA) pathways in the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae). Insect Mol. Biol. 22: 635-647.

Youngman, R.R., Day, E.R. (1993). Incidence of western corn rootworm beetles (Coleoptera: Chrysomelidae) on corn in Virginia from 1987 to 1992. J. Entomol. Sci. 28: 136–41.

Youssef, R.M., Kim, K.H., Haroon, S.A. and Matthews, B.F. (2013). Post-transcriptional gene silencing of the gene encoding aldolase from soybean cyst nematode by transformed soybean roots. Exp. Parasitol. 134: 266–274.

Zhao, Y.Y., Yang, G., Wang-Pruski, G., You, M.S. (2008). *Phyllotreta striolata* (Coleoptera: Chrysomelidae): arginine kinase cloning and RNAi-based pest control. European Journal of Entomology. 105: 815–822.

Zhou, X.G., Wheeler, M.M., Oi, F.M., Scharf, M.E. (2008). RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA. Insect Biochemistry and Molecular Biology. 38: 805–815.

Zhu, F., Cui, Y., Walsh, D.B., Lavine, L.C. (2014) Application of RNAi toward insecticide resistance management. In: Short Views on Insect Biochemistry and Molecular Biology (Chandrasekar, R., Tyagi, B.K. and Gui, Z.Z. RG.) Academic Publisher, 595–619.

Zotti, M.J., Smagghe, G. (2015). RNAi Technology for Insect Management and Protection of Beneficial Insects from Diseases: Lessons, Challenges and Risk Assessments. Neotrop. Entomol. 44: 197–213.

Zotti, M., dos Santos, E.A., Caligari, D., Christiaens, O., Taning, C.N.T., Smagghe, G. (2018). RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. Pest Manag. Sci. 74: 1239-1250.

Zubok, M. (2019). Generic Agrochemical Market 2017. AgriFutura <https://agribusinessintelligence.informa.com/resources/product-content/agri-futura-the-generic-agrochemical-market-2017>. Accessed on 19th of April, 2019.

Zukoff, S.N., Zukoff, A.L. (2017). Host Recognition Responses of Western (Family: Chrysomelidae) Corn Rootworm Larvae to RNA Interference and Bt Corn. Journal of Insect Science. 17(2): 59; 1-7.