 doi:10.20944/preprints202210.0034.v1

Review

Current scenario of Exogenously Induced RNAi for Lepidopteran Agricultural Pest Control: from dsRNA design to topical application

Vívian Lucena-Leandro [1], Emanuel Abreu [1], Leonardo Vidal [1,2], Caroline Torres [1,2], Camila Junqueira [1,2], Juliana Almeida [1], Érika Albuquerque [1,*]

[1] Embrapa Genetic Resources and Biotechnology, Brasília-DF 70770-917, Brazil;
[2] University of Brasília - UnB - Brasília, DF, 70910-9002, Brazil;
* Correspondence: erika.albuquerque@embrapa.br

Abstract: Global crop yields are estimated to be reduced by 30–40% per year on account of plant pests and pathogens. Agricultural insect pests raise concerns about constraining global food security and climate changes contributing to the rise of infestation. The current management relies on plant breeding, associated or not with transgenes and chemical pesticides. Both approaches face serious technology obsolescence on the field due to resistance breakdown or development of insecticide resistance. The need for new Modes of Action (MoA) approaches in managing crop health grows each year, driven by market demands to reduce economic losses and phytosanitary requirements to meet the consumer perception. Disabling pest genes by sequence-specific expression silencing is considered a promising tool in the development of environment and health respectful biopesticides. The specificity conferred by long dsRNA-base solutions give support to minimizing effects on off-targeted genes in the insect pest genome and the target gene in non-target organisms (NTOs). In this review, we summarize the current status of gene silencing by RNA interference (RNAi) for agricultural control. More specifically, we focus on the engineering, development and application of gene silencing to control Lepidoptera by the employment of non-transforming dsRNA technologies. Despite some delivery and stability drawbacks of topical applications, we reviewed works showing convincing proof-of-concept results that point to imminent innovative solutions. Considerations about the regulamentation of the ongoing research on dsRNA-based pesticides to produce commercialized products for exogenous application are discussed. Academic and industry initiatives reveal a worthy effort to accomplish controlling Lepidoptera pests with this new mode of action to provide more sustainable and reliable technologies to field management. New data on genomics of this taxon encourage the increment of a customized target genes portfolio. As a case of study, we illustrate how dsRNA and associated methodologies could be applied to control an important Lepidopteran coffee pest.

Keywords: insect; genome; biopesticide; silencing; topical; gene target; validation

1. Introduction

Insects play an important role in global crop loss, whether feeding on plants or acting as vectors of other diseases, or both [1]. It is estimated that insects are responsible for reducing world food production by 20%, resulting in losses valued at US$470 billion [2]. They also reduce food security at the household at the post-harvest level [3]. Adaptive interventions are required, otherwise this damage can increase within the climate change scenario [4].

Conventional pesticides are currently used to control insects related to crop threat. However, resistant populations are frequently reported to be selected by continuous exposure to a given molecule (e.g., chlorantraniliprole)[5]. This context prompted the research on alternative ways to mitigate the negative impacts of insects in crops without

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

using genetic transformation, which is favored by the increasing knowledge concerning plant-pest interactions. Pest management strategies that utilize novel Modes of Action (MoA), such as RNA interference (RNAi), are welcome to bypass pesticide resistance and avoid chemical pesticides [6].

The currently called RNAi technique evolved from the report of expression disruption of two target genes in the nematode Caenorhabditis elegans after injections of antisense RNA molecules [7]. The RNAi pathway has been reported as highly conserved in almost all eukaryotes [8] and described as a fine-tuned form of gene regulation [9,10] and defensive barrier [11–13]. Additionally, RNA-silencing contribute to suppression of transposable elements [14], DNA elimination [15], heterochromatin formation [16], and posttranscriptional repression of cellular genes [17].

In insects, the double-stranded RNA (dsRNA) mediated gene silencing technique was initially used in the study Drosophila melanogaster functional genomics [18]. As other insects were being studied and, consequently, the mechanism of gene silencing via RNAi was being unveiled, it was clear that RNAi efficiency is variable among different families. Furthermore, it depends on the insect's ability to trigger the gene silencing machinery, including steps like cellular uptake, dsRNA degradation, inter- and intracellular transports and processing of dsRNA to short/small interfering RNA (siRNA) [19].

At first, *D. melanogaster* was used as a model [18]. Afterwards, several insects were used to test RNAi efficiency to silencing essential genes by RNAi. Gene silencing is a promising technology able to contribute to the control of several insects of agronomic interest, especially in its topic version, also called spray-induced gene silencing (SIGS) [20].

Compared to conventional pesticides, the SIGS approach presents the advantage of high specificity towards the target organism and fast environmental degradation into innocuous compounds [21,22]. Therefore, this novel pest management procedure has the potential to reduce the employing of conventional insecticides without the regulatory restrictions inherent to Genetically Modified Organisms (GMOs), whereas dsRNAs can be delivered via topical methods while still maintaining target specificity [23].

One major concern is that the development of a biopesticide containing the SIGS technology starts with basic studies covering the genome of the target insect. The more sequences of the target insect are available, the better is the verification of genes belonging to the RNAi machinery and also the identification of essential genes to impair by silencing.

Preliminary tests in greenhouses and final field trials require hard work in formulation and scaling of dsRNAs for exogenous application. The first successful report of SIGS against insects for commercial use, Ledprona, has reported results in advanced Technology Readiness Levels (TRL) to control the Colorado Potato Beetle (CPB, *Leptinotarsa decemlineata*). Ledprona is now pending registration [24].

2. dsRNA-mediated silencing by the siRNA pathway in insects

The RNAi gene silencing pathways are highly conserved in insects and operate basically through three distinct ways, according to the kind of small RNA responsible for triggering the silencing effect: siRNAs, mediating the siRNA pathway for endo/exogenous dsRNAs; microRNAs (miRNAs), mediating the miRNA pathway [19]; P-element induced wimpy testis (Piwi)-interacting RNAs (piRNAs), mediating the piRNA pathway [25,26].

In this paper, we highlight the siRNA pathway for exogenous double-stranded RNA (dsRNA) taking into account that it may represent the next phase of species-specific pest management [27].

The first step for triggering the dsRNA-mediated RNAi pathway is the uptake of dsRNA molecules from the external environment, which depends on transmembrane channel-mediated and/or an endocytosis-mediated mechanisms [28], followed by interaction of dsRNA with degradation machinery located inside the cells. In terms of ability to spread the dsRNA signal, two types of events are described: the cell-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

autonomous, in which a single cell undergoes the effect of dsRNA presence and the non-cell-autonomous route, in which the interfering effects travel towards tissues/cells/organs distinct from that were the dsRNA molecules were applied or produced. The non-cell-autonomous is desirable for the development of new MoA assets, RNAi based, to pest control. For deeper details on dsRNA uptake in several species and the progress of this knowledge over time, we recommend the following reviews [29,30].

Several proteins implicated in dsRNA uptake have been studied and described. In the model organism *C. elegans*, four proteins belonging to the *Sid* family (SID-1, SID-2, SID-3 and SID-5) were related to dsRNA uptake efficiency (reviewed by[30]). SID-1 is a channel protein that binds to dsRNA molecules required for systemic interfering [31]. SID-1 orthologs are reported in lepidopteran pests [20,32]. Another class of proteins connected with dsRNA uptake from the insect gut clathrin, which promotes clathrin-dependent endocytosis [33]. In *Acyrthosiphon pisum*, the pea aphid, genes involved in the clathrin-dependent pathway are induced 12 h after feeding dsRNA [34]. Pattern recognition receptors (PRRs) interacts with membrane receptors having an important mission on dsRNA uptake by endocytosis pathway [35,36]. PRRs in the lepidopteran pests *Helicoverpa armigera*, *Plutella xylostella*, and *Spodoptera exigua* were revised [37].

2.1. dsRNA-mediated silencing machinery

Upon uptake of dsRNA by the target insect, in general, the first step for intracellular siRNA- the mediated pathway is the cleavage of the exogenous dsRNA by a specific endonuclease from the *Dcr2* family [19]. The fragments generated by *DCR2* are used as a template by *R2D2*, an RNA polymerase RNA dependent, leading to amplification of the stimulus of silencing. Next, the siRNA molecules produced by the complex DCR2/R2D2 guide the activity of an RNA-induced silencing complex (RISC) formed by Argonaute 2 (AGO2) and its associated proteins which together are responsible for the homologous RNA degradation [38]. As a consequence, the next requirement for a successful RNAi-based strategy is the presence of working machinery in the target insect because there is a positive co-relationship between RNAi efficiency and the pattern of expression of core RNAi pathway genes.

Insects present different levels of susceptibility to dsRNA. Some orders like Coleoptera perform strongly while others, including Lepidoptera and Diptera, exhibit highly variable outcomes in response to dsRNA treatment thus its applications in entomology and agriculture are currently limited [39]. Once *DCR2*, *R2D2* and AGO2 are considered the core elements of the siRNA pathway they have been chosen to elucidate this mechanism in some species.

In the case of fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), the basic transcription rate of the core elements of RNAi machinery presents similar levels to those registered to western corn rootworm (*Diabrotica virgifera virgifera*) Coleoptera and southern green stink bug (*Nezara viridula*) Hemiptera [40]). All of them are high RNAi efficient species [41–43] although represent phylogenetic orders whose response to dsRNAi varies. The impact of dsRNA exposition via injection or feeding in modulation of core genes, remains unclear. This kind of study was made with European Corn Borer (ECB), *Ostrinia nubilalis* (Hübner), a low RNAi efficient species [6]. A recent report revealed one transcript for each of three core RNAi pathway genes *Dcr2*, *R2D2*, and *Ago2* here denominated *OnDcr2*, *OnR2D2*, and *OnAgo2*. Expression levels of these genes were addressed after dsRNA injection or by feeding of dsRNA in an artificial diet assay. Gene expression of the core RNAi machinery was steady during injection assay while only *OnDcr2* was upregulated in the feeding one. Besides the presence of the core genes and its expression levels, the analysis of domains within the predicted protein sequences may provide functional information concerning protein performance of *OnAgo2*, *OnR2D2*, and OnDcr2 and in this case there are conformational differences that could justify the low efficiency of RNAi apparatus in ECB [6].

2.2. dsRNA designing

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

The employment of RNAi for pest control purposes was improved with the advances in high-throughput sequencing (genomic and transcriptomic) and bioinformatic tools, allowing reliable information about patterns of insect gene expression [44]. The mastering of this information provided the identification of essential genes of the target insect. For dsRNA designing and its use with commercial motivation, the first step is to develop a preliminary pipeline through software settings for each specific trait vs. organism analyzed [45–48]. After this, select functional siRNA sequence with minimum off-target effects, eliminating near-perfect match is essential [49]. Carefully designed dsRNA makes it possible to obtain broad-spectrum or extremely specific molecules to the same gene in different insect species or even within species of the same genus [50]. Hence, for the choice of a promising target gene for pest control, the level of homology between dsRNA and target is critical.

The precaution of exclusion of cross-kingdom sRNAs (ck-sRNAs) is important to avoid undesirable effects on non-target species once those sequences are shared by several organisms and could harm beneficial insects and ecological balance of populations. For this purpose, when preparing samples for the sequencing step, it is necessary to avoid contamination with other organisms (plant cells, endogenous microbiota, parasites)[51].

Furthermore, it is required to determine the number of biological replicates and necessary number of reads to achieve sufficient genome coverage (typically three biological replicates with 5–10 million reads each). Sequencing can be carried out using a wide variety of high-throughput technologies [48,52]. The most used alternatives to sequencing include the Next Generation Sequencing (NGS) technologies such as Illumina systems (MiSeq and HighSeq), PacBio and Roche 454 sequencing depending on the output range and total reads per run required [53]. FastQC is the most frequently used program to realize a quality control analysis [52]. Those NGS technologies also allow direct sequencing of cDNA produced from messenger RNA (RNA-seq) enabling the de novo construction of the transcriptome without an anchoring genome [54,55].

Illumina's RNA-seq and digital gene expression tag profile (DGE-tag) was used to screen optimal RNAi targets from Asian corn borer (ACB) (Ostrinia furnalalis). Larval-stage specific expression genes were selected for RNAi testing by spraying dsRNA on larvae, reaching mortalities of 73% to 100% at 5 day after treatment [56]. The combination of DGE-tag with RNA-seq is a rapid way to select candidate target genes for RNAi [57].

siRNA-mediated silencing requires high pairing between the target RNA and siRNA. The siRNA length homology must be observed for more successful RNAi silencing, considering lepidopteran siRNA populations 20 nt long were observed in some species [58]. BLAST search is an important tool, but not so accurate for short sequences like siRNAs. Other software to select functional siRNAs like PFRED [59] and siRNA-Finder (si-Fi)[60] are available.

2.3. Molecular data on Lepidopteran pests to the development of RNAi control

The order Lepidoptera comprises butterflies and moths whose life cycle comprise: egg, 1st to 4th instar larvae, pupa and sexually dimorphic adult. In this way, its development is determined as a holometabolous [61]. The egg and pupa stages last about five days, while the larval stages take about 12 days, considering a temperature of 25°C. [62]. It is important to note that lepidopterans have their cycle affected by temperature, relative humidity and precipitation. During the dry season the attack is usually more severe. [63,64]. According to [65], 157,424 species of lepidopterans are recognized, being the second most diverse order, and including the most devastating agricultural pests in the world [66].

Fully sequenced insect pest genomes endorse the assertivity of gene target selection to dsRNA designing [44]. According to the NCBI, the Insecta class (**Figure 2a**) has 3,091 deposited genomes. From this data, 1,831 are reference genomes, while 220 were annotated using the NCBI RefSeq. For the Lepidoptera order (**Figure 2b**) we found 1,540 total genomes, 836 of which were reference genomes and 34 Annotated by NCBI RefSeq.

 doi:10.20944/preprints202210.0034.v1

The superfamily Yponomeutidae (**Figure 1c**) contains 16 deposited genomes, with four reference genomes and only one genome annotated by NCBI RefSeq, at the Plutellidae family (**Figure 1d**) containing the well-studied *Plutella xylostella* that has genome assembly at the chromosome level. However, other families lack genomic data, such as the cosmopolitan Lyonetiidae family (**Figure 1E**), which has about 200-210 species described.

The Lyonetiids encompasses important pests which are usually leaf and branch mining and parasitize dicotyledons [67], causing wilting and defoliation to: fruit bushes, as apple and pear plants by *L. malifoliella* in Europe and Asia [68]; ornamental plants, such as *L. albella* [69], *L. laburnella*, *L. lathyrifoliella* and *L. orobi* [70,71]; medicinal plants by *L. lustratella* [72]; trees, like willow (*Salix spp*.) and poplar (*Populus spp*.), by *L. sinuella* [73]; shrubs, as *Cytisus scoparius*, attacked by *L. spartifoliella* in European and Australian swamps and forests [74]. In the *Phyllobrostis* genus, there are 12 recognized species that occur in Europe, Middle East and southern Africa, whose larvae feed on leaves forming mines and can be borers on the branches of ornamental plants of the Thymelaeaceae family [75]. The genus *Lyonetia* has the leafminer species *L. clerkella* that attacks peach, apricot, cherry, apple, pear and hawthorn trees that occur in China, South Korea and Japan. It causes severe defoliation and directly impacts productivity [76]. *L. prunifoliella* has a habit of mining apple tree leaves [77]. *L. pulverulentella* feeds on willow leaves, causing mines in Russia, Ukraine, Norway and Italy [78]. The last species of the genus according to the NCBI is *L. saliciella*, and no biological or genomic information was found for this species.

Another widespread leafminer in the Neotropical region is *L. coffeella* (Guérin-Méneville) or Coffee Leaf Miner (CLM)[79]. Opposite to other harmful pests, it feeds exclusively on plants of the genus *Coffea spp*. The leaf damage caused by the CLM attack can cause productivity losses estimated at 87% and defoliation of up to 75%, depending on the season [80,81]. Recently obtained sequences of *L. coffeella* generated large genome, transcriptome and proteome information at the molecular level [82], contributing to the development of RNAi research in Lepidoptera by better understanding of the RNAi machinery and selecting highly specific gene targets.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

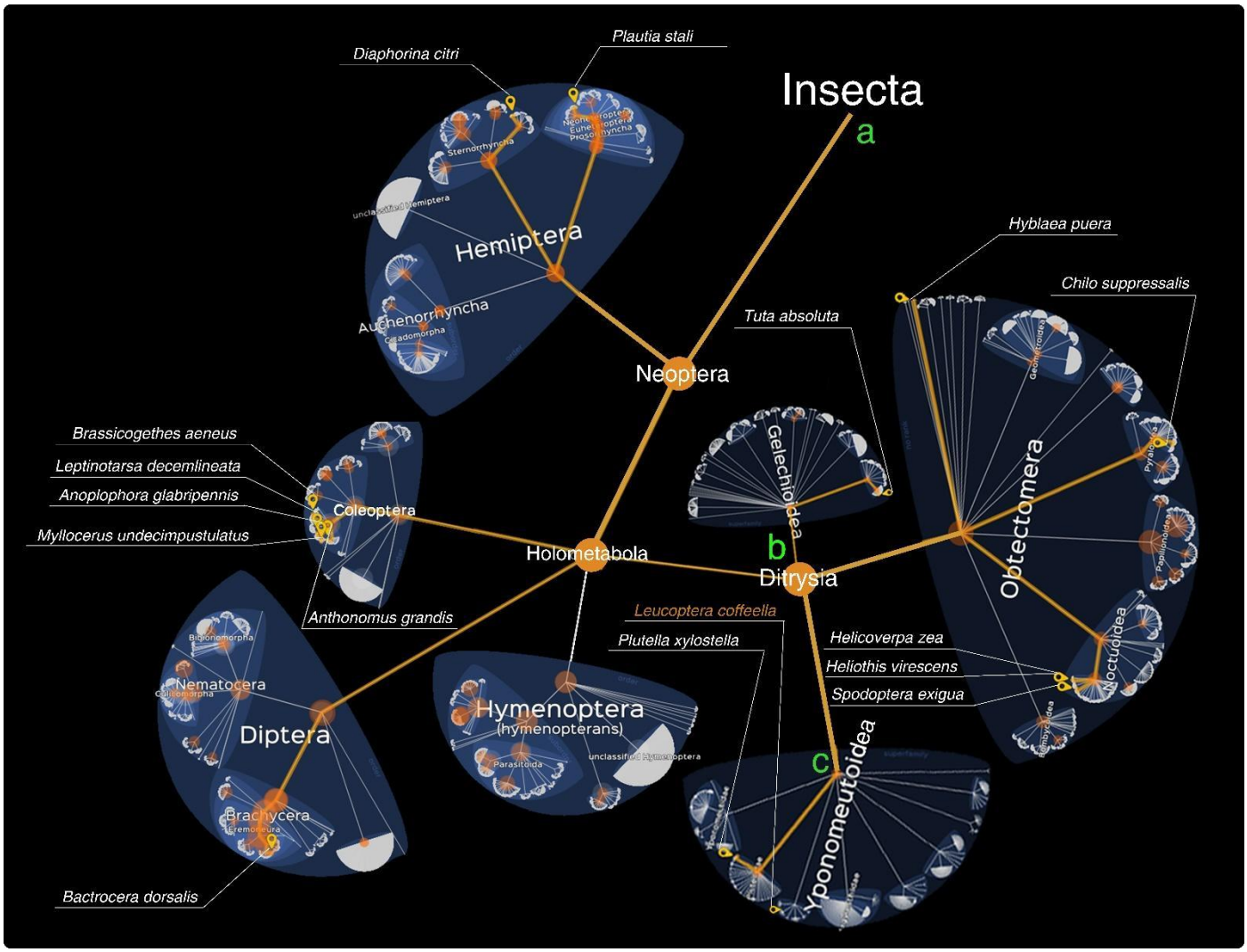

Figure 1. Main taxonomic levels are depicted in a tree (modified from https://lifemap-ncbi.univ-lyon1.fr/#) showing in the Insecta class (a), the Ditrysia clade (b) and the Yponomeutoidae (c) families. Orange spheres highlight the levels with fully sequenced genomes data. Insect species listed in tables 1 and 2 (written in white) are marked by a yellow tag. The *L. coffeella* in the Lyonetiidae family is written in orange.

2.4. Insect target genes for dsRNA silencing

In an attempt to reduce or eliminate the use of chemical pesticides harmful to health and the environment, RNAi has been increasingly developed and tested in agricultural pests. In **Table 1**, we show some validated genes that are not used *in planta* silencing (non-GMOs) . Insect gene silencing papers reported before 2017 and some related to pests occurring in the Neotropical Region were compiled and reviewed elsewhere [50,83].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

Table 1. dsRNA validated genes for insects silencing by non-transformative methods

Year	Species (Order)	NCBI:txid	Target	Delivery	Mortality	Reference
2017	*Anthonomus grandis* (Coleoptera)	7044	*Agra*CHS2	microinjection	100%	[84]
2019	*Bactrocera dorsalis* (Diptera)	27457	*Tssk1* *Tektin1*	artificial diet	58,99% 64,49%	[85]
2019	*Myllocerus undecimpustulatus undatus* (Coleoptera)	1811735	*Prosα2* *RPS13* *Snf7* *V-ATPase A*	injection and feeding	78,60% 64,10% 92,70% 43,10%	[86]
2020	*Anoplophora glabripennis* (Coleoptera)	217634	*IAP* *SNF7* *SSK*	artificial diet	90% 75% 80%	[87]
2021	*Diaphorina citri* (Hemiptera)	121845	DcCP64	soaking	72%	[88]
2021	*Leptinotarsa decemlineata* (Coleoptera)	7539	Ledprona	leaf soaking	50-90%	[89]
2021	*Plautia stali* (Hemiptera)	106108	vATPase IAP MCO2 Snf7	injection and feeding	100%	[90]
2022	*Brassicogethes aeneus* (Coleoptera)	1431903	*αCOP*	leaf soaking	62%	[91]

The *Agra*CHS2 gene in the coleoptera *Anthonomus grandis* (cotton boll weevil) by microinjection method in the insect, resulting in 100% adult mortality. This gene is essential for PM biosynthesis, intestinal epithelium and for nutrient assimilation [84]. The *Tssk1* and *Tekin1* genes (important for male fertility) were silenced, causing the death of 58-64% of *Bactrocera dorsalis* diptera individuals by artificial diet [85].

In the Sri Lanka Weevil (*Myllocerus undecimpustulatus undatus*, Coleoptera) silencing was validated through injection and feeding the genes *Prosα2* (proteasome subunit alpha type 2) [92], RPS13 (structural element of the 40S subunit) [93], Snf7 (endosomal sorting complex required for transport III- ESCRT-III) [94] and *V-ATPase A* (transmembrane ATP-driven proton pump) [95]. Mortality rate ranged from 43.1% to 92.7%.

Anoplophora glabripennis (Coleoptera: Cerambycidae) is the Asian long-horned beetle (ALB). Considered a serious invasive forest pest in several countries, it is a polyphagous wood-boring species of native wood. Bioassays of feeding larvae with dsRNA resulted in the death of 75-90% of the individuals, upon silencing the inhibitor of apoptosis (*IAP*), *SNF7* and snake skin (*SSK*) genes [87].

Diaphorina citri (Asian citrus psyllid-ACP) is a vector that transmits *Candidatus Liberibacter asiaticus* (CLas), which causes Citrus Huanglongbing disease (HLB). This hemipteran was tested with dsRNA to silence the *DcCP64* gene, responsible for the synthesis of the 64-like cuticle protein. The silencing caused 72% of the psyllid's death [88].

The hemipteran *Plautia stali* (brown-winged green stink bug) was submitted to tests based on RNAi, using the genes: *vATPase*, *IAP*, *MCO2* and *Snf7*, where 100% of the insects

 doi:10.20944/preprints202210.0034.v1

died. The pest is known for infesting various fruits and crop plants. (https://doi.org/10.1371/journal.pone.0245081).

The pollen beetle (*Brassicogethes aeneus*) is an important pest of *Brassica napus*, an oilseed rape. Silencing the αCOP, a subunit of coatomer protein complex-I (COPI) gene, resulted in a 72% mortality rate to this Coleoptera (https://doi.org/10.1038/s42003-021-01975-9).

The Colorado potato beetle *(L. decemlineata*) was subjected to PSMB5 gene silencing as part of the ubiquitin/proteasome machinery. The study ended up generating an RNA-Based Biopesticide, the Ledprona, which is being reviewed for registration at the United States Environmental Protection Agency (EPA). The mortality rate ranged from 50-90% of the individuals tested [24].

2.4.1 dsRNA silencing in Lepidoptera

The order Lepidoptera contains several highly destructive representatives that generally show low mortality rates when subjected to RNAi-based tests [96]. Despite the limitations of the gene silencing effect in lepidopterans, potential solutions may still exist, as there are possibilities that have not yet been tested [97]. In **Table 2,** we listed works published between 2018-2022 referring to the validation of lepidopteran pests genes silenced by exogenous application.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

Table 2. Lepidopteran target genes validated for dsRNA silencing - the position of the taxonomic representation depicted in figure 2.

Year	Species (Lepidoptera order)	NCBI: txid	Target	Delivery	Mortality	Reference
2018	*Helicoverpa zea* (Noctuidae)	7113	*TipE* *GluCl* *Para* *Notch*	microinjection	12-16% 12-16% 12-16% 12-16%	[98]
2018	*Plutella xylostella* (Plutellidae)	51655	*AChE*	topical foliar application	69-74%	[99]
2018	*Heliothis virescens* (Noctuidae) *Helicoverpa zea* (Noctuidae)	7102 7113	*PBAN*	topical foliar application and Injection	50-60% 50-60%	[100]
2021	*Hyblaea puera* (Hyblaeidae)	268502	*HpEcR* *HpCHS1* *HpChi-h*	topical foliar application	46% 30% 32%	[101]
2021	*Spodoptera exigua* (Noctuidae)	7107	GNAF	feeding	48%	[102]
2021	*Chilo suppressalis* (Crambidae)	168631	*ND* *GPDH* *MSL3*	topical foliar application	50% 50% 50%	[103]
2021	*Tuta absoluta* (Gelechiidae)	702717	*v-ATPase B* *JHBP*	topical foliar application	70% 70%	[104]

The *AChE* gene was silenced by soaking in the lepidopteran *P. xylostella*, one of the main pests of crucifers. This gene is responsible for the synthesis of acetylcholine esterase, which interrupts the action of neurotransmitters, being the primary target of commercial insecticides. In order to increase stability, they tested a concatemerized form of the molecule. The mortality rate was up to 72%, higher than that observed with the non-concatemerized control. [99].

The lepidopteran *Helicoverpa zea* (corn caterpillar) was subjected to tests involving RNAi using the genes that are targets of commercial insecticides: *Para* (paralytic effect), *TipE* (temperature-induced paralysis), *GluCl* (glutamate chloride channel) and *Notch* (encodes proteins that make up neuronal cells). Three delivery methods were used: microinjection, egg immersion and larval feeding. Microinjection of eggs of the *GluCl*, *Para* and *TipE* genes reduced hatching rates, while the *Notch* gene showed no difference. None of the genes was effective for larval feeding and egg immersion methods [98].

The most aggressive tomato pest in South America, Africa and Asia is *Tuta absoluta*. This lepidopteran that feeds on the mesophyll was subjected to tests with RNAi, whose genes: *v-ATPase B* (keep the midgut lumen alkaline by increasing amino acid absorption) and *JHBP* (essential for development and reproductive maturation). Topical application on the leaf surface resulted in 70% of larval mortality for the two target genes [104].

The rice stem borer is one of the main crop pests in the world. As chemical control is expensive, the RNAi technique became an option to test three selected genes: *ND* (NADH

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

dehydrogenase), *GPDH* (glycerol 3-phosphate dehydrogenase) and *MSL3* (male specific lethal 3). Rice leaves were brushed with bacterial dsRNA solution that contained newly hatched *Chilo supressalis* larvae. The insect mortality rate was 50% for each gene. [103]).

Corn caterpillar (*H. zea*) and tobacco caterpillar (*Heliothis virescens*) were subjected to control tests containing dsRNA of the *PBAN* target gene. DsRNA delivery by artificial larval diet or pupae injection caused a mortality rate that ranged from 30 to 60%. It was also observed a delay in larval development and some interference in the development of pupae in the two agricultural pests analyzed[100].

The teak (*Tectona grandis*) is of paramount importance in forestry directed to the wood commercialization. The pest *Hyblaea puera* causes severe defoliation, having a great impact on the tree development. Sequences from the *HpCHS1*, *HpChi-h* and *HpEcR* genes, related to chitin metabolism, were used in the construction of dsRNAs that were offered to the larvae by topical application on the leaves. It was found that 30-46% of the treated larvae died, in addition to the deformed pupae [101].

Assuming that gene silencing can occur in Lepidoptera species, we believe that *L. coffeella* can be also controlled by exogenous dsRNA technologies. Aiming to develop biopesticide solutions to control the CLM, our research group has performed the full genome PACBio and paired-end Illumina combined DNA sequencing from pupae samples. The generated data allowed nuclear genome, transcriptome and proteome analyzes as a basis for the discovery of RNAi mechanisms particular to the Lyonetiidae family, as well as the selection of target genes. This information is essential to the RNAi development, as the closest complete genome is at a large distance in the Yponomeutoidea taxon [82].

3. dsRNA candidate validation to Proof-of-concept

After synthesizing the dsRNA molecules in the laboratory, validation in a controlled rearing environment is required before field application. According to our literature searches, delivery can be made directly to the insect or indirectly by application to the plant for subsequent ingestion of the insect.

Microinjection can be a dsRNA carrier vehicle for preliminary laboratory testing. Tests performed with cotton boll weevil (*Anthonomus grandis*) showed a 93% reduction in oviposition and death of 100% of adults whose larvae (1 mm in length) were microinjected for silencing the *Agra*CHS2 gene [84]. Bioassays performed with *H. zea* (corn earworm) based on the egg microinjection technique (1 mm in diameter) resulted in 12-16% mortality of larvae for genes: *Para* (paralyticts), *TipE* (temperature-induced paralysis locus E), *GluCl* (glutamate-gated chloride channel), and *Notch* [98]. However, microinjection of dsRNA may be limited to small-sized individuals and leaf miners distubed by the larvae removal from the mines like the microlepidoptera *L. coffeella*. Preliminary tests revealed the death of the larvae after removing them from their mines.

Immature stages can also be tested by i) detached leaves: the beet armyworm (*S. exigua*) received dsRNA (**Table 2**) through *in vivo* feeding, where the leaves of *Galanthus nivalis* were treated with the solution, and then offered to the larvae. The mortality obtained was 48% [102], and ii) artificial diet: oral feeding bioassays of *N. viridula* nymphs with artificial diet were performed, containing dsRNA for the genes: *vATPase A*, *αCop*, *40SrpS13*, *60SrpL19* and *PP1-beta*. Treatments for the *vATPase A* and *αCop* genes resulted in 43% and 45% mortality after 14 days of treatment, respectively. There was a 57% and 60% reduction in the weight of the nymphs that survived for the *vATPase A* and *αCop* genes, respectively. The other genes *40SrpS13*, *60SrpL19* and *PP1-beta* caused mortality of 12.5%, 17.5% and 25%, respectively [105].

The soaking method can be performed to validate the efficiency of RNAi in the laboratory and in the greenhouse. According to https://doi.org/10.1002/ps.4549, dsRNA was produced and diluted in RNAse free water at various concentrations. In the laboratory, the nymphs of *D. citri* (Asian Citrus Psyllid) were immersed for 5 minutes and after this period they were removed. After receiving the treatment, the nymphs were

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

placed on filter paper for drying and transferred to "Valencia" orange leaves, kept in a greenhouse. Mortality was 68% after the eighth day of treatment, silencing the *DcMP20* gene (muscle protein 20). Soaking was also efficient in *D. citri*, reaching 72%, silencing the *DcCP64* gene [88].

3.1. dsRNA-Induced Suppression insect treatment

Microinjection can be a dsRNA carrier vehicle for preliminary laboratory testing. Tests performed with cotton boll weevil (*Anthonomus grandis*) showed a 93% reduction in oviposition and death of 100% of adults whose larvae (1 mm in length) were microinjected for gene *Agra*CHS2 (https://doi.org/10.1016/j.biori.2017.04.001). Bioassays performed with corn earworm based on the egg microinjection technique (1 mm in diameter) resulted in 12-16% mortality of larvae for genes: *Para* (paralyticts), *TipE* (temperature-induced paralysis locus E), *GluCl* (glutamate-gated chloride channel), and Notch [98].

The specific dsRNA for seven genes: *heros*, *PP1-beta*, *26Sprs6B*, *vATPase A*, *40SrpS13*, *60SrpL19*, *αCop* and *USP* from *N. viridula* (southern green stink bug) were microinjected into the nymphs and then the insects were placed on bean leaves and evaluated daily by 14 days. The average mortality rate for the genes was 90%, while for the negative controls of the treatments it was less than 20% [105]. Another example for *N. viridula* using the *Actin* target gene microinjected into nymphs, resulted in close to 100% mortality of nymphs, in two independent experiments with only 9 days of treatment [106].

This method of microinjection of dsRNA is very promising for insects, however, for *L. coffeella* it can be quite limited, due to its small size and the difficulty of proving that the mortality was caused by the dsRNA and not by the fact that the larvae were removed of the mines. Preliminary tests revealed the death of the larvae after removing them from their mines. Perhaps the pupal stage can be tested.

3.2. dsRNA-Induced Suppression plant treatment

An interesting study provided important insights on how to exogenously apply dsRNA to plants. The exogenous treatment to suppress the neomycin phosphotransferase II (*NPTII*) gene in *Arabidopsis thaliana* [107] was essayed under different physiological conditions (plant age, time of day, soil moisture, high salinity, heat, and cold stresses) and varied application media (brush spreading, spraying, infiltration, inoculation, needle injection, and pipetting). Better results followed the application at night. Low moisture soil presented better results than soggy soil. Among the application methods, the brush spreading, spraying and pipetting treatments showed greater suppression of *NPTII*.

The infiltration procedure is performed by syringe without needle, pressing the solution with dsRNA under the abaxial face of the leaf. It may be a viable method to validate the dsRNA in plants, as it is possible to visualize the entry of the solution in the leaf. Some time later, the liquid diffuses inside the plant tissues and is no longer visible. In sunflower, the infiltration process is carried out [108]. Even if it is an easy method, it is quite variable in effectiveness, depending on the plant anatomy.

4. Formulation of dsRNA-based products with nanocarriers

Once the target genes to impair the insect proliferation are chosen and preliminary assays are realized in laboratory and greenhouse conditions, other constraints inherent to dsRNAi approach should be addressed. The stability of dsRNA molecules after field application and in the insect gut conditions is crucial to extend the material half-life length. It is known that to control Lepidopteran pests via dsRNA-based products, avoiding dsRNA degradation by the RNases and the high pH rates is a *sine qua non* situation [109]. The fate of dsRNA is fast both in soil and aquatic environments [21,110]. Microbial nucleases present in the soil and on leaves, UV-radiation, and run-off due to dew and rain can significantly limit the availability of dsRNA to the pest [110,111].

However, most of those problems have been overcome gradually with the improvement and recent findings in chemistry and nanotechnology. Thus, dsRNA formulations containing protection wetting and agents, surfactants and diluents may be required [112]. Many nanomaterials have been used to form complexes with dsRNA, as

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

presented below, especially the cationic ones that present positive extremities, able to bond with the negative parts of dsRNA molecules.

4.1. Peptides

The peptide transduction domain (PTD) has been fused to dsRNA binding domain molecules, allowing a more efficient internalization by the cell and, consequently, enhancing the gene silencing at *Anthonomus grandis*. Although the PTD-DRBD complex, turned the dsRNA more stable and efficient in the presence of nucleases in the *A. grandis* gut [113], it is harder to be synthesized. An alternative is using branched amphiphilic peptide capsules (BAPCs) that are more easily synthesized than PTD to protect dsRNAs [114]. These nano capsules are made of natural amino acids, water soluble, resistant to detergents, proteases, and chaotropic agents. The association of BAPCs to dsRNA led to premature deaths of pea aphid (*Acyrthosiphon pisum*) when fed with a diet containing BAPCs-dsRNA complex [115].

4.2. Lipofectamine

Lipofectamine 2000 (Invitrogen) is a transfection reagent, able to inhibit the nuclease activity, and characterized by the presence of cations at the phospholipid bilayer. These characteristics allow Lipofectamine to overcome the repulsion relation between cell membranes and nucleic acids, becoming a good candidate to be added to dsRNA formulations [114]. When used to treat *Euschistus heros*, lipofectamine was able to increase the mortality the second-instar nymphs by 15% after 14 days of artificial feeding, in comparison with the naked dsRNA molecules that caused 33% of mortality [116].

Another transfection reagent, Cellfactin II (CFII), was tested complexed with dsRNA against *S. frugiperda*. One group of larvae was fed with CFII-dsRNA complex on the diet, and the other group received naked dsRNA. The diet without CFII caused 25% of mortality, while the group that received CFII-dsRNA registered 55%. These results indicate that CFII was able to protect dsRNA molecules from the high pH and the nucleases of the hemolymph and midgut lumen, increasing dsRNA final efficiency [117].

4.3. Macromolecular polymers

Star polycation (SPc) is a cationic amino acid dendrimer nanocarrier that is able to enhance gene transfection efficiency. Its dsRNA association capability, due to its positive charge, allowed RNAi silencing of the CYP6CY3 gene up to 84.3% of mortality in *Aphis gossypii* [118]. SPc was also used in association with dsRNA to verify its capability in the control of *Myzus persicae* [119]. To avoid water repellency a 0,1% detergent solution was added to the formulation. The penetration efficiency at the aphid, measured by fluorescence tests, showed that with SPc, dsRNA molecules were able to get to the whole aphid's body. The *vestigial* (vg) and *Ultrabithorax* (Ubx) genes were downregulated in 44.0% and 36.5%, respectively, 24h after treatment.

SPc polymerized with DMAEMA (2-N-(dimethyl aminoethyl) methacrylate) was made in association with dsRNA to control *Chilo suppressalis* by artificial feeding. The SPc protection showed an increase of 60% to the larvae mortality [120]. To *S. frugiperda*, the SPc complexed with dsRNA was tested in Sf9 cells, hemolymph and midgut lumen contents collected from *S. frugiperda* larvae. The performance of the complex and naked molecules was measured by UV tests at 488nm, showing that SPc was able to enhance dsRNA molecules' stability. After 12h of incubation, the UV signal was much stronger in cells that absorbed the complex dsRNA/SPc than in naked dsRNA, showing that SPc can promote dsRNA uptake by the cells. Also, SPc was able to protect dsRNA molecules from being degraded by RNAse A and the insect hemolymph [121].

Polymers containing guanidine were developed to turn the dsRNA molecules stable in very alkaline environments and to protect these molecules from nucleases. Since dsRNA molecules are very sensitive to high pH environments because of the hydrolyzation caused to double-stranded molecules [33], it is mandatory to prevent degradation in Lepidopterans' gut, that can reach pH 12 [122]. dsRNA can form complexes with the guanylate polymer due to its positive charge. In laboratory ex vivo

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

tests, a guanylate polymer was able to avoid dsRNA degradation for over 30 hours at pH11, while the naked molecules were degraded after 10 minutes at *S. exigua* gut juice. The *in vivo* bioassays have shown that the mortality associated to the dsRNA complexed with the guanylate polymer is around 53.3%, while the naked molecules caused 16.7% of mortality [123].

Chitin can be deacetylated and become a natural material called chitosan. This cationic nanopolymer is biodegradable and biocompatible molecules [124]. Chitosan nanoparticles (CNPs) were complexed to dsRNA molecules and sprayed over chickpea leaves to control *Helicoverpa armigera*. The complex CNPs/dsRNA showed a reduction in length and weight of the larvae when compared to naked-molecule silencing [125]. Chitosan was also able to improve the delivery in mosquito assays with *Anopheles gambiae* and *Aedes aegypti*. The mortality rate was also improved by the chitosan complex [126,127]. Chitosan (CS) can be further improved by other materials, such as sodium tripolyphosphate (STPP). STPP is a nanosized cross-linker able to increase the protection of the chitosan-dsRNA complex, forming a CS-STPP-dsRNA complex. When used against mosquitos *A. aegypti*, the CS-STPP-dsRNA complex has shown an increase of 60% in mortality when compared to naked dsRNA molecules [128].

4.4. Other Materials

Layered double hydroxide (LDH) clay nanosheets have a flat hexagonal positively charged structure that interact with dsRNA molecules, forming a complex named BioClay. This structure allows the dsRNA detection at leaves' surface even 30 days after application via foliar spray. Moreover, it also enabled a better permanence of the molecules over the leaves, avoiding removal by application of other products [129].

Carbon Quantum Dots (CQD) is another possible material to be used in association with dsRNA molecules. When associated with dsRNA molecules, CQD was able to improve gene depression in 41% in the gut, 45% in other tissues, and 43% in the whole organism, in comparison to naked dsRNA, which hasn't shown any reduction at expression levels. This reduction of the gene expression resulted in a 70% mortality rate 6 days after diet feeding *C. suppressalis*. The CQD also showed a very good stability result in comparison to chitosan, lipofectamine2000, and naked molecules of dsRNA when tested on midgut homogenates [130].

5. dsRNA production costs

The dsRNA production methods have been continuously optimized in recent years to adapt to production needs and promote the application of this technology. The main limiting factor for the use of dsRNA as a topical bioinsecticide is the need to produce dsRNA molecules on a large-scale at low cost. dsRNA can be produced using both *in vitro* transcription and *in vivo* expression in bacteria, yeast, microalgae and other species [131–135]. Production of a large amount of dsRNA is required for silencing the expression of a gene *in vivo*. The bacterial system is the most common between *in vivo* dsRNA expressing alternatives [136]. This production involves the efficient release of the dsRNA from the cells without affecting RNA integrity during the extraction process [136]. In experimental settings three main approaches are used to produce dsRNA: chemical synthesis of NTPs, *in vitro* synthesis through RNA-dependent RNA polymerases and fermentation through microorganisms. In 2008, the cost of dsRNA was approximately US$12,000/g, dropping to US$60/g in 2018 [137]. RNAGri had the ability to produce tons of dsRNA at a cost of US$1/g, Greenlight's GreenWorX™ system can further reduce the cost of dsRNA synthesis to < US$0.5/g [50,138–141]).

In the context, the *in vitro* synthesis strategy can produce high-purity dsRNA, but the cost is relatively high (Li et al 2021). On the other hand, *in vitro* chemical RNA production is best suited for the synthesis of short dsRNAs [142]. The *in vivo* synthesis strategy produces low-cost dsRNA in high yields, but this strategy requires later purification of the product and inactivation of the engineered microbial strain [143,144]. To long dsRNAs need to be synthesized biologically, the production these molecules using the bacterial

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

system can be effectively employed to produce a large amount of dsRNA at relatively low cost to enable the development of products based on RNAi technology for the control of Lepidoptera species and for other pests [42,145]. In shrimp production farms the *Yellow head virus* (YHV) is an important pest that causes highly lethal and contagious disease. Study using dsRNA at the dose of 25 μg per shrimp was injected into shrimp in order to knock down YHV replication and reduce mortality [146]. It should be noted that the cost of production of 30 mg dsRNA by the *in vivo* system was approximately one-third of the *in vitro* methodology. RNAi for pest control requires the development of effective delivery strategies of dsRNA. To overcome these barriers, great efforts have been spent on the development and optimization of biological production systems that have shown to be the most economically viable alternative.

5.1. Production of dsRNA in recombinant microorganisms

Systemic and large-scale delivery of dsRNA is imperative to restrict an insect pest and reduce the selection of resistant individuals [29]. The delivery of dsRNA using bacteria has many advantages in comparison with plant-mediated dsRNA delivery, other microorganisms, or *in vitro* synthesized dsRNA [35]. *E. coli* HT115 (DE3) were first used as a heterologous dsRNA production system by [147] and become since then a useful tool for functional studies in invertebrate physiology [131,136,148,149]. Still, other kind of bacteria, like *Bacillus thuringiensis* (Bt) [134,135,150], *B. subtilis* [151,152], symbiotic bacteria [153–157], *Pseudomonas syringae*, *Corynebacterium glutamicum*, and *Chlamydomonas reinhardtii* are effective for biopesticide production strains that have been widely used for control of several pests. The effectiveness at inducing knockdowns in insects and other animals through feeding with bacteria expressing dsRNA has been demonstrated [136]. HT115 (DE3) bacteria transformed with L4440 vector expressing dsRNA of five different target genes, were orally supplied to Colorado potato beetle (*L. decemlineata*) to induce RNAi and caused significant mortality at larval phase 12 days after feeding [158]. An immune gene of the noctuid moth *S. littoralis* was silenced after feeding with bacteria expressing a homologous dsRNA [134]. In other lepidopteran insects, such as *S. exigua* [159,160], *C. infuscatellus* [57] and *Tuta absoluta* [161]. RNAi effects were observed. In Lepidoptera order, delivery of dsRNA to hemocoel needs high amounts of dsRNA to achieve significant knockdowns. In field conditions, the quantity required is even higher [42,162].

Bacteria can also be used as an alternative to produce large amounts of dsRNA at low cost [163]. In addition, eukaryotic species *Saccharomyces cerevisiae* has also been used as a relevant dsRNA production system. *S. cerevisiae* does not contain the core genes Dicer2 and Argonaute-2 of the RNAi pathway [132], which allows efficient dsRNA synthesis in *S. cerevisiae* compared with *E. coli* and other bacterial species [164]. Fungi [133] and viruses [165,166] have also been engineered to produce dsRNA.

6. Field application of dsRNA assets

dsRNA is a great resource to become a powerful tool for pest control of field crops. To become a true and useful product in the market, the foliar spray is one of the most efficient delivery methods because, with time, it became a low-cost production way and a very practical and convenient application method [167]. Trunk injection is another application method that can be especially useful to be applied in the field, in particular for those crops that are perennial plants and those that present woody trunks, such as citrus, coffee, and pine, among others [50].

As well, dsRNA is a great resource, it is a sensible molecule, making its direct application face a few difficulties. For both application methods, foliar spray and trunk injection, dsRNA molecules require some kind of protection or association with other compounds to be used as an active principle to any product, to avoid being degraded before it gets to its final destination and allow the molecule to reach its maximum potential [139].

6.1. Foliar Spray

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

To become useful in the field, dsRNA must present at least two characteristics, be cheap and efficient. The application via foliar spray has been demonstrated to be this solution, the price of synthesis for this application method costs around 0,5 and 1 USD per gram on a cell-free bioprocessing platform [139]. To be efficient, at this cost, it will be needed to apply between 2 and 10 grams per hectare [137,168,169]. Considering these facts, the easiest way to turn it into an applicable product will be to apply it via foliar spray [139].

The convenience of this method to its field usability due to its practicality to be a method already present in most productive areas allowed a few companies to start the development of commercial products that are starting to be commercialized to the farmers.

The most advanced commercial product to be launched is a foliar spray, dsRNA based, Ledprona®. Its active ingredient is a dsRNA artificially synthesized and applied via foliar spray. It has been demonstrated to be a very promising product in field trials, reaching from 50% up to 90% depending on the life stage of the CPB [89].

However, one of the biggest challenges and determining factors for the success of pests being controlled by dsRNA spraying is its uptake by the insect. After the application to the plants, the dsRNA molecule must get to the target gene inside the insect cells [170]. The primary constraint of using molecules ingested by insects is degradation in the gut. Most of the dsRNases present in Lepidoptera insect's guts are basophilic, presenting an optimum pH of around 9.0 [33]. dsRNA stability is influenced by high pH values due to its chemical structure. At alkaline conditions, dsRNA can be hydrolyzed in some regions [33]. Noticeably, the drawbacks in Lepidoptera are even more significant [171] because pH at Lepidoptera can reach 12 [122]. This factor is probably the biggest challenge to making dsRNA applicable to Lepidoptera insect control [171]. Beyond the gut, dsRNAases present high activity in other body fluids such as saliva and hemolymph, which cause the degradation before the dsRNA has been processed by the insect [31].

Another factor that makes RNAi a difficult strategy to be applied is the uptake of the product when applied via foliar spray. Many leaves are recovered by any kind of cover, most of them are recovered by some wax or, in some cases, the leaf has a hydrophobic cuticle, which makes the absorption more difficult, also the presence of trichomes and stomata density and position interfere directly on the dsRNA absorption by the plants [167].

Moreover, the dsRNA strategy faces other drawbacks to be applicable at the moment such as the low pH that many plants present on the leaf surface, causing the dsRNA degradation. Also, UV light can reduce the biological activity of the dsRNA. Rain, which most of the time is an excellent factor, in dsRNA application sight, can become a threat to the stability of the molecules, these crucial factors that dsRNA strategies have to overcome to reach a good market position [172,173].

6.2. Trunk Injection

The low efficiency of dsRNA delivery methods is a situation that has been circumvented by attempts to deliver through injection into the trunk, a promising method that reduces environmental impacts. The process takes into account the fact that phloem is a channel where dsRNA could remain stable for a long time due to the sap that runs through the vessel being a medium free of RNase, besides the advantage that, once present in the sap, the material can dissipate throughout the plant [174].

The process was successful for the first time in citrus trees and vines that were exposed to a single injection of 2g of dsRNA diluted in 15L of water. The delivery was confirmed after the detection of dsRNA in plants 6 years of age and from the demonstration that two hemipteran insects that fed on sap also absorbed dsRNA, evidencing that this method would be an excellent choice for pests that have this feeding behavior [175]. It is important, however, to note that the trunk injection strategy requires the mass production of dsRNA, which can make the method expensive [35].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 October 2022 doi:10.20944/preprints202210.0034.v1

Currently, the Arbojet® product is available for purchase on the market bringing the injection proposal directly into the trunk from the insertion of drilling plug [174].

Another similar one also available in the market, ChemJet® is a rechargeable and environmentally correct injector, and can be used for fruit trees and as control of various diseases such as silver leaf in apple and pear orchards, mango flower malformation in hoses, phytophthora, among others. Unlike Arboject®, ChemJet® does not use plugs, its solution is inserted from the previous realization of a hole with the aid of drill or similar in the trunk of the plant (https://www.chemjet.com.au/)

To the coffee miner, for example, it is important to evaluate what type of injection favors the circulation of dsRNA, so that the insect finds it in the palisade parenchyma of leaf mesophyll, the place from which the pest removes its food. It is also important to evaluate the size of the orifice and the presence or not of fixed plug in the stem of the plant, since the open orifice becomes an entrance port for pathogens, in addition to the risk of generating embolism in the vessels or compartmentalization of the vascular system of the plant. In addition, an experiment of injection of the trunk of apple trees, plant similar to coffee, shows that the injection was efficient in the systemic conduction of essential oils through the injection of emulsions with perforation of 1 mm wide and 1 cm deep. Three injections per trunk were performed, each perforation was performed at equal distances from each other (every 120° of the stem radius). Still, each injector was tilted upwards of 60° relative to the horizontal axis of the trunk. [176]

One example of a large crop to use the trunk injection methods is coffee to the CLM dsRNA-based control. Similarly, to the apple tree, coffee is a perennial bush that could be treated even by trunk injection or SIGS to fit exogenous RNAi aiming at the CLM larval stage feeding on the leaf mesophyll, as illustrated in **Figure 2**.

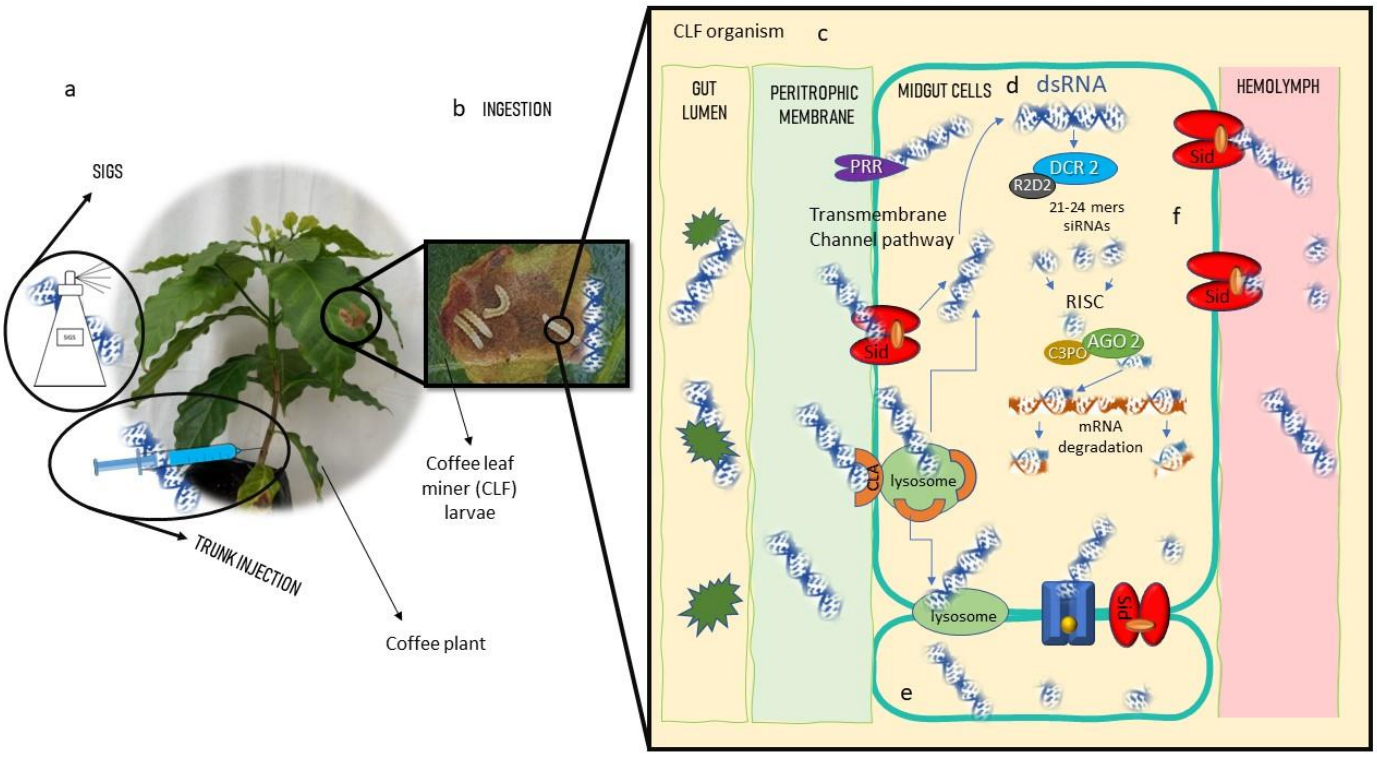

Figure 2. Schematic strategy of RNAi to control *L. coffeella* by long dsRNA induced silencing of coffee plants: (a) dsRNA delivery by SIGS or trunk injection; (b) dsRNA oral ingestion by feeding of the leaf parenchyma; (c) dsRNA transfer from insect gut lumen to midgut cells by the endocytic pathway mediated by clathrin (CLA), pattern recognition receptors (PRRs) and/or RNA-binding proteins (RBPs); (d) siRNA-guided silencing in the cytoplasm. Targeted mRNA molecules may be processed after dsRNA is sliced by Dicer (DCR 2) with *R2D2*. An RNA-induced silencing complex

 doi:10.20944/preprints202210.0034.v1

(RISC) formed by Argonaute (AGO) and C3PO that determines the degradation of the passenger strand. Systemic RNAi may occur by the transferring of dsRNA and siRNA to other midgut cells and the hemolymph via endocytosis or RBPs.

7. Limitations RNAi Technology in Lepidopteran

During the last decade, many studies have proven that RNAi technology is efficient in pest control mainly via transgenic plants, feeding, trunk injection or spraying [56,57,177,178]. Lepidopteran insects, however, present variable RNAi efficiency due to several reasons but the successful uptake is a preponderant one once injection, transfection or transgenic assays normally show good response to dsRNA stimulus [35,96] Studies concerning persistence of dsRNA in the digestive tract indicate that dsRNA can be quickly degraded by nucleases present in the saliva, hemolymph and gut juice [179,180]. It means that the successful usage of dsRNA in a SIGS approach relies on the protection of the dsRNA from degradation in the field and in insect gut. Formulation technologies can also be used to improve cellular internalization of dsRNA and to protect dsRNA against nucleolytic degradation, hence improving overall delivery to the pest [109,123]. Nevertheless, formulation contents might present a risk on their own and the impact of the formulation itself on the environment and non-target organisms (NTO) should be assessed as well.

Other aspects to consider for commercial application of RNAi are cost-efficiency, safety, the delivery to the site of action in the target organism and adverse effects in NTOs. The exposure of NTO is dependent on several parameters, including application rate, timing of application, application method, number of applications, off-site movement of the dsRNA, and stability and persistence of the dsRNA [181,182].

Hence, RNAi efficiency varies greatly among different insect species, and the major limitations for efficient RNAi include dsRNA instability, refractory gene targets, low efficiency of dsRNA cellular internalization, deficient core RNAi machinery and impaired systemic spreading of dsRNA, which constrains the application of RNAi-based pest management [121].

8. Final considerations

RNAi-based biopesticides have less harmful effects than most of the conventional chemical pesticides and no pest resistance development is expected in the field when using long dsRNA strategies. These molecules are processed in many different siRNAs to silence the target genes, minimizing the probability of acquired RNAi-resistance. Also, non-transformative strategies likely prevent insects from adapting and circumventing resistance to the dsRNA silencing. First, for the intermittent presence of the dsRNAS represents lower selection pressure, in contrast to a constant supply provided by HIGS [183].

RNAi-based pesticide control of Lepidopteran pests encompass environmental stability and low silencing efficiency may be surmounted by nanocarriers and adjuvants associated to dsRNAs [184].

From a regulamentation perspective, exogenous delivery methods are more likely to be accepted for commercialization due to its biosafety appeal. These products offer pest gene control without introducing GMOs plants in the environment. Moreover, minimal impact of the topical application of dsRNAs is expected from its fast environmental fate, low non-target and off-target risks, and other advantages over HIGS solutions.

In this way, as the importance of research and commercial interests in exogenous RNAi technology has been strikingly raising in recent years, many forums to discuss the regulatory frameworks for pesticide authorization in the U.S. and in the EU are being formed among the European Food Safety Authority (EFSA), the Organization for Economic Co-operation and Development (OECD) European Co-operation in Science and Technology (COST) and the scientific community [44]. At the current stage, while the dsRNA-based insecticides are being generated, documents from these initiatives are gathering knowledge and outlining the classification and authorization procedures to the

environmental risk assessment (ERA) guidelines to this new MoA, like introduction and mobility of dsRNA within target species, the environmental fate, predicting and determining off-target and non-target effects, resistance development [44,184].

Author Contributions: All authors contributed to the writing and reviewing of the manuscript.

Funding: This work was supported by the CONCAFE consortium (project funding and fellowships to V.L.L. and C.T.), Embrapa (fellowship L.A.V.), CNPq (fellowship to C.J.) and FAPDF (publication grant).

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: In this section, you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. García-Lara, S.; Serna-Saldivar, S. Insect Pests. *Encyclopedia of Food and Health* **2016**, doi:10.1016/B978-0-12-384947-2.00396-2.
2. Sharma, S.; Kooner, R.; Arora, R. Insect Pests and Crop Losses. In *Breeding Insect Resistant Crops for Sustainable Agriculture*; Arora, R., Sandhu, S., Eds.; Springer Singapore: Singapore, 2017; pp. 45–66 ISBN 978-981-10-6055-7.
3. Savary, S.; Willocquet, L.; Pethybridge, S.J.; Esker, P.; McRoberts, N.; Nelson, A. The Global Burden of Pathogens and Pests on Major Food Crops. *Nat Ecol Evol* **2019**, *3*, 430–439, doi:10.1038/s41559-018-0793-y.
4. IPPC Secretariat *Scientific review of the impact of climate change on plant pests: A global challenge to prevent and mitigate plant-pest risks in agriculture, forestry and ecosystems*; FAO on behalf of the IPPC Secretariat: Rome, Italy, 2021; ISBN 978-92-5-134435-4.
5. Gould, F.; Brown, Z.S.; Kuzma, J. Wicked Evolution: Can We Address the Sociobiological Dilemma of Pesticide Resistance? *Science* **2018**, *360*, 728–732, doi:10.1126/science.aar3780.
6. Cooper, A.M.W.; Song, H.; Shi, X.; Yu, Z.; Lorenzen, M.; Silver, K.; Zhang, J.; Zhu, K.Y. Characterization, Expression Patterns, and Transcriptional Responses of Three Core RNA Interference Pathway Genes from Ostrinia Nubilalis. *Journal of Insect Physiology* **2021**, *129*, 104181, doi:10.1016/j.jinsphys.2020.104181.
7. Fire, A.; Albertson, D.; Harrison, S.W.; Moerman, D.G. Production of Antisense RNA Leads to Effective and Specific Inhibition of Gene Expression in C. Elegans Muscle. *Development* **1991**, *113*, 503–514, doi:10.1242/dev.113.2.503.
8. Hannon, G.J. RNA Interference. *Nature* **2002**, *418*, 244–251, doi:10.1038/418244a.
9. Carthew, R.W.; Sontheimer, E.J. Origins and Mechanisms of MiRNAs and SiRNAs. *Cell* **2009**, *136*, 642–655, doi:10.1016/j.cell.2009.01.035.
10. Gebert, L.F.R.; MacRae, I.J. Regulation of MicroRNA Function in Animals. *Nat Rev Mol Cell Biol* **2019**, *20*, 21–37, doi:10.1038/s41580-018-0045-7.
11. Ding, S.-W.; Han, Q.; Wang, J.; Li, W.-X. Antiviral RNA Interference in Mammals. *Current Opinion in Immunology* **2018**, *54*, 109–114, doi:10.1016/j.coi.2018.06.010.
12. Ding, S.-W. RNA-Based Antiviral Immunity. *Nat Rev Immunol* **2010**, *10*, 632–644, doi:10.1038/nri2824.
13. Bronkhorst, A.W.; van Rij, R.P. The Long and Short of Antiviral Defense: Small RNA-Based Immunity in Insects. *Curr Opin Virol* **2014**, *7*, 19–28, doi:10.1016/j.coviro.2014.03.010.
14. Chapman, E.; Taglini, F.; Bayne, E.H. Separable Roles for RNAi in Regulation of Transposable Elements and Viability in the Fission Yeast Schizosaccharomyces Japonicus. *PLOS Genetics* **2022**, *18*, e1010100, doi:10.1371/journal.pgen.1010100.
15. Kataoka, K.; Mochizuki, K. Programmed DNA Elimination in Tetrahymena: A Small RNA-Mediated Genome Surveillance Mechanism. *Adv Exp Med Biol* **2011**, *722*, 156–173, doi:10.1007/978-1-4614-0332-6_10.
16. Martienssen, R.; Moazed, D. RNAi and Heterochromatin Assembly. *Cold Spring Harb Perspect Biol* **2015**, *7*, a019323, doi:10.1101/cshperspect.a019323.
17. Ashfaq, M.A.; Dinesh Kumar, V.; Soma Sekhar Reddy, P.; Anil Kumar, C.; Sai Kumar, K.; Narasimha Rao, N.; Tarakeswari, M.; Sujatha, M. Post-Transcriptional Gene Silencing: Basic Concepts and Applications. *J Biosci* **2020**, *45*, 128, doi:10.1007/s12038-020-00098-3.
18. Kennerdell, J.R.; Carthew, R.W. Use of DsRNA-Mediated Genetic Interference to Demonstrate That Frizzled and Frizzled 2 Act in the Wingless Pathway. *Cell* **1998**, *95*, 1017–1026, doi:10.1016/S0092-8674(00)81725-0.
19. Zhu, K.Y.; Palli, S.R. Mechanisms, Applications, and Challenges of Insect RNA Interference. *Annu. Rev. Entomol.* **2020**, *65*, 293–311, doi:10.1146/annurev-ento-011019-025224.

20. Saxena, S.; Reddy, K.R.K.; Rajam, M.V. DsRNA-Mediated Silencing of Chitin Synthase A (CHSA) Affects Growth and Development of Leucinodes Orbonalis, Brinjal Fruitand Shoot Borer. *Journal of Asia-Pacific Entomology* **2022**, *25*, 101908, doi:10.1016/j.aspen.2022.101908.
21. Dubelman, S.; Fischer, J.; Zapata, F.; Huizinga, K.; Jiang, C.; Uffman, J.; Levine, S.; Carson, D. Environmental Fate of Double-Stranded RNA in Agricultural Soils. *PLOS ONE* **2014**, *9*, e93155, doi:10.1371/journal.pone.0093155.
22. Albright III, V.C.; Wong, C.R.; Hellmich, R.L.; Coats, J.R. Dissipation of Double-Stranded RNA in Aquatic Microcosms. *Environmental Toxicology and Chemistry* **2017**, *36*, 1249–1253, doi:10.1002/etc.3648.
23. Zhang, H.; Zhang, H.; Demirer, G.S.; González-Grandío, E.; Fan, C.; Landry, M.P. Engineering DNA Nanostructures for SiRNA Delivery in Plants. *Nat Protoc* **2020**, *15*, 3064–3087, doi:10.1038/s41596-020-0370-0.
24. Rodrigues, T.B.; Mishra, S.K.; Sridharan, K.; Barnes, E.R.; Alyokhin, A.; Tuttle, R.; Kokulapalan, W.; Garby, D.; Skizim, N.J.; Tang, Y.; et al. First Sprayable Double-Stranded RNA-Based Biopesticide Product Targets Proteasome Subunit Beta Type-5 in Colorado Potato Beetle (Leptinotarsa Decemlineata). *Frontiers in Plant Science* **2021**, *12*.
25. Zhou, X.; Liao, Z.; Jia, Q.; Cheng, L.; Li, F. Identification and Characterization of Piwi Subfamily in Insects. *Biochemical and Biophysical Research Communications* **2007**, *362*, 126–131, doi:10.1016/j.bbrc.2007.07.179.
26. Kolliopoulou, A.; Santos, D.; Taning, C.N.T.; Wynant, N.; Vanden Broeck, J.; Smagghe, G.; Swevers, L. PIWI Pathway against Viruses in Insects. *WIREs RNA* **2019**, *10*, e1555, doi:10.1002/wrna.1555.
27. Darlington, M.; Reinders, J.D.; Sethi, A.; Lu, A.L.; Ramaseshadri, P.; Fischer, J.R.; Boeckman, C.J.; Petrick, J.S.; Roper, J.M.; Narva, K.E.; et al. RNAi for Western Corn Rootworm Management: Lessons Learned, Challenges, and Future Directions. *Insects* **2022**, *13*, 57, doi:10.3390/insects13010057.
28. Barnard, A.-C.; Nijhof, A.M.; Fick, W.; Stutzer, C.; Maritz-Olivier, C. RNAi in Arthropods: Insight into the Machinery and Applications for Understanding the Pathogen-Vector Interface. *Genes* **2012**, *3*, 702–741, doi:10.3390/genes3040702.
29. Huvenne, H.; Smagghe, G. Mechanisms of DsRNA Uptake in Insects and Potential of RNAi for Pest Control: A Review. *Journal of Insect Physiology* **2010**, *56*, 227–235, doi:10.1016/j.jinsphys.2009.10.004.
30. Vélez, A.M.; Fishilevich, E. The Mysteries of Insect RNAi: A Focus on DsRNA Uptake and Transport. *Pesticide Biochemistry and Physiology* **2018**, *151*, 25–31, doi:10.1016/j.pestbp.2018.08.005.
31. Liu, S.; Jaouannet, M.; Dempsey, D.A.; Imani, J.; Coustau, C.; Kogel, K.-H. RNA-Based Technologies for Insect Control in Plant Production. *Biotechnology Advances* **2020**, *39*, 107463, doi:10.1016/j.biotechadv.2019.107463.
32. Meng, F.; Yang, M.; Li, Y.; Li, T.; Liu, X.; Wang, G.; Wang, Z.; Jin, X.; Li, W. Functional Analysis of RNA Interference-Related Soybean Pod Borer (Lepidoptera) Genes Based on Transcriptome Sequences. *Frontiers in Physiology* **2018**, *9*.
33. Kunte, N.; McGraw, E.; Bell, S.; Held, D.; Avila, L.-A. Prospects, Challenges and Current Status of RNAi through Insect Feeding. *Pest Management Science* **2020**, *76*, 26–41, doi:10.1002/ps.5588.
34. Ye, C.; Hu, X.-S.; Wang, Z.-W.; Wei, D.; Smagghe, G.; Christiaens, O.; Niu, J.; Wang, J.-J. Involvement of Clathrin-Dependent Endocytosis in Cellular DsRNA Uptake in Aphids. *Insect Biochemistry and Molecular Biology* **2021**, *132*, 103557, doi:10.1016/j.ibmb.2021.103557.
35. Joga, M.R.; Zotti, M.J.; Smagghe, G.; Christiaens, O. RNAi Efficiency, Systemic Properties, and Novel Delivery Methods for Pest Insect Control: What We Know So Far. *Frontiers in Physiology* **2016**, *7*.
36. Brubaker, S.W.; Bonham, K.S.; Zanoni, I.; Kagan, J.C. Innate Immune Pattern Recognition: A Cell Biological Perspective. *Annual Review of Immunology* **2015**, *33*, 257–290, doi:10.1146/annurev-immunol-032414-112240.
37. Lin, Z.; Wang, J.-L.; Cheng, Y.; Wang, J.-X.; Zou, Z. Pattern Recognition Receptors from Lepidopteran Insects and Their Biological Functions. *Developmental & Comparative Immunology* **2020**, *108*, 103688, doi:10.1016/j.dci.2020.103688.
38. Liu, S.; Geng, S.; Li, A.; Mao, Y.; Mao, L. RNAi Technology for Plant Protection and Its Application in Wheat. *aBIOTECH* **2021**, *2*, 365–374, doi:10.1007/s42994-021-00036-3.
39. Cooper, A.M.; Silver, K.; Zhang, J.; Park, Y.; Zhu, K.Y. Molecular Mechanisms Influencing Efficiency of RNA Interference in Insects. *Pest Management Science* **2019**, *75*, 18–28, doi:10.1002/ps.5126.
40. Davis-Vogel, C.; Allen, B.V.; Hemert, J.L.V.; Sethi, A.; Nelson, M.E.; Sashital, D.G. Identification and Comparison of Key RNA Interference Machinery from Western Corn Rootworm, Fall Armyworm, and Southern Green Stink Bug. *PLOS ONE* **2018**, *13*, e0203160, doi:10.1371/journal.pone.0203160.
41. Dhandapani, R.K.; Gurusamy, D.; Palli, S.R. Protamine–Lipid–DsRNA Nanoparticles Improve RNAi Efficiency in the Fall Armyworm, Spodoptera Frugiperda. *J. Agric. Food Chem.* **2022**, *70*, 6634–6643, doi:10.1021/acs.jafc.2c00901.
42. Wan, X.-S.; Shi, M.-R.; Xu, J.; Liu, J.-H.; Ye, H. Interference Efficiency and Effects of Bacterium-Mediated RNAi in the Fall Armyworm (Lepidoptera: Noctuidae). *Journal of Insect Science* **2021**, *21*, 8, doi:10.1093/jisesa/ieab073.
43. Souza, D.; Christensen, S.A.; Wu, K.; Buss, L.; Kleckner, K.; Darrisaw, C.; Shirk, P.D.; Siegfried, B.D. RNAi-Induced Knockdown of White Gene in the Southern Green Stink Bug (Nezara Viridula L.). *Sci Rep* **2022**, *12*, 10396, doi:10.1038/s41598-022-14620-0.
44. Christiaens, O.; Sweet, J.; Dzhambazova, T.; Urru, I.; Smagghe, G.; Kostov, K.; Arpaia, S. Implementation of RNAi-Based Arthropod Pest Control: Environmental Risks, Potential for Resistance and Regulatory Considerations. *J Pest Sci* **2022**, *95*, 1–15, doi:10.1007/s10340-021-01439-3.
45. Dai, X.; Zhuang, Z.; Zhao, P.X. Computational Analysis of MiRNA Targets in Plants: Current Status and Challenges. *Briefings in Bioinformatics* **2011**, *12*, 115–121, doi:10.1093/bib/bbq065.

46. Srivastava, P.K.; Moturu, T.R.; Pandey, P.; Baldwin, I.T.; Pandey, S.P. A Comparison of Performance of Plant MiRNA Target Prediction Tools and the Characterization of Features for Genome-Wide Target Prediction. *BMC Genomics* **2014**, *15*, 348, doi:10.1186/1471-2164-15-348.
47. Li, P.; Piao, Y.; Shon, H.S.; Ryu, K.H. Comparing the Normalization Methods for the Differential Analysis of Illumina High-Throughput RNA-Seq Data. *BMC Bioinformatics* **2015**, *16*, 347, doi:10.1186/s12859-015-0778-7.
48. Conesa, A.; Madrigal, P.; Tarazona, S.; Gomez-Cabrero, D.; Cervera, A.; McPherson, A.; Szcześniak, M.W.; Gaffney, D.J.; Elo, L.L.; Zhang, X.; et al. A Survey of Best Practices for RNA-Seq Data Analysis. *Genome Biology* **2016**, *17*, 13, doi:10.1186/s13059-016-0881-8.
49. Naito, Y.; Ui-Tei, K. SiRNA Design Software for a Target Gene-Specific RNA Interference. *Frontiers in Genetics* **2012**, *3*.
50. Cagliari, D.; Dias, N.P.; Galdeano, D.M.; dos Santos, E.Á.; Smagghe, G.; Zotti, M.J. Management of Pest Insects and Plant Diseases by Non-Transformative RNAi. *Frontiers in Plant Science* **2019**, *10*.
51. Willow, J.; Taning, C.N.T.; Cook, S.M.; Sulg, S.; Silva, A.I.; Smagghe, G.; Veromann, E. RNAi Targets in Agricultural Pest Insects: Advancements, Knowledge Gaps, and IPM. *Frontiers in Agronomy* **2021**, *3*.
52. Zanini, S.; Šečić, E.; Jelonek, L.; Kogel, K.-H. A Bioinformatics Pipeline for the Analysis and Target Prediction of RNA Effectors in Bidirectional Communication During Plant–Microbe Interactions. *Frontiers in Plant Science* **2018**, *9*.
53. Andrews, S. FastQC: A Quality Control Tool for High Throughput Sequence Data. **2010**.
54. Wang, X.-B.; Wu, Q.; Ito, T.; Cillo, F.; Li, W.-X.; Chen, X.; Yu, J.-L.; Ding, S.-W. RNAi-Mediated Viral Immunity Requires Amplification of Virus-Derived SiRNAs in Arabidopsis Thaliana. *Proceedings of the National Academy of Sciences* **2010**, *107*, 484–489, doi:10.1073/pnas.0904086107.
55. Haas, B.J.; Zody, M.C. Advancing RNA-Seq Analysis. *Nat Biotechnol* **2010**, *28*, 421–423, doi:10.1038/nbt0510-421.
56. Wang, Y.; Zhang, H.; Li, H.; Miao, X. Second-Generation Sequencing Supply an Effective Way to Screen RNAi Targets in Large Scale for Potential Application in Pest Insect Control. *PLOS ONE* **2011**, *6*, e18644, doi:10.1371/journal.pone.0018644.
57. Zhang, H.; Li, H.-C.; Miao, X.-X. Feasibility, Limitation and Possible Solutions of RNAi-Based Technology for Insect Pest Control. *Insect Science* **2013**, *20*, 15–30, doi:10.1111/j.1744-7917.2012.01513.x.
58. Santos, D.; Mingels, L.; Vogel, E.; Wang, L.; Christiaens, O.; Cappelle, K.; Wynant, N.; Gansemans, Y.; Van Nieuwerburgh, F.; Smagghe, G.; et al. Generation of Virus- and DsRNA-Derived SiRNAs with Species-Dependent Length in Insects. *Viruses* **2019**, *11*, 738, doi:10.3390/v11080738.
59. Sciabola, S.; Xi, H.; Cruz, D.; Cao, Q.; Lawrence, C.; Zhang, T.; Rotstein, S.; Hughes, J.D.; Caffrey, D.R.; Stanton, R.V. PFRED: A Computational Platform for SiRNA and Antisense Oligonucleotides Design. *PLOS ONE* **2021**, *16*, e0238753, doi:10.1371/journal.pone.0238753.
60. Lück, S.; Kreszies, T.; Strickert, M.; Schweizer, P.; Kuhlmann, M.; Douchkov, D. SiRNA-Finder (Si-Fi) Software for RNAi-Target Design and Off-Target Prediction. *Frontiers in Plant Science* **2019**, *10*.
61. Motta, I.O.; Dantas, J.; Vidal, L.; Bílio, J.; Pujol-Luz, J.R.; Albuquerque, É.V. The Coffee Leaf Miner, Leucoptera Coffeella (Lepidoptera: Lyonetiidae): Identification of the Larval Instars and Description of Male and Female Genitalia. *Revista Brasileira de Entomologia* **2021**, *65*.
62. Katiyar, K.P.; Ferrer, F. Rearing Technique, Biology and Sterilization of the Coffee Leaf Miner, Leucoptera Coffeella Guer. (Lepidoptera: Lyonetiidae). **1968**.
63. Costa, J.N.M.; Teixeira, C.A.D.; Júnior, J.R.V.; Rocha, R.B.; Fernandes, C. de F. Informações para facilitar a identificação das diferentes fases do bicho-mineiro (Leucoptera coffeella) em campo. **2012**, 4.
64. Wolcott, G.N. A Quintessence of Sensitivity: The Coffee Leaf-Miner. *1* **1947**, *31*, 215–219, doi:10.46429/jaupr.v31i3.12848.
65. Mitter, C.; Davis, D.R.; Cummings, M.P. Phylogeny and Evolution of Lepidoptera. *Annu. Rev. Entomol.* **2017**, *62*, 265–283, doi:10.1146/annurev-ento-031616-035125.
66. *Biocontrol of Lepidopteran Pests: Use of Soil Microbes and Their Metabolites*; Sree, K.S., Varma, A., Eds.; Soil Biology; Springer International Publishing: Cham, 2015; Vol. 43; ISBN 978-3-319-14498-6.
67. Fazekas, I. Distribution and Bionomy of Lyonetia Ledi Wocke, 1859 - a Review with Notes (Lepidoptera, Lyonetiidae). *Lepidopterologica Hungarica* **2022**, *18(1)*, 5–21, doi:10.24386/LepHung.2022.18.1.5.
68. Davis, E.E.; Schober, N.; Gómez, N.N.; Venette, R.C. Mini Risk Assessment: Pear Leaf Blister Moth, Leucoptera Malifoliella Costa [Lepidoptera: Lyonetiidae]. *Cooperative Agricultural Pest Survey Program.* **2009**.
69. Lindquist, O.H. The Cottonwood Leaf Miner, Leucoptera Albella Cham. *Bi-m. Progr. Rep. For. Ent. Path. Br. Dep. For. Can.* **1963**, *19*.
70. Dello Jacovo, E.; Valentine, T.A.; Maluk, M.; Toorop, P.; Lopez del Egido, L.; Frachon, N.; Kenicer, G.; Park, L.; Goff, M.; Ferro, V.A. Towards a Characterisation of the Wild Legume Bitter Vetch (Lathyrus Linifolius L.(Reichard) Bässler): Heteromorphic Seed Germination, Root Nodule Structure and N-fixing Rhizobial Symbionts. *Plant Biology* **2019**, *21*, 523–532.
71. Kaila, L.; Wikstroem, B. Leucoptera Lathyrifoliella (Stainton, 1866) and L-Orobi (Stainton, 1870): Two Distinct Species (Lyonetiidae). *Nota lepidopterologica* **2004**, *27*, 187–192.
72. van Nieukerken, E. Records of Mining Lepidoptera in Belgium with Nine Species New to the Country (Nepticulidae, Opostegidae, Tischeriidae, Lyonetiidae). *Phegea* **2006**, *34*, *4*, 125–144.
73. Sandoval, A.; Ide, S.; Rothmann, S.; Zuñiga, E.; Bosch, P.; Peragallo, M. Detección de Leucoptera sinuella (Reutti) (Lepidoptera: Lyonetiidae) en Chile,
con la identificación de algunos parasitoides asociados . *Revista Chilena de Entomología* **2019**, *45*.

74. Syrett, P.; Fowler, S.; Coombs, E.; Hosking, J.; Markin, G.; Paynter, Q.; Sheppard, A. The Potential for Biological Control of Scotch Broom (Cytisus Scoparius) (Fabaceae) and Related Weedy Species. *Biocontrol News and Information* **1999**, *20*, 17N-34N.
75. Mey, W. Phyllobrostis Minoica Sp. n. from Crete (Greece) – an Expected Discovery (Lepidoptera, Yponomeutoidea, Lyonetiidae). *Nota Lepidopterologica* **2014**, *37*, 161–165, doi:10.3897/nl.37.8098.
76. Yuan, S.; Chenyihang, L.; Zhen, Z.; Zhijie, S.; Tao, L.; Jianyu, D. Advances in Biological Characteristics and Control of Lyonetia Clerkella. 浙江农林大学学报 **2022**, *39*, 687–694.
77. Choi, H.-S.; Kim, G.-J.; Shin, H.-J. Biocontrol of Moth Pests in Apple Orchards: Preliminary Field Study of Application Potential for Mass Trapping. *Biotechnol Bioproc E* **2011**, *16*, 153–157, doi:10.1007/s12257-010-0127-7.
78. Baryshnikova, S.V. A Review of the Lyonetiid Moths (Lepidoptera, Lyonetiidae): II. The Subfamilies Lyonetiinae and Bedelliinae. *Entmol. Rev.* **2007**, *87*, 361–367, doi:10.1134/S0013873807030098.
79. Dantas, J.; Motta, I.O.; Vidal, L.A.; Nascimento, E.F.M.B.; Bilio, J.; Pupe, J.M.; Veiga, A.; Carvalho, C.; Lopes, R.B.; Rocha, T.L.; et al. A Comprehensive Review of the Coffee Leaf Miner Leucoptera Coffeella (Lepidoptera: Lyonetiidae)—A Major Pest for the Coffee Crop in Brazil and Others Neotropical Countries. *Insects* **2021**, *12*, 1130, doi:10.3390/insects12121130.
80. Neves, M.F. Análise Dos Benefícios Econômicos e Sociais Da Utilização Do Carbofurano No Controle de Nematoides, Bicho Mineiro (Leucoptera Coffeella) e Cigarra Do Cafeeiro (Quesada Gigas e Fidicina. *Univ. Sao Paulo* **2006**, *29*.
81. Souza, J.C. de; Reis, P.R.; Rigitano, R.L. de O.; Ciociola Júnior, A.I. Eficiência de thiamethoxam no controle do bicho-mineiro do cafeeiro. II - Influência na época de aplicação via irrigação por gotejamento. **2006**.
82. Martins, N.; Nascimento, E.; Vidal, L.; Lucena-Leandro, V.; Junqueira, C.; Soares, F.; Viana, M.; Nobrega, P.; Fontes, W.; Luz, I.; et al. Paving the Way for Gene Silencing in Lepidoptera: Integrated Sequencing Data Unveil the Rnai Core Machinery of Leucoptera Coffeella. 2022.
83. Dias, N.P.; Cagliari, D.; dos Santos, E.A.; Smagghe, G.; Jurat-Fuentes, J.L.; Mishra, S.; Nava, D.E.; Zotti, M.J. Insecticidal Gene Silencing by RNAi in the Neotropical Region. *Neotrop Entomol* **2020**, *49*, 1–11, doi:10.1007/s13744-019-00722-4.
84. Macedo, L.L.P.; Antonino de Souza Junior, J.D.; Coelho, R.R.; Fonseca, F.C.A.; Firmino, A.A.P.; Silva, M.C.M.; Fragoso, R.R.; Albuquerque, E.V.S.; Silva, M.S.; de Almeida Engler, J.; et al. Knocking down Chitin Synthase 2 by RNAi Is Lethal to the Cotton Boll Weevil. *Biotechnology Research and Innovation* **2017**, *1*, 72–86, doi:10.1016/j.biori.2017.04.001.
85. Sohail, S.; Tariq, K.; Zheng, W.; Ali, M.W.; Peng, W.; Raza, M.F.; Zhang, H. RNAi-Mediated Knockdown of Tssk1 and Tektin1 Genes Impair Male Fertility in Bactrocera Dorsalis. *Insects* **2019**, *10*, 164, doi:10.3390/insects10060164.
86. Pinheiro, D.H.; Taylor, C.E.; Wu, K.; Siegfried, B.D. Delivery of Gene-Specific DsRNA by Microinjection and Feeding Induces RNAi Response in Sri Lanka Weevil, Myllocerus Undecimpustulatus Undatus Marshall. *Pest Management Science* **2020**, *76*, 936–943, doi:10.1002/ps.5601.
87. Rk, D.; Jj, D.; Sr, P. Orally Delivered DsRNA Induces Knockdown of Target Genes and Mortality in the Asian Long-Horned Beetle, Anoplophora Glabripennis. *Archives of insect biochemistry and physiology* **2020**, *104*, doi:10.1002/arch.21679.
88. Yu, H.; Yi, L.; Lu, Z. Silencing of Chitin-Binding Protein with PYPV-Rich Domain Impairs Cuticle and Wing Development in the Asian Citrus Psyllid, Diaphorina Citri. *Insects* **2022**, *13*, 353, doi:10.3390/insects13040353.
89. Pallis, S.J. Effects of a Novel DsRNA-Based Insecticide on the Colorado Potato Beetle, The University of Maine, 2021.
90. Nishide, Y.; Kageyama, D.; Tanaka, Y.; Yokoi, K.; Jouraku, A.; Futahashi, R.; Fukatsu, T. Effectiveness of Orally-Delivered Double-Stranded RNA on Gene Silencing in the Stinkbug Plautia Stali. *PLOS ONE* **2021**, *16*, e0245081, doi:10.1371/journal.pone.0245081.
91. Willow, J.; Soonvald, L.; Sulg, S.; Kaasik, R.; Silva, A.I.; Taning, C.N.T.; Christiaens, O.; Smagghe, G.; Veromann, E. RNAi Efficacy Is Enhanced by Chronic DsRNA Feeding in Pollen Beetle. *Commun Biol* **2021**, *4*, 1–8, doi:10.1038/s42003-021-01975-9.
92. Sümegi, M.; Hunyadi-Gulyás, É.; Medzihradszky, K.F.; Udvardy, A. 26S Proteasome Subunits Are O-Linked N-Acetylglucosamine-Modified in Drosophila Melanogaster. *Biochemical and Biophysical Research Communications* **2003**, *312*, 1284–1289, doi:10.1016/j.bbrc.2003.11.074.
93. Rugjee, K.N.; Chaudhury, S.R.; Al-Jubran, K.; Ramanathan, P.; Matina, T.; Wen, J.; Brogna, S. Fluorescent Protein Tagging Confirms the Presence of Ribosomal Proteins at Drosophila Polytene Chromosomes. *PeerJ* **2013**, *1*, e15, doi:10.7717/peerj.15.
94. Ramaseshadri, P.; Segers, G.; Flannagan, R.; Wiggins, E.; Clinton, W.; Ilagan, O.; McNulty, B.; Clark, T.; Bolognesi, R. Physiological and Cellular Responses Caused by RNAi- Mediated Suppression of Snf7 Orthologue in Western Corn Rootworm (Diabrotica Virgifera Virgifera) Larvae. *PLOS ONE* **2013**, *8*, e54270, doi:10.1371/journal.pone.0054270.
95. Forgac, M. Vacuolar ATPases: Rotary Proton Pumps in Physiology and Pathophysiology. *Nat Rev Mol Cell Biol* **2007**, *8*, 917–929, doi:10.1038/nrm2272.
96. Terenius, O.; Papanicolaou, A.; Garbutt, J.S.; Eleftherianos, I.; Huvenne, H.; Kanginakudru, S.; Albrechtsen, M.; An, C.; Aymeric, J.-L.; Barthel, A.; et al. RNA Interference in Lepidoptera: An Overview of Successful and Unsuccessful Studies and Implications for Experimental Design. *Journal of Insect Physiology* **2011**, *57*, 231–245, doi:10.1016/j.jinsphys.2010.11.006.
97. Bramlett, M.; Plaetinck, G.; Maienfisch, P. RNA-Based Biocontrols—A New Paradigm in Crop Protection. *Engineering* **2020**, *6*, 522–527, doi:10.1016/j.eng.2019.09.008.
98. Wang, J.; Gu, L.; Knipple, D.C. Evaluation of Some Potential Target Genes and Methods for RNAi-Mediated Pest Control of the Corn Earworm Helicoverpa Zea. *Pesticide Biochemistry and Physiology* **2018**, *149*, 67–72, doi:10.1016/j.pestbp.2018.05.012.
99. Sharath Chandra, G.; Asokan, R.; Manamohan, M.; Krishna Kumar, N. Enhancing RNAi by Using Concatemerized Double-Stranded RNA. *Pest Manag Sci* **2019**, *75*, 506–514, doi:10.1002/ps.5149.

100. Choi, M.-Y.; Vander Meer, R.K. Phenotypic Effects of PBAN RNAi Using Oral Delivery of DsRNA to Corn Earworm (Lepidoptera: Noctuidae) and Tobacco Budworm Larvae. *Journal of Economic Entomology* **2019**, *112*, 434–439, doi:10.1093/jee/toy356.
101. Kottaipalayam-Somasundaram, S.R.; Jacob, J.P.; Aiyar, B.; Merzendorfer, H.; Nambiar-Veetil, M. Chitin Metabolism as a Potential Target for RNAi-Based Control of the Forestry Pest Hyblaea Puera Cramer (Lepidoptera: Hyblaeidae). *Pest Management Science* **2022**, *78*, 296–303, doi:10.1002/ps.6634.
102. Martinez, Z.; De Schutter, K.; Van Damme, E.J.M.; Vogel, E.; Wynant, N.; Vanden Broeck, J.; Christiaens, O.; Smagghe, G. Accelerated Delivery of DsRNA in Lepidopteran Midgut Cells by a Galanthus Nivalis Lectin (GNA)-DsRNA-Binding Domain Fusion Protein. *Pesticide Biochemistry and Physiology* **2021**, *175*, 104853, doi:10.1016/j.pestbp.2021.104853.
103. Jin, H.; Abouzaid, M.; Lin, Y.; Hull, J.J.; Ma, W. Cloning and RNAi-Mediated Three Lethal Genes That Can Be Potentially Used for Chilo Suppressalis (Lepidoptera: Crambidae) Management. *Pesticide Biochemistry and Physiology* **2021**, *174*, 104828, doi:10.1016/j.pestbp.2021.104828.
104. Ramkumar, G.; Asokan, R.; Prasannakumar, N.R.; Kariyanna, B.; Karthi, S.; Alwahibi, M.S.; Elshikh, M.S.; Abdel-Megeed, A.; Ghaith, A.; Senthil-Nathan, S.; et al. RNA Interference Suppression of V-ATPase B and Juvenile Hormone Binding Protein Genes Through Topically Applied DsRNA on Tomato Leaves: Developing Biopesticides to Control the South American Pinworm, Tuta Absoluta (Lepidoptera: Gelechiidae). *Frontiers in Physiology* **2021**, *12*.
105. Sharma, R.; Christiaens, O.; Taning, C.N.; Smagghe, G. RNAi-Mediated Mortality in Southern Green Stinkbug Nezara Viridula by Oral Delivery of DsRNA. *Pest Management Science* **2021**, *77*, 77–84, doi:10.1002/ps.6017.
106. Riga, M.; Denecke, S.; Livadaras, I.; Geibel, S.; Nauen, R.; Vontas, J. Development of Efficient RNAi in Nezara Viridula for Use in Insecticide Target Discovery. *Archives of Insect Biochemistry and Physiology* **2020**, *103*, e21650, doi:10.1002/arch.21650.
107. Kiselev, K.V.; Suprun, A.R.; Aleynova, O.A.; Ogneva, Z.V.; Dubrovina, A.S. Physiological Conditions and DsRNA Application Approaches for Exogenously Induced RNA Interference in Arabidopsis Thaliana. *Plants (Basel)* **2021**, *10*, 264, doi:10.3390/plants10020264.
108. Hewezi, T.; Alibert, G.; Kallerhoff, J. Local Infiltration of High- and Low-Molecular-Weight RNA from Silenced Sunflower (Helianthus Annuus L.) Plants Triggers Post-Transcriptional Gene Silencing in Non-Silenced Plants. *Plant Biotechnology Journal* **2005**, *3*, 81–89, doi:10.1111/j.1467-7652.2004.00103.x.
109. de Schutter, K.; Christiaens, O.; Taning, C.N.T.; Smagghe, G. Boosting DsRNA Delivery in Plant and Insect Cells with Peptide- and Polymer-Based Carriers: Case-Based Current Status and Future Perspectives. *RNAi for plant improvement and protection* **2021**, 102–116, doi:10.1079/9781789248890.0011.
110. Bachman, P.; Fischer, J.; Song, Z.; Urbanczyk-Wochniak, E.; Watson, G. Environmental Fate and Dissipation of Applied DsRNA in Soil, Aquatic Systems, and Plants. *Frontiers in Plant Science* **2020**, *11*.
111. Parker, K.M.; Barragán Borrero, V.; van Leeuwen, D.M.; Lever, M.A.; Mateescu, B.; Sander, M. Environmental Fate of RNA Interference Pesticides: Adsorption and Degradation of Double-Stranded RNA Molecules in Agricultural Soils. *Environ. Sci. Technol.* **2019**, *53*, 3027–3036, doi:10.1021/acs.est.8b05576.
112. Lim, F.-H.; Rasid, O.A.; As'wad, A.W.M.; Vadamalai, G.; Parveez, G.K.A.; Wong, M.-Y. The Future Is Now: Revolution of RNA-Mediated Gene Silencing in Plant Protection against Insect Pests and Diseases. *Plant Biotechnol Rep* **2020**, *14*, 643–662, doi:10.1007/s11816-020-00640-7.
113. Gillet, F.-X.; Garcia, R.A.; Macedo, L.L.P.; Albuquerque, E.V.S.; Silva, M.C.M.; Grossi-de-Sa, M.F. Investigating Engineered Ribonucleoprotein Particles to Improve Oral RNAi Delivery in Crop Insect Pests. *Frontiers in Physiology* **2017**, *8*, 256, doi:10.3389/fphys.2017.00256.
114. Yang, W.; Wang, B.; Lei, G.; Chen, G.; Liu, D. Advances in Nanocarriers to Improve the Stability of DsRNA in the Environment. *Frontiers in Bioengineering and Biotechnology* **2022**, *10*.
115. Avila, L.A.; Chandrasekar, R.; Wilkinson, K.E.; Balthazor, J.; Heerman, M.; Bechard, J.; Brown, S.; Park, Y.; Dhar, S.; Reeck, G.R.; et al. Delivery of Lethal DsRNAs in Insect Diets by Branched Amphiphilic Peptide Capsules. *Journal of Controlled Release* **2018**, *273*, 139–146, doi:10.1016/j.jconrel.2018.01.010.
116. Castellanos, N.L.; Smagghe, G.; Sharma, R.; Oliveira, E.E.; Christiaens, O. Liposome Encapsulation and EDTA Formulation of DsRNA Targeting Essential Genes Increase Oral RNAi-Caused Mortality in the Neotropical Stink Bug Euschistus Heros. *Pest Management Science* **2019**, *75*, 537–548, doi:10.1002/ps.5167.
117. Gurusamy, D.; Mogilicherla, K.; Shukla, J.N.; Palli, S.R. Lipids Help Double-Stranded RNA in Endosomal Escape and Improve RNA Interference in the Fall Armyworm, Spodoptera Frugiperda. *Archives of Insect Biochemistry and Physiology* **2020**, *104*, e21678, doi:10.1002/arch.21678.
118. Linyu, W.; Lianjun, Z.; Ning, L.; Xiwu, G.; Xiaoning, L. Effect of RNAi Targeting CYP6CY3 on the Growth, Development and Insecticide Susceptibility of Aphis Gossypii by Using Nanocarrier-Based Transdermal DsRNA Delivery System. *Pesticide Biochemistry and Physiology* **2021**, *177*, 104878, doi:10.1016/j.pestbp.2021.104878.
119. Zhang, Y.-H.; Ma, Z.-Z.; Zhou, H.; Chao, Z.-J.; Yan, S.; Shen, J. Nanocarrier-Delivered DsRNA Suppresses Wing Development of Green Peach Aphids. *Insect Science* **2022**, *29*, 669–682, doi:10.1111/1744-7917.12953.
120. Sun, Y.; Wang, P.; Abouzaid, M.; Zhou, H.; Liu, H.; Yang, P.; Lin, Y.; Hull, J.J.; Ma, W. Nanomaterial-Wrapped DsCYP15C1, a Potential RNAi-Based Strategy for Pest Control against Chilo Suppressalis. *Pest Management Science* **2020**, *76*, 2483–2489, doi:10.1002/ps.5789.

121. Ma, Z.; Zheng, Y.; Chao, Z.; Chen, H.; Zhang, Y.; Yin, M.; Shen, J.; Yan, S. Visualization of the Process of a Nanocarrier-Mediated Gene Delivery: Stabilization, Endocytosis and Endosomal Escape of Genes for Intracellular Spreading. *Journal of Nanobiotechnology* **2022**, *20*, 124, doi:10.1186/s12951-022-01336-6.
122. Dow, J.A. Extremely High PH in Biological Systems: A Model for Carbonate Transport. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **1984**, *246*, R633–R636, doi:10.1152/ajpregu.1984.246.4.R633.
123. Christiaens, O.; Tardajos, M.G.; Martinez Reyna, Z.L.; Dash, M.; Dubruel, P.; Smagghe, G. Increased RNAi Efficacy in Spodoptera Exigua via the Formulation of DsRNA With Guanylated Polymers. *Frontiers in Physiology* **2018**, *9*, 316, doi:10.3389/fphys.2018.00316.
124. Mujtaba, M.; Khawar, K.M.; Camara, M.C.; Carvalho, L.B.; Fraceto, L.F.; Morsi, R.E.; Elsabee, M.Z.; Kaya, M.; Labidi, J.; Ullah, H.; et al. Chitosan-Based Delivery Systems for Plants: A Brief Overview of Recent Advances and Future Directions. *International Journal of Biological Macromolecules* **2020**, *154*, 683–697, doi:10.1016/j.ijbiomac.2020.03.128.
125. Kolge, H.; Kadam, K.; Galande, S.; Lanjekar, V.; Ghormade, V. New Frontiers in Pest Control: Chitosan Nanoparticles-Shielded DsRNA as an Effective Topical RNAi Spray for Gram Podborer Biocontrol. *ACS Appl. Bio Mater.* **2021**, *4*, 5145–5157, doi:10.1021/acsabm.1c00349.
126. Zhang, X.; Zhang, J.; Zhu, K.Y. Chitosan/Double-Stranded RNA Nanoparticle-Mediated RNA Interference to Silence Chitin Synthase Genes through Larval Feeding in the African Malaria Mosquito (Anopheles Gambiae). *Insect Molecular Biology* **2010**, *19*, 683–693, doi:10.1111/j.1365-2583.2010.01029.x.
127. Ramesh Kumar, D.; Saravana Kumar, P.; Gandhi, M.R.; Al-Dhabi, N.A.; Paulraj, M.G.; Ignacimuthu, S. Delivery of Chitosan/DsRNA Nanoparticles for Silencing of Wing Development Vestigial (vg) Gene in Aedes Aegypti Mosquitoes. *International Journal of Biological Macromolecules* **2016**, *86*, 89–95, doi:10.1016/j.ijbiomac.2016.01.030.
128. Dhandapani, R.K.; Gurusamy, D.; Howell, J.L.; Palli, S.R. Development of CS-TPP-DsRNA Nanoparticles to Enhance RNAi Efficiency in the Yellow Fever Mosquito, Aedes Aegypti. *Sci Rep* **2019**, *9*, 8775, doi:10.1038/s41598-019-45019-z.
129. Mitter, N.; Worrall, E.A.; Robinson, K.E.; Li, P.; Jain, R.G.; Taochy, C.; Fletcher, S.J.; Carroll, B.J.; Lu, G.Q. (Max); Xu, Z.P. Clay Nanosheets for Topical Delivery of RNAi for Sustained Protection against Plant Viruses. *Nature Plants* **2017**, *3*, 1–10, doi:10.1038/nplants.2016.207.
130. Wang, K.; Peng, Y.; Chen, J.; Peng, Y.; Wang, X.; Shen, Z.; Han, Z. Comparison of Efficacy of RNAi Mediated by Various Nanoparticles in the Rice Striped Stem Borer (Chilo Suppressalis). *Pesticide Biochemistry and Physiology* **2020**, *165*, 104467, doi:10.1016/j.pestbp.2019.10.005.
131. Timmons, L.; Court, D.L.; Fire, A. Ingestion of Bacterially Expressed DsRNAs Can Produce Specific and Potent Genetic Interference in Caenorhabditis Elegans. *Gene* **2001**, *263*, 103–112, doi:10.1016/S0378-1119(00)00579-5.
132. Drinnenberg, I.A.; Weinberg, D.E.; Xie, K.T.; Mower, J.P.; Wolfe, K.H.; Fink, G.R.; Bartel, D.P. RNAi in Budding Yeast. *Science* **2009**, *326*, 544–550, doi:10.1126/science.1176945.
133. Chen, X.; Li, L.; Hu, Q.; Zhang, B.; Wu, W.; Jin, F.; Jiang, J. Expression of DsRNA in Recombinant Isaria Fumosorosea Strain Targets the TLR7 Gene in Bemisia Tabaci. *BMC Biotechnology* **2015**, *15*, 64, doi:10.1186/s12896-015-0170-8.
134. Caccia, S.; Astarita, F.; Barra, E.; Di Lelio, I.; Varricchio, P.; Pennacchio, F. Enhancement of Bacillus Thuringiensis Toxicity by Feeding Spodoptera Littoralis Larvae with Bacteria Expressing Immune Suppressive DsRNA. *J Pest Sci* **2020**, *93*, 303–314, doi:10.1007/s10340-019-01140-6.
135. Kang, S.; Sun, D.; Qin, J.; Guo, L.; Zhu, L.; Bai, Y.; Wu, Q.; Wang, S.; Zhou, X.; Guo, Z.; et al. Fused: A Promising Molecular Target for an RNAi-Based Strategy to Manage Bt Resistance in Plutella Xylostella (L.). *J Pest Sci* **2022**, *95*, 101–114, doi:10.1007/s10340-021-01374-3.
136. Verdonckt, T.-W.; Vanden Broeck, J. Methods for the Cost-Effective Production of Bacteria-Derived Double-Stranded RNA for in Vitro Knockdown Studies. *Frontiers in Physiology* **2022**, *13*.
137. Zotti, M.; Santos, E.A. dos; Cagliari, D.; Christiaens, O.; Taning, C.N.T.; Smagghe, G. RNA Interference Technology in Crop Protection against Arthropod Pests, Pathogens and Nematodes. *Pest Management Science* **2018**, *74*, 1239–1250, doi:10.1002/ps.4813.
138. Suhag, A.; Yadav, H.; Chaudhary, D.; Subramanian, S.; Jaiwal, R.; Jaiwal, P.K. Biotechnological Interventions for the Sustainable Management of a Global Pest, Whitefly (Bemisia Tabaci). *Insect Science* **2021**, *28*, 1228–1252, doi:10.1111/1744-7917.12853.
139. Taning, C.N.; Arpaia, S.; Christiaens, O.; Dietz-Pfeilstetter, A.; Jones, H.; Mezzetti, B.; Sabbadini, S.; Sorteberg, H.-G.; Sweet, J.; Ventura, V.; et al. RNA-Based Biocontrol Compounds: Current Status and Perspectives to Reach the Market. *Pest Management Science* **2020**, *76*, 841–845, doi:10.1002/ps.5686.
140. Guan, R.; Chu, D.; Han, X.; Miao, X.; Li, H. Advances in the Development of Microbial Double-Stranded RNA Production Systems for Application of RNA Interference in Agricultural Pest Control. *Frontiers in Bioengineering and Biotechnology* **2021**, *9*.
141. Li, T.; Huang, L.; Yang, M. Lipid-Based Vehicles for SiRNA Delivery in Biomedical Field. *Curr Pharm Biotechnol* **2020**, *21*, 3–22, doi:10.2174/1389201020666190924164152.
142. Kanwal, F.; Chen, T.; Zhang, Y.; Simair, A.; Rujie, C.; Sadaf Zaidi, N. us S.; Guo, X.; Wei, X.; Siegel, G.; Lu, C. Large-Scale in Vitro Transcription, RNA Purification and Chemical Probing Analysis. *CPB* **2018**, *48*, 1915–1927, doi:10.1159/000492512.
143. Mendiola, S.Y.; Civitello, D.J.; Gerardo, N.M. An Integrative Approach to Symbiont-Mediated Vector Control for Agricultural Pathogens. *Current Opinion in Insect Science* **2020**, *39*, 57–62, doi:10.1016/j.cois.2020.02.007.

144. Li, W.; O'Neill, K.R.; Haft, D.H.; DiCuccio, M.; Chetvernin, V.; Badretdin, A.; Coulouris, G.; Chitsaz, F.; Derbyshire, M.K.; Durkin, A.S.; et al. RefSeq: Expanding the Prokaryotic Genome Annotation Pipeline Reach with Protein Family Model Curation. *Nucleic Acids Res* **2021**, *49*, D1020–D1028, doi:10.1093/nar/gkaa1105.
145. Li, J.; Qian, J.; Xu, Y.; Yan, S.; Shen, J.; Yin, M. A Facile-Synthesized Star Polycation Constructed as a Highly Efficient Gene Vector in Pest Management. *ACS Sustainable Chem. Eng.* **2019**, *7*, 6316–6322, doi:10.1021/acssuschemeng.9b00004.
146. Saksmerprome, V.; Charoonnart, P.; Gangnonngiw, W.; Withyachumnarnkul, B. A Novel and Inexpensive Application of RNAi Technology to Protect Shrimp from Viral Disease. *Journal of Virological Methods* **2009**, *162*, 213–217, doi:10.1016/j.jviromet.2009.08.010.
147. Timmons, L.; Fire, A. Specific Interference by Ingested DsRNA. *Nature* **1998**, *395*, 854–854, doi:10.1038/27579.
148. Tenllado, F.; Martínez-García, B.; Vargas, M.; Díaz-Ruíz, J.R. Crude Extracts of Bacterially Expressed DsRNA Can Be Used to Protect Plants against Virus Infections. *BMC Biotechnol* **2003**, *3*, 3, doi:10.1186/1472-6750-3-3.
149. Voloudakis, A.E.; Holeva, M.C.; Sarin, L.P.; Bamford, D.H.; Vargas, M.; Poranen, M.M.; Tenllado, F. Efficient Double-Stranded RNA Production Methods for Utilization in Plant Virus Control. In *Plant Virology Protocols: New Approaches to Detect Viruses and Host Responses*; Uyeda, I., Masuta, C., Eds.; Methods in Molecular Biology; Springer: New York, NY, 2015; pp. 255–274 ISBN 978-1-4939-1743-3.
150. Park, M.G.; Kim, W.J.; Choi, J.Y.; Kim, J.H.; Park, D.H.; Kim, J.Y.; Wang, M.; Je, Y.H. Development of a Bacillus Thuringiensis Based DsRNA Production Platform to Control Sacbrood Virus in Apis Cerana. *Pest Management Science* **2020**, *76*, 1699–1704, doi:10.1002/ps.5692.
151. Lezzerini, M.; Ven, K. van de; Veerman, M.; Brul, S.; Budovskaya, Y.V. Specific RNA Interference in Caenorhabditis Elegans by Ingested DsRNA Expressed in Bacillus Subtilis. *PLOS ONE* **2015**, *10*, e0124508, doi:10.1371/journal.pone.0124508.
152. Saelim, H.; Loprasert, S.; Phongdara, A. Bacillus Subtilis Expressing DsVP28 Improved Shrimp Survival from WSSV Challenge. *ScienceAsia* **2020**, *46S*, 19, doi:10.2306/scienceasia1513-1874.2020.S003.
153. Taracena, M.L.; Oliveira, P.L.; Almendares, O.; Umaña, C.; Lowenberger, C.; Dotson, E.M.; Paiva-Silva, G.O.; Pennington, P.M. Genetically Modifying the Insect Gut Microbiota to Control Chagas Disease Vectors through Systemic RNAi. *PLOS Neglected Tropical Diseases* **2015**, *9*, e0003358, doi:10.1371/journal.pntd.0003358.
154. Whitten, M.M. Novel RNAi Delivery Systems in the Control of Medical and Veterinary Pests. *Current Opinion in Insect Science* **2019**, *34*, 1–6, doi:10.1016/j.cois.2019.02.001.
155. Whitten, M.M.A.; Facey, P.D.; Del Sol, R.; Fernández-Martínez, L.T.; Evans, M.C.; Mitchell, J.J.; Bodger, O.G.; Dyson, P.J. Symbiont-Mediated RNA Interference in Insects. *Proceedings of the Royal Society B: Biological Sciences* **2016**, *283*, 20160042, doi:10.1098/rspb.2016.0042.
156. Whitten, M.; Dyson, P. Gene Silencing in Non-Model Insects: Overcoming Hurdles Using Symbiotic Bacteria for Trauma-Free Sustainable Delivery of RNA Interference: Sustained RNA Interference in Insects Mediated by Symbiotic Bacteria: Applications as a Genetic Tool and as a Biocide. *Bioessays* **2017**, *39*, doi:10.1002/bies.201600247.
157. Asghari, S.; Harighi, B.; Ashengroph, M.; Clement, C.; Aziz, A.; Esmaeel, Q.; Ait Barka, E. Induction of Systemic Resistance to Agrobacterium Tumefaciens by Endophytic Bacteria in Grapevine. *Plant Pathology* **2020**, *69*, 827–837, doi:10.1111/ppa.13175.
158. Zhu, F.; Xu, J.; Palli, R.; Ferguson, J.; Palli, S.R. Ingested RNA Interference for Managing the Populations of the Colorado Potato Beetle, Leptinotarsa Decemlineata. *Pest Management Science* **2011**, *67*, 175–182, doi:10.1002/ps.2048.
159. Tian, H.; Peng, H.; Yao, Q.; Chen, H.; Xie, Q.; Tang, B.; Zhang, W. Developmental Control of a Lepidopteran Pest Spodoptera Exigua by Ingestion of Bacteria Expressing DsRNA of a Non-Midgut Gene. *PLOS ONE* **2009**, *4*, e6225, doi:10.1371/journal.pone.0006225.
160. Vatanparast, M.; Kim, Y. Optimization of Recombinant Bacteria Expressing DsRNA to Enhance Insecticidal Activity against a Lepidopteran Insect, Spodoptera Exigua. *PLOS ONE* **2017**, *12*, e0183054, doi:10.1371/journal.pone.0183054.
161. Bento, F.M.; Marques, R.N.; Campana, F.B.; Demétrio, C.G.; Leandro, R.A.; Parra, J.R.P.; Figueira, A. Gene Silencing by RNAi via Oral Delivery of DsRNA by Bacteria in the South American Tomato Pinworm, Tuta Absoluta. *Pest Management Science* **2020**, *76*, 287–295, doi:10.1002/ps.5513.
162. Xu, J.; Wang, X.-F.; Chen, P.; Liu, F.-T.; Zheng, S.-C.; Ye, H.; Mo, M.-H. RNA Interference in Moths: Mechanisms, Applications, and Progress. *Genes* **2016**, *7*, 88, doi:10.3390/genes7100088.
163. Hashiro, S.; Yasueda, H. RNA Interference-Based Pesticides and Antiviral Agents: Microbial Overproduction Systems for Double-Stranded RNA for Applications in Agriculture and Aquaculture. *Applied Sciences* **2022**, *12*, 2954, doi:10.3390/app12062954.
164. Zhong, C.; Smith, N.A.; Zhang, D.; Goodfellow, S.; Zhang, R.; Shan, W.; Wang, M.-B. Full-Length Hairpin RNA Accumulates at High Levels in Yeast but Not in Bacteria and Plants. *Genes* **2019**, *10*, 458, doi:10.3390/genes10060458.
165. Dubreuil, G.; Magliano, M.; Dubrana, M.P.; Lozano, J.; Lecomte, P.; Favery, B.; Abad, P.; Rosso, M.N. Tobacco Rattle Virus Mediates Gene Silencing in a Plant Parasitic Root-Knot Nematode. *J Exp Bot* **2009**, *60*, 4041–4050, doi:10.1093/jxb/erp237.
166. Kumar, P.; Pandit, S.S.; Baldwin, I.T. Tobacco Rattle Virus Vector: A Rapid and Transient Means of Silencing Manduca Sexta Genes by Plant Mediated RNA Interference. *PLOS ONE* **2012**, *7*, e31347, doi:10.1371/journal.pone.0031347.
167. Hoang, B.T.L.; Fletcher, S.J.; Brosnan, C.A.; Ghodke, A.B.; Manzie, N.; Mitter, N. RNAi as a Foliar Spray: Efficiency and Challenges to Field Applications. *International Journal of Molecular Sciences* **2022**, *23*, 6639, doi:10.3390/ijms23126639.
168. Rodrigues, T.B.; Petrick, J.S. Safety Considerations for Humans and Other Vertebrates Regarding Agricultural Uses of Externally Applied RNA Molecules. *Frontiers in Plant Science* **2020**, *11*.

169. Mezzetti, B.; Smagghe, G.; Arpaia, S.; Christiaens, O.; Dietz-Pfeilstetter, A.; Jones, H.; Kostov, K.; Sabbadini, S.; Opsahl-Sorteberg, H.-G.; Ventura, V.; et al. RNAi: What Is Its Position in Agriculture? *J Pest Sci* **2020**, *93*, 1125–1130, doi:10.1007/s10340-020-01238-2.
170. Rebijith, K.B.; Asokan, R.; Hande, H.R.; Kumar, N.K.K. The First Report of MiRNAs from a Thysanopteran Insect, Thrips Palmi Karny Using High-Throughput Sequencing. *PLOS ONE* **2016**, *11*, e0163635, doi:10.1371/journal.pone.0163635.
171. Christiaens, O.; Swevers, L.; Smagghe, G. DsRNA Degradation in the Pea Aphid (Acyrthosiphon Pisum) Associated with Lack of Response in RNAi Feeding and Injection Assay. *Peptides* **2014**, *53*, 307–314, doi:10.1016/j.peptides.2013.12.014.
172. Christiaens, O.; Whyard, S.; Vélez, A.M.; Smagghe, G. Double-Stranded RNA Technology to Control Insect Pests: Current Status and Challenges. *Frontiers in Plant Science* **2020**, *11*.
173. San Miguel, K.; Scott, J.G. The next Generation of Insecticides: DsRNA Is Stable as a Foliar-Applied Insecticide. *Pest Management Science* **2016**, *72*, 801–809, doi:10.1002/ps.4056.
174. Yan, S.; Ren, B.; Zeng, B.; Shen, J. Improving RNAi Efficiency for Pest Control in Crop Species. *BioTechniques* **2020**, *68*, 283–290, doi:10.2144/btn-2019-0171.
175. Andrade, E.C. de; Hunter, W.B. *RNA Interference – Natural Gene-Based Technology for Highly Specific Pest Control (HiSPeC)*; IntechOpen, 2016; ISBN 978-953-51-2272-2.
176. Werrie, P.-Y.; Burgeon, C.; Le Goff, G.J.; Hance, T.; Fauconnier, M.-L. Biopesticide Trunk Injection Into Apple Trees: A Proof of Concept for the Systemic Movement of Mint and Cinnamon Essential Oils. *Frontiers in Plant Science* **2021**, *12*.
177. Mao, Y.-B.; Cai, W.-J.; Wang, J.-W.; Hong, G.-J.; Tao, X.-Y.; Wang, L.-J.; Huang, Y.-P.; Chen, X.-Y. Silencing a Cotton Bollworm P450 Monooxygenase Gene by Plant-Mediated RNAi Impairs Larval Tolerance of Gossypol. *Nat Biotechnol* **2007**, *25*, 1307–1313, doi:10.1038/nbt1352.
178. Baum, J.A.; Bogaert, T.; Clinton, W.; Heck, G.R.; Feldmann, P.; Ilagan, O.; Johnson, S.; Plaetinck, G.; Munyikwa, T.; Pleau, M.; et al. Control of Coleopteran Insect Pests through RNA Interference. *Nat Biotechnol* **2007**, *25*, 1322–1326, doi:10.1038/nbt1359.
179. Garbutt, J.S.; Bellés, X.; Richards, E.H.; Reynolds, S.E. Persistence of Double-Stranded RNA in Insect Hemolymph as a Potential Determiner of RNA Interference Success: Evidence from Manduca Sexta and Blattella Germanica. *Journal of Insect Physiology* **2013**, *59*, 171–178, doi:10.1016/j.jinsphys.2012.05.013.
180. Guan, R.-B.; Li, H.-C.; Fan, Y.-J.; Hu, S.-R.; Christiaens, O.; Smagghe, G.; Miao, X.-X. A Nuclease Specific to Lepidopteran Insects Suppresses RNAi. *Journal of Biological Chemistry* **2018**, *293*, 6011–6021, doi:10.1074/jbc.RA117.001553.
181. Arora, A.K.; Chung, S.H.; Douglas, A.E. Non-Target Effects of DsRNA Molecules in Hemipteran Insects. *Genes* **2021**, *12*, 407, doi:10.3390/genes12030407.
182. Christiaens, O.; Dzhambazova, T.; Kostov, K.; Arpaia, S.; Joga, M.R.; Urru, I.; Sweet, J.; Smagghe, G. Literature Review of Baseline Information on RNAi to Support the Environmental Risk Assessment of RNAi-Based GM Plants. *EFSA Supporting Publications* **2018**, *15*, 1424E, doi:10.2903/sp.efsa.2018.EN-1424.
183. Arpaia, S.; Christiaens, O.; Giddings, K.; Jones, H.; Mezzetti, B.; Moronta-Barrios, F.; Perry, J.N.; Sweet, J.B.; Taning, C.N.T.; Smagghe, G.; et al. Biosafety of GM Crop Plants Expressing DsRNA: Data Requirements and EU Regulatory Considerations. *Frontiers in Plant Science* **2020**, *11*.
184. Dietz-Pfeilstetter, A.; Mendelsohn, M.; Gathmann, A.; Klinkenbuß, D. Considerations and Regulatory Approaches in the USA and in the EU for DsRNA-Based Externally Applied Pesticides for Plant Protection. *Frontiers in Plant Science* **2021**, *12*.