forests

MDPI

Article

Foliar Application of dsRNA to Induce Gene Silencing in Emerald Ash Borer: Systemic Distribution, Persistence, and Bioactivity †

Flávia Pampolini ‡ and Lynne K. Rieske *

Department of Entomology, University of Kentucky, Lexington, KY, 40546-0091, USA; flapampo@gmail.com
* Correspondence: lrieske@uky.edu; Tel.: +1-859-257-1167
† This paper is a part of the Ph.D. dissertation of Flavia Pampolini, presented at the University of Kentucky, Lexington, KY, USA.
‡ Current address: Renaissance Bioscience Corp., Vancouver, BC V6T 1Z3, Canada.

Abstract: RNA interference (RNAi) is a cellular mechanism triggered by double-stranded RNA (dsRNA), in which gene expression is reduced in a sequence-specific manner, allowing development of pest-specific control strategies. Effective delivery of the dsRNA is a hurdle, particularly in systems with endophagous insects such as the emerald ash borer (EAB) *Agrilus planipennis*, an invasive phloem-feeding beetle that develops beneath the bark of ash trees, *Fraxinus* spp., causing rapid tree death. We evaluated uptake and bioactivity of dsRNA in green ash (*F. pennsylvanica*) growing in a greenhouse to investigate the feasibility of delivery through the host plant. To assess dsRNA persistence and distribution, seedlings were exposed to EAB-specific dsRNA using foliar spray and sectioned into different tissue types at selected time-points; RT-PCR and Sanger sequencing were used to detect the exogenous dsRNA. We found dsRNA persistence in plant tissues 21 days after treatment. To evaluate bioactivity, neonate EAB were exposed to treated seedlings and assessed for gene expression and feeding behavior. Results demonstrate gene silencing and a 24% ($p = 0.03$) reduction in cambial consumption. Our findings provide proof-of-concept for delivery of RNAi to the target insect through the host plant, suggesting the feasibility of RNAi functioning as a sustainable approach for tree protection against EAB.

Keywords: RNA interference; spray induced gene silencing; dsRNA delivery; forest pest management

Citation: Pampolini, F.; Rieske, L.K. Foliar Application of dsRNA to Induce Gene Silencing in Emerald Ash Borer: Systemic Distribution, Persistence, and Bioactivity. *Forests* **2023**, *14*, 1853. https://doi.org/10.3390/f14091853

Academic Editor: Sergio Angeli

Received: 17 August 2023
Revised: 7 September 2023
Accepted: 9 September 2023
Published: 12 September 2023

1. Introduction

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive pest of forest, shade, and ornamental ash (*Fraxinus* spp.) that caused extensive tree mortality throughout its invaded range in North America [1]. EAB is among the most impactful of North American invasive pests, with unprecedented ecological and economic impacts [2] that include extensive tree mortality in urban and wildland forests, accompanying losses in biodiversity and ecosystem services, and debilitating costs to municipalities for suppression programs and tree removal [3,4]. Furthermore, naturalized areas in urban environments provide human health benefits by improving air quality, increasing physical activity, and reducing stress. The loss of the urban tree canopy associated with the EAB invasion caused tangible effects on human health and well-being that include cardiovascular deaths and respiratory disease [5]. The costs associated with the EAB invasion in North America are estimated at USD 10B per year [6].

All North American ash are susceptible to EAB [7], though white and green ash (*F. americana* L. and *F. pennsylvanica* Marshall) are highly preferred. In the eastern US, ash is a significant component of wildland forests [8], but is also prevalent as street, park, and landscape trees. Current management strategies in urban situations include trunk injection or soil drench of insecticides, removal of infested trees, and classical biological

control [4]; municipalities are frequently encouraged to develop long-range management plans encompassing all three [9]. Despite intensive management efforts, EAB continues to expand its range in North America and is projected to colonize wherever ash occurs. Clearly, innovative suppression strategies are needed.

An emerging pest control technology that shows tremendous potential for EAB management is gene silencing through RNA interference (RNAi). RNAi is a naturally occurring cellular immune response triggered by the introduction of double-stranded RNA (dsRNA). The RNAi pathway can silence specific genes, disrupt protein function, and can lead to insect mortality when essential genes are silenced [10,11]. By carefully designing dsRNAs targeting specific essential genes, manipulating the RNAi pathway can be used as a pest management strategy to kill insects. Since dsRNAs can be translocated through plant vascular systems [12,13], topical applications on leaves or soil for root absorption could be utilized as delivery options. Systemic spread of topically applied dsRNAs in woody plants via foliar spray, root drenching, or trunk injections was demonstrated in citrus trees and grapevines [12,14], deciduous white oak, *Q. alba* L., seedlings [15], and seedlings of the coniferous loblolly pine, *Pinus taeda* L. [16].

RNAi is emerging as a viable next-generation pest control strategy to manage insect pests in herbaceous agricultural crops [17], including the corn rootworm complex, *Diabrotica* spp., in corn [18], and the Colorado potato beetle, *Leptinotarsa decemlineata* Say, in potatoes [19]. Beyond its application in crops, RNAi technology shows efficacy against the emerald ash borer [20,21], as well as other forest pests such as the southern and mountain pine beetles (*Dendroctonus frontalis* Zimmermann and *D. ponderosae* (Hopkins)) [22–24] and the Asian longhorned beetle (*Anoplophora glabripennis* Motschulsky) [25,26].

RNAi works in EAB; effective target genes that cause rapid and extensive mortality were identified [21]. Investigations into the safety of RNAi for tree protection demonstrated its specificity to EAB, with no observable effects on non-target organisms [27]. Furthermore, proof of concept of dsRNA movement through the vascular tissue of small ash seedlings (~5 cm) and excised twigs was demonstrated, as has bioactivity in ash cotyledons [28]. However, a significant barrier to deployment of RNAi technology against EAB is the development of a practical and reliable method of dsRNA delivery [29,30].

Here, we investigate the efficacy of foliar spray as a dsRNA delivery option for EAB suppression. Significant mortality of neonate larvae and adult beetles can be induced in laboratory bioassays following oral ingestion of dsRNA targeting the gene heat shock 70-kDa protein (*hsp*) [21]; thus, it was selected for our study of *in planta* behavior of dsRNA to assess the systemic distribution, persistence, and bioactivity of a spray application of dsRNA in greenhouse grown green ash seedlings.

2. Materials and Methods

2.1. Ash Seedlings

Dormant green ash seedlings (~70 cm from root tip to terminal bud and ~1 cm root collar diameter (RCD)) were received from the Pennsylvania Department of Conservation and Natural Resources, Spring Mills, PA and immediately stored in darkness at 4 °C. Seedlings were potted in general purpose Promix BX growing medium (Premier Tech Horticulture, 92 Rivière-du-Loup, QC, Canada) in 10.16 × 35.56 cm tall tree pots (Stuewe & Sons Inc., Tangent, OR, USA) 40–45 days before use, and maintained in the greenhouse (~18–22 °C, 15:9 L:D) for the duration of the experiments. To ensure developmental uniformity between replicates, seedlings were potted in groups of ~30.

2.2. Insects

In April 2021, EAB-infested green ash trees from Upton, KY (37°26′13.7″ N 85°51′53.6″ W) were felled and stems were immediately transported to the University of Kentucky Forest Entomology Lab, Lexington, KY, and stored at 4 °C in constant darkness. In late May, stems were sectioned into 75 cm lengths and placed in rearing bins (55.6 × 62.7 × 81.3 cm) at room temperature (~24 °C, 15:9 L:D) and monitored for emergence of adult beetles.

Rearing colonies were set up by grouping 5–7 adult beetles into rearing containers consisting of 1 L plastic cups covered by a plastic mesh screen and a standard white paper coffee filter (Kroger, Cincinnati OH) placed directly on top and secured by rubber bands [31]. The plastic mesh mimicked the tree bark crevices, and the filter paper provided a substrate for oviposition. Eggs were collected on the filter paper and allowed to develop at room temperature (~24 °C) for two weeks before use in the bioassay. Rearing colonies were checked twice weekly and provided with new filter paper and fresh tropical ash (*F. uhdei*) foliage.

2.3. Gene Selection

Due to its efficacy triggering gene silencing in EAB, the heat shock protein gene *hsp* [21] was selected for evaluation of uptake, translocation and distribution, and persistence of topically sprayed dsRNA in ash tissues and in the subsequent bioassay. Elongation factor β (*ef1β*) [32] targeting an ash-specific gene was chosen as an endogenous and quality control gene for the methods used to detect dsRNA uptake in ash; green fluorescent protein (*gfp*) was selected as the negative control.

2.4. dsRNA Synthesis

Total RNA was isolated from adult EAB using Trizole reagent (ThermoFisher, Waltham, MA, USA), and the quantity and quality were checked by electrophoresis and a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and cDNA was synthesized using M-MLV reverse transcriptase (ThermoFisher, USA) according to the manufacturer's instructions.

PCR templates for in vitro synthesis of dsRNA were generated using *hsp*-specific primers [21]. PCR cycle conditions were 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 45 s at 72 °C, finishing with an extension step at 72 °C for 10 min. The PCR template was purified using a PCR purification kit (Qiagen Inc., Valencia, CA, USA). After PCR purification, dsRNA synthesis was performed using the MEGAscript RNAi Kit (Ambion Inc., Foster City, CA, USA), following the manufacturer's instructions. The quality of the dsRNA was checked by electrophoresis and quantified using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.5. dsRNA Exposure

Before dsRNA application, the top three leaves of each seedling were protected with a plastic bag (26.8 × 27.3 cm) to prevent direct exposure to the dsRNA treatment, and the remaining leaves and stem were fully sprayed to runoff with 200 µg of EAB dsHSP diluted in 10 mL of dd H_2O or sprayed with water only to serve as a negative control. Seedlings were randomly assigned to receive either the dsRNA treatment or the water negative control, as well as the different sampling time intervals of 3, 7, 14, and 21 days post-dsRNA application. Seedlings were maintained in the greenhouse (18–22 °C, 15:9 L:D) for the duration of the assay and watered twice weekly or as needed. There were three seedlings per time interval per replicate for the dsHSP treatment (n = 12 per replication) and one negative control seedling per time interval (n = 4 total). The experiment was replicated three times at approximately 2-week intervals in June and July 2021.

2.6. Plant Processing and RNA Isolation

At each predetermined sampling interval, designated seedlings were gently removed from the growing medium, rinsed thoroughly, and measured (total seedling length (cm) from root tip to the apical meristem, and root collar diameter (mm)) [15]. Seedlings were then sectioned into (a) root; (b) woody stem, representing the previous year's growth; (c) soft stem, representing the stem tissue grown during the current season; (d) treated leaf; and (e) untreated leaf, comprising the leaves covered with the plastic bag and therefore, unsprayed. Following sectioning, each tissue type was soaked in a 1% bleach solution for 30 s to remove any remaining dsRNA, profusely rinsed in dd H_2O, and processed for RNA isolation.

Using liquid nitrogen and a mortar and pestle, tissues were ground to a fine powder, and ~200 mg of each tissue type was transferred to a 1.5 mL microcentrifuge tube and stored at −80 °C until RNA extraction. Following homogenization, total RNA was isolated using previously published protocols [15] and used for the cDNA synthesis.

2.7. Recovery of Exogenously Applied dsRNA

2.7.1. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Gel Electrophoresis

The presence of the topically applied dsRNA was assessed in each tissue type at the predetermined time points by RT-PCR. RNA quality and concentration were checked by a spectrophotometer at absorbances of 260/280 and 260/230, and 1000 ng of RNA was used for cDNA synthesis using a M-MLV reverse transcriptase kit (ThermoFisher, USA). To increase the specificity of the reverse transcription, we used a combination of both Oligo (dT) and EAB-*hsp* reverse primers. Each cDNA sample served as a template for PCR targeting the EAB *hsp* gene and the ash *ef1β* gene that served as an endogenous control to confirm the success of RNA extraction, cDNA synthesis, and PCR amplification. To increase the sensitivity of the PCR, we used nested primers flanking a 250 bp region inside the original *hsp* sequence (468 bp) to build the dsRNA (primer sequences in Supplementary Table S1). PCR cycle conditions were 2 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 1 min at 50 °C, and 1 min at 68 °C, finishing with an extension step at 68 °C for 5 min. PCR amplification was visualized by gel electrophoresis to assess the presence of the target amplicons in the different tissues and time points.

The dsRNA recovery in each sample was treated as a binary dependent variable, with successful recovery equal to 1 and unsuccessful recovery equal to 0. A logistic regression model was used to estimate the factors that influenced the successful recovery of dsRNA in treated tissues. For the logistic regression model, recovery of dsRNA served as the response variable, and sample interval, RCD, and height were considered as continuous variables, and replicate and tissue type were treated as categorical.

2.7.2. Sanger Sequencing

PCR samples representing different tissue types (root, woody stem, soft stem, leaf (treated), and leaf (untreated)) were chosen randomly and Sanger sequenced. There were 10 PCR samples resulting in 20 reads (10 forward and 10 reverse). Consensus reads were generated using the online tool Benchling [Biology Software] and Emboss Stretcher [33] was used to create a pairwise alignment between the resulting consensus sequence and EAB *hsp* to assess the similarities between the recovered material and annotated sequence.

2.8. Biological Activity of Exogenous dsRNA

2.8.1. Bioassay

EAB eggs were obtained from the laboratory colony. Green ash seedlings (~112 cm in length (root tip to apical bud) and 1.12 cm RCD) were artificially infested with EAB eggs (n = 7/seedling) at 5 cm increments along the length of the stem and secured using 2.5 cm-wide strips of parafilm [34]. Each seedling was then individually sprayed either with dsRNA targeting *hsp*, or *gfp* as a control at a concentration of 500 µg of dsRNA/seedling diluted in 10 mL of water. Each seedling hosting seven EAB eggs/neonates was considered one biological replicate (n = 2) for this assay, which was conducted in August 2021.

Concurrently, a subsample of EAB eggs were kept in Petri dishes with moistened filter paper under the same greenhouse conditions; when the eggs in the Petri dish started hatching, it was assumed that the eggs attached to the stems in the bioassay were also hatching. Neonates were allowed to feed and develop on the treated ash seedlings for seven days, after which stems were excised and carefully debarked with a scalpel, and the fate of individual EAB larvae was recorded. All living EAB larvae were collected and evaluated for gene expression. Larval galleries were measured by wrapping each experimental seedling in translucent plastic and tracing over the galleries with a black permanent marker. Gallery tracings were scanned and ImageJ (Rasband NIH, version 1.53q, March 2022) software

was used to calculate the total area of cambial tissue consumed on each seedling [34]. A one-tailed *t* test was used for statistical analysis to compare the means of a single variable.

2.8.2. Gene Expression

EAB larvae recovered from treated seedlings were evaluated for gene expression using quantitative real-time PCR (RT-qPCR). Following total RNA isolation, cDNA was synthesized from 500 ng of RNA using M-MLV reverse transcriptase (ThermoFisher, USA) and served as a template for gene expression studies. Gene expression analyses were conducted using SYBR Green PCR Master Mix. The PCR mixture contained 1 µL of cDNA, 0.2 µL of each primer (10 mM) (Supplementary Table S1), 5 µL of the SYBR green PCR master mix, and 3.6 µL of ddH2O, totaling 10 µL. RT-qPCR was performed using the QuantStudio 3 Real Time PCR System (ThermoFisher Scientific, Waltham, MA, USA) under the following conditions: one cycle of 20 s at 95 °C, followed by 40 cycles of denaturation at 95 °C (3 s), annealing, and extension for 30 s at 60 °C, ending with generation of a melting curve to confirm a single peak and rule out nonspecific product and primer dimer formations. The reference genes used were *tef1α* and *β-tub* [35], and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of the target gene compared to controls [36]. A two-tailed t test was used for statistical analysis to compare the means of a single variable.

3. Results

3.1. Seedling Measurements

Treated seedlings across all replicates (n = 36) had an average root collar diameter (RCD) of 0.97 cm ± 0.02 (X ± SE) and an average height of 96.38 cm ± 1.61. RCD did not differ among replicates ($F_{2,33}$ = 0.88, p = 0.42) nor among time intervals ($F_{3,33}$ = 0.41, p = 0.74); similarly, seedling height did not differ among replicates ($F_{2,33}$ = 0.92, p = 0.40) or time intervals ($F_{3,33}$ = 0.91, p = 0.44).

3.2. Recovery of Exogenous dsRNA

3.2.1. Gel Imaging and Logistic Regression Modeling

Recovery of exogenously applied dsRNA was assessed through end-point PCR and gel electrophoresis (Figure 1), in which the presence of an amplicon corresponding to the length of the EAB target sequence (250 bp) indicated recovery of the sprayed dsRNA, and the absence of amplicon indicated the lack of the dsRNA in a given sample.

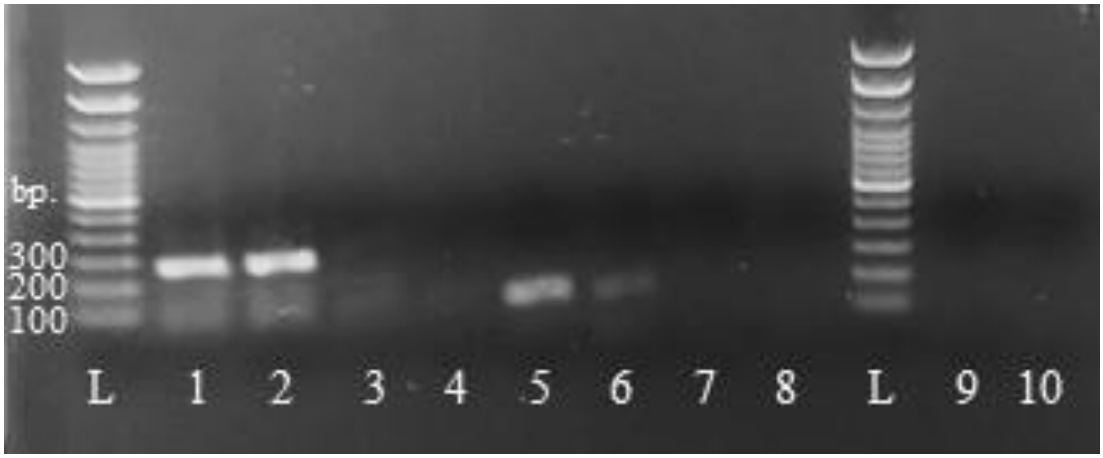

Figure 1. Gel demonstrating successful amplification of EAB-specific *hsp* from ash-treated tissue (lanes 1 and 2), absence of the specific amplicon in tissues from each dsRNA in lanes 3 and 4, where it was not recovered, amplification of our endogenous control, the ash gene e*f1β* (lanes 5 and 6), no amplification of our target gene (*hsp*) from the water control seedlings (lanes 7 and 8), and negative controls showing RT-PCR mixture without RNA in lane 9 and the PCR mixture with the non-template RT-PCR in lane 10. Lanes labeled with L represent 100 bp DNA ladder.

Non-specific products or primer dimers were present in some samples but they did not compromise the analysis of the results. Overall exogenous dsRNA was recovered from 98.3% of the samples (Table 1).

Table 1. Recovery of EAB-specific dsHSP in ash seedling tissues 3, 7, 14, and 21 days post-treatment, showing percentage and total count (incidence of recovery in numerator and total number of tissue samples in denominator).

	Day 3		Day 7		Day 14		Day 21		All Time Points	
Root	100%	(9/9)	100%	(9/9)	77.7%	(7/9)	100%	(9/9)	94.4	(34/36)
Woody stem	100%	(9/9)	100%	(9/9)	100%	(9/9)	88.8%	(8/9)	97.2%	(35/36)
Soft stem	100%	(9/9)	100%	(9/9)	100%	(9/9)	100%	(9/9)	100%	(36/36)
Treated leaf	100%	(9/9)	100%	(9/9)	100%	(9/9)	100%	(9/9)	100%	(36/36)
Untreated leaf	100%	(9/9)	100%	(9/9)	100%	(9/9)	100%	(9/9)	100%	(36/36)
All tissues	100%	(45/45)	100%	(45/45)	93.3%	(42/45)	95.5%	(43/45)	98.3%	(177/180)

There was a slight decrease in dsRNA recovery after 14 and 21 days (Figure 2A); however, recovery across time points did not differ statistically ($\chi^2_{1,180} = 1.69$, $p = 0.19$). Recovery between tissue types differed across time (Figure 2B), but a chi-square test of independence showed no significant association between recovery and tissue type ($\chi^2_{4,180} = 5.29$, $p = 0.20$).

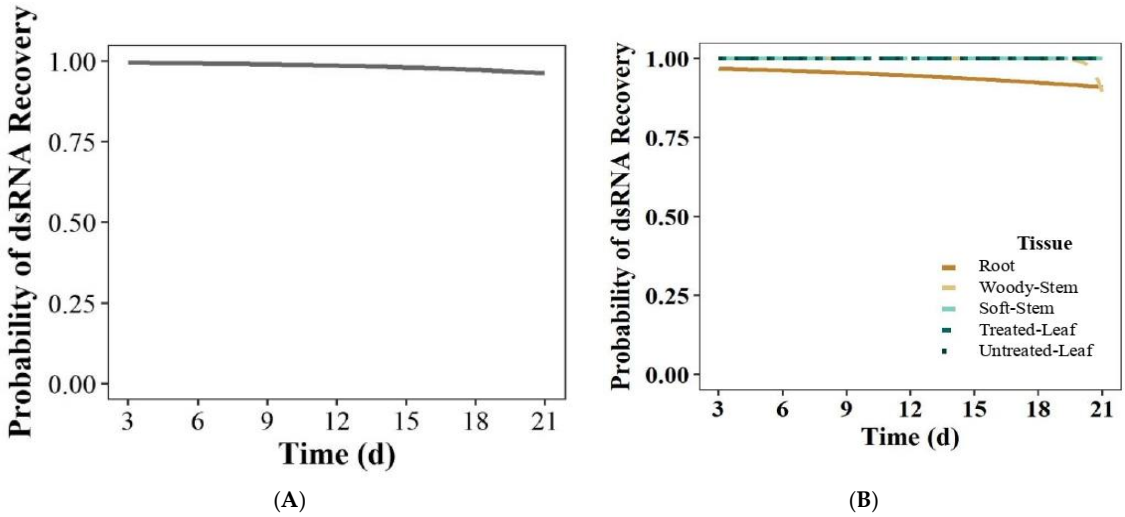

Figure 2. Projected probabilities of dsRNA recovery based on logistic regression models (**A**) across time points for the whole model including all predictors, and (**B**) each tissue type over time.

Logistic regression modeling showed no association between dsRNA recovery and the multiple predictors, which included time, RCD, height, tissue, and replicate ($\chi^2_{9,180} = 11.07$ $p = 0.27$). When assessing each predictor variable individually, none of the parameters were significant (Table 2). Although the odds ratios were <1.0, indicating a negative relationship between the predictor and the response, the *p*-values were non-significant in our model.

Table 2. Logistic regression model representing the association of each individual categorical with the response variable, dsRNA recovery.

Characteristic	Odds Ratio	Confidence Interval	*p*-Value
Time (d)	0.88	(0.68, 1.06)	0.18
RCD (mm)	0.05	(0.00, 4637)	0.59
Height (cm)	0.97	(0.82, 1.13)	0.69
Tissue	N/A	N/A	0.19
Replicate	N/A	N/A	0.21

3.2.2. Sanger Sequencing

The sequence alignment of our multiple samples resulted in a consensus sequence of a 233 bp length fragment. Pairwise alignments comparing the consensus sequence of the amplicons obtained from dsRNA-treated seedlings resulted in 99.6% similarity between the recovered material and the EAB *hsp* annotated gene sequence, with one single gap (0.4%) in the alignment.

3.3. dsRNA Bioactivity

EAB larvae were recovered from each gallery within the debarked experimental seedlings, and all larvae recovered were viable and morphologically indistinguishable between dsHSP-treated and control seedlings. However, gallery area differed significantly between the dsHSP-sprayed seedlings and the control group ($t_{(17)} = 1.9$, $p = 0.03$), with a ~24% reduction in cambial tissue consumption on treated seedlings (Figure 3A,B). Additionally, EAB larvae experienced a significant reduction in gene expression after exposure to the dsHSP-sprayed seedlings (Figure 3C).

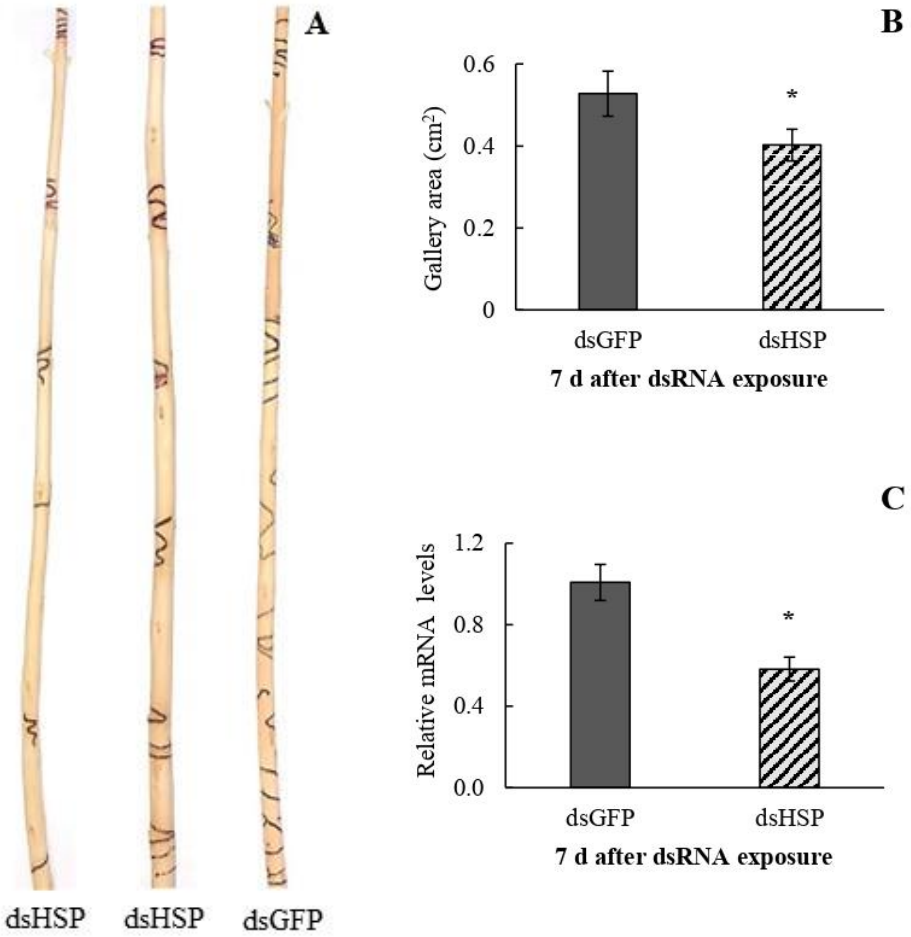

Figure 3. (**A**) Stems showing larval galleries indicating cambial tissue consumption by neonate EAB larvae on two ash seedlings sprayed with 500 µg of dsRNA targeting the EAB gene hsp. One seedling sprayed with 500 µg of dsGFP was used as a negative control. (**B**) Average areas were measured 7 days post treatment from six galleries in each of the two treated seedlings (0.40 $cm^2 \pm 0.03$) and seven galleries in the control seedling (0.52 $cm^2 \pm 0.05$). Means ± SE (n = 6–7) with an asterisk indicates significant differences (t-test, one-tailed $p = 0.03$). (**C**) Transcript levels of the hsp gene in EAB larvae recovered from sprayed seedlings. Relative mRNA levels were normalized using tef1α and β-tub as reference genes. Means ± SE (n = 4) with an asterisk indicates significant differences (t-test, two-tailed $p = 0.003$).

4. Discussion

RNAi-induced gene silencing is a powerful tool that is increasingly utilized for crop protection against pathogens and insects, offering an efficient pest suppression strategy while minimizing negative environmental and human health concerns [37]. The technology has proven to be effective against numerous forest pests [21–23] and has tremendous potential as a tree protection strategy and for integration into forest management efforts. Here, we provide evidence that an appropriately timed foliar application of dsRNA designed to silence genes and induce mortality could potentially provide season-long protection against EAB. However, the success of RNAi technology is reliant on practical and effective delivery of efficacious, pest-specific dsRNAs. Hunter et al. (2012) [12] first demonstrated exogenous application of dsRNA and delivery in non-transformed woody plants, and subsequently, there has been intense interest in non-transformed strategies, including trunk injection [13], root drench [12–14], and spray-induced gene silencing (SIGS), which is touted as an effective delivery method in terms of cost, time, and labor for many agricultural settings [38]. Here, we assess SIGS in green ash seedlings; we evaluate the systemic movement and retention of EAB-specific dsRNA sprayed on leaves of green ash seedlings, followed by an assessment of bioactivity against EAB larvae. This builds on previous work providing proof-of-concept of plant uptake and delivery of exogenous dsRNA to EAB larvae and adult beetles through excised ash twigs and leaves [28]. In planta movement is particularly relevant in our system. Adult EAB feeds on ash foliage prior to oviposition in bark crevices and larvae are confined to cambial tissues; systemic movement allows dsRNAs to reach tissues exploited by both feeding life stages. To investigate uptake, systemic distribution, and retention of SIGS-associated RNAs in ash seedlings, we evaluated different tissues for the presence of exogenous dsRNA, including sprayed and unsprayed distal leaf, stem, and root tissues over a 21 d greenhouse assay.

RT-PCR and subsequent visualization using gel electrophoresis was our initial confirmation of the presence of the exogenous dsRNAs in plant tissues. Following total RNA extraction, each sample from each tissue type and each time point served as a template for cDNA synthesis and was evaluated with primers targeting both the EAB-specific *hsp* gene to assess for the presence of exogenous dsRNA, and the plant-specific *ef1β* gene, which served as a positive control for our protocol and methods. Using semi-quantitative RT-PCR, we detected the sprayed dsRNA in all tissue types and time points up to 21 days after a single foliar application, confirming systemic movement from source to sink and long-term persistence. Sanger sequencing also provided evidence that the recovered material was our EAB-specific dsHSP.

The mechanisms for foliar dsRNA uptake and subsequent entry into plant cells is not fully understood [38]. Stomata have been suggested as an entry point [39], with two main routes. The first is the symplastic pathway, where dsRNA molecules permeate the cell wall and plasma membrane and are processed into small RNAs by the plants' RNAi machinery and transported via plasmodesmata to adjacent cells, to the phloem, and finally move longer distances to additional plant parts [40]. A disadvantage of the symplastic path is that plant-processed RNAi may be less efficient in triggering gene silencing compared to long unprocessed dsRNA molecules [41]. In contrast, the apoplastic route suggests that unprocessed dsRNA remains intact in the apoplast and travels to the vascular tissues for distal translocation [13,42]. The plant vascular system is a nuclease-free environment [43,44], so any dsRNA accessing this tissue should remain stable and available for target pest consumption for long periods.

Research suggests that the mechanism may be taxon dependent. Following spray application of dsRNA to the cereal *Hordeum vulgare* L., RT-qPCR and confocal imaging revealed both plant-processed siRNAs and unprocessed dsRNAs, demonstrating simultaneous symplastic and apoplastic routes of dsRNA movement in the graminaceous host [42]. In contrast, herbaceous and woody plants appear to utilize apoplastic translocation; Dalakouras et al. (2018) [13] evaluated systemic movement of exogenous dsRNAs using Northern blot and confocal imagery and showed that hairpin RNA (hpRNA) is prevented from reach-

ing the cell cytoplasm and remains unprocessed by plant RNAi machinery, suggesting transport exclusively in the xylem. In our study, we did not assess the presence of small RNAs, which could elucidate plant processing of exogenous dsRNAs. However, similar studies detected both dsRNA and hpRNA through confocal imaging solely in the xylem of both ash and apple [13,28]. dsRNA and hpRNA are large molecules whose size may initially confine them to the xylem [15]. Our RT-PCR and Sanger sequencing results clearly show that unprocessed dsRNA was recovered from ash tissues, demonstrating that the dsRNA moved systemically through plant tissues and was not degraded.

Additionally, our logistic regression shows no association between ash tissue type, sample time point, and recovery of dsRNA, implying that dsRNA applied via foliar spray could potentially be recovered from any tissue type sampled from 3 to 21 days. We observed a slight decline in dsRNA detected 14 and 21 days post-application; however, it was not statistically significant ($p = 0.27$), and the overall recovery was 93.3% and 95.5% for days 14 and 21, respectively. The assessment of dsRNA recovery per tissue type shows 97.2% detection in woody stem and 100% detection in soft stem and leaf tissues over time, indicating the presence of exogenous dsHSP in both tissue types utilized by different EAB feeding stages. Adult beetles live for about 3 weeks [31] and require approximately one week of maturation feeding on ash foliage before mating begins [2]. The persistence of EAB dsHSP in ash tissues for up to 21 days suggests that a single foliar spray application could potentially provide season-long protection against EAB with appropriately timed applications. Hunter et al. (2012) [12] and Ghosh et al. (2018) [14] evaluated dsRNA uptake and distribution in citrus trees following foliar application; using qPCR, a technique with greater sensitivity than the RT-PCR we used, they detected dsRNA on untreated leaves from 3–4 h to 7 weeks after spray application, demonstrating rapid uptake and systemic transport of exogenous dsRNA and the long-lasting effects of foliar application. Our results corroborate their findings, suggesting the suitability of foliar spray as a delivery method for RNAi technology targeting EAB.

Our previous work demonstrated mortality of neonates and adult EAB following dsRNA exposure in controlled laboratory bioassays [21,28]. Here, using a more applied approach, we conducted greenhouse bioassays to assess the bioactivity of dsRNAs sprayed to ash seedlings and delivered to neonate EABs through the host plant. Our results show that larvae exposed to dsRNA-sprayed seedlings experience significant gene knockdown (Figure 3C), confirming our assertion that dsRNA remains stable and bioactive in ash plant tissues.

Beyond silencing genes, our data show a 24% reduction in cambial tissue consumption (Figure 3A,B) in treated seedlings. Reduced feeding associated with a reduction in gene expression could lead to delayed larval development, with several consequences. It could benefit the host by allowing a more rapid host defense against early larval instars. It could also potentially arrest larval growth rate, preventing larvae from reaching the later instars when feeding is more detrimental to the host. Finally, delayed larval development provides additional opportunities for natural enemies to inflict mortality. Our experimental design did not allow evaluation of larval development and mortality over the course of the experiment, as seedlings were destructively sampled for data collection. However, our results demonstrate dsRNA stability and provide evidence for bioactivity under greenhouse conditions and suggest the potential for SIGS in protecting ash from EAB. Several studies demonstrated SIGS efficacy in crop systems targeting both pathogens [45–47] and insects [12,19,41,42], confirming the potential of this technology for plant protection, but this is the first published greenhouse trial demonstrating successful SIGS to a wood-boring insect in a deciduous tree.

We demonstrated that dsRNA technology targeting EAB is efficacious [21,28], highly specific, and harmonious with existing biocontrol efforts [27], and here we show that the RNAi pathway can be successfully induced in EAB using SIGS in greenhouse-grown seedlings. However, moving this technology to field scales poses challenges that must be addressed. Quantification of dsRNA in various ash tissues, optimization of dsRNA

concentration for field application, and the bioactivity of sprayable dsRNA in adult beetles are logical next steps. Additionally, a better understanding of plant mechanisms for dsRNA uptake and systemic transport to distal tissues will be crucial to help determine if the dsRNA is being processed by plant RNAi machinery and accumulating in the form of siRNAs, and how it might impact the efficiency of SIGS targeting EAB.

5. Conclusions

RNAi is emerging as a next-generation biopesticide, offering an innovative and sustainable approach for pest management. Development of RNAi-based technologies for plant protection underwent significant advances over the last decades, with increasing emphasis on delivery approaches without plant transformation. Production costs are decreasing as biotech companies improve dsRNA production techniques, leading to marketability of non-transformed products utilizing RNAi technology [48].

Here, we demonstrate that topically applied dsRNA is internalized in ash tissues and systemically transported to distal plant parts, remaining stable and available for pest consumption for 21 days. Our bioassay provides evidence that exogenous dsRNA is delivered to EAB larvae leading, to reduced feeding and gene knockdown. Our results demonstrate the feasibility of SIGS for providing season-long protection of ash trees against EAB and confirm the potential for RNAi to be implemented as an additional and sustainable tool to manage EAB.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14091853/s1, Table S1: Primer sequences for dsRNA synthesis and PCR amplification.

Author Contributions: F.P. and L.K.R. conceived the experiments; F.P. conducted the experiments; F.P. and L.K.R. analyzed the results; F.P. and L.K.R. prepared the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported through funds provided by USDA APHIS AP20PPQS & T00C0032, the University of Kentucky, and the Kentucky Agricultural Experiment Station under McIntire-Stennis 2351197000. This publication is from the Kentucky Agricultural Experiment Station and is published with the approval of the director.

Data Availability Statement: The data supporting the conclusions of this paper will be made available by the authors upon request.

Acknowledgments: The authors thank Bruce Downie and Lynnette M.A. Dirk for fundamental technical assistance, Zachary Bragg for statistical assistance, Beth Kyre, Mary Wallace, Kylie Bickler and Anna Hawse (University of Kentucky) for laboratory assistance, and Morris Farm (Upton, Kentucky) for providing experimental insects.

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

References

1. USDA APHIS. Emerald Ash Borer. 2022. Available online: https://www.aphis.usda.gov/aphis/ourfocus/planthealth/plant-pest-and-disease-programs/pests-and-diseases/emerald-ash-borer (accessed on 15 October 2022).
2. Herms, D.A.; McCullough, D.G. Emerald ash borer invasion of North America: History, biology, ecology, impacts, and management. *Annu. Rev. Entomol.* **2014**, *59*, 13–30. [CrossRef]
3. Aukema, J.E.; Leung, B.; Kovacs, K.; Chivers, C.; Britton, K.O.; Englin, J.; Frankel, S.J.; Haight, R.G.; Holmes, T.P.; Liebhold, A.M.; et al. Economic impacts of non-native forest insects in the continental United States. *PLoS ONE* **2011**, *6*, e24587. [CrossRef] [PubMed]
4. McCullough, D.G. Challenges, tactics and integrated management of emerald ash borer in North America. *Forestry Int. J. For. Res.* **2020**, *93*, 197–211. [CrossRef]
5. Donovan, G.H.; Butry, D.T.; Michael, Y.L.; Prestemon, J.P.; Liebhold, A.M.; Gatziolis, D.; Mao, M.Y. The relationship between trees and human health: Evidence from the spread of the emerald ash borer. *Am. J. Prev. Med.* **2013**, *44*, 139–145. [CrossRef] [PubMed]
6. Kovacs, K.F.; Mercader, R.J.; Haight, R.G.; Siegert, N.W.; McCullough, D.G.; Liebhold, A.M. The influence of satellite populations of emerald ash borer on projected economic costs in U.S. communities, 2010–2020. *J. Environ. Manag.* **2011**, *92*, 2170–2181. [CrossRef] [PubMed]

7. Liu, H.; Bauer, L.S.; Miller, D.L.; Zhao, T.; Gao, R.; Song, L.; Luan, Q.; Jin, R.; Gao, C. Seasonal abundance of *Agrilus planipennis* (Coleoptera: Buprestidae) and its natural enemies *Oobius agrili* (Hymenoptera: Encyrtidae) and *Tetrastichus planipennisi* (Hymenoptera: Eulophidae) in China. *Biol. Control.* **2007**, *42*, 61–71. [CrossRef]
8. Wharton, M.E.; Barbour, R.W. *Trees and Shrubs of Kentucky*; University Press of Kentucky: Lexington, KY, USA, 1993; 582p.
9. Sadof, C.S.; Hughes, G.P.; Witte, A.R.; Peterson, D.J.; Ginzel, M.D. Tools for staging and managing emerald ash borer in the urban forest. *Arboric. Urban For.* **2017**, *43*, 15–26. [CrossRef]
10. Huvenne, H.; Smagghe, G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. *J. Insect. Physiol.* **2010**, *56*, 227–235. [CrossRef]
11. Zhang, J.; Khan, S.A.; Heckel, D.G.; Bock, R. Next-generation insect-resistant plants: RNAi-mediated crop protection. *Trends Biotechnol.* **2017**, *35*, 871–882. [CrossRef]
12. Hunter, W.B.; Glick, E.; Paldi, N.; Bextine, B.R. Advances in RNA interference: dsRNA treatment in trees and grapevines for insect pest suppression. *Southwest Entomol.* **2012**, *37*, 85–87. [CrossRef]
13. Dalakouras, A.; Jarausch, W.; Buchholz, G.; Bassler, A.; Braun, M.; Manthey, T.; Krczal, G.; Wassenegger, M. Delivery of hairpin RNAs and small RNAs into woody and herbaceous plants by trunk injection and petiole absorption. *Front. Plant Sci.* **2018**, *9*, 1253. [CrossRef]
14. Ghosh, S.K.; Hunter, W.B.; Park, A.L.; Gundersen-Rindal, D.E. Double-stranded RNA oral delivery methods to induce RNA interference in phloem and plant-sap-feeding hemipteran insects. *J. Vis. Exp.* **2018**, *135*, e57390. [CrossRef]
15. Bragg, Z.; Rieske, L.K. Feasibility of systemically applied dsRNAs for pest-specific RNAi-induced gene silencing in white oak. *Front. Plant Sci.* **2022**, *13*, 830226. [CrossRef] [PubMed]
16. Bragg, Z.; Rieske, L.K. Spatial distribution and retention in loblolly pine seedlings of exogenous dsRNAs applied through roots. *Int. J. Mol. Sci.* **2022**, *23*, 9167. [CrossRef]
17. Mamta, B.; Rajam, M.V. RNAi technology: A new platform for crop pest control. *Physiol. Mol. Biol. Plants* **2017**, *23*, 487–501. [CrossRef] [PubMed]
18. Baum, J.; 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. [CrossRef]
19. Rodrigues, T.B.; Mishra, S.K.; Sridharan, K.; Barnes, E.R.; Alyokhin, A.; Tuttle, R.; Kokulapalan, W.; Garby, D.; Skizim, N.J.; Tang, Y.-W.; et al. First sprayable double-stranded RNA-based biopesticide product targets *Proteasome* subunit beta type-5 in Colorado potato beetle (*Leptinotarsa decemlineata*). *Front. Plant Sci.* **2021**, *12*, 728652. [CrossRef]
20. Zhao, C.; Alvarez-Gonzales, M.A.; Poland, T.M.; Mittapalli, O. Core RNAi machinery and gene knockdown in the emerald ash borer (*Agrilus planipennis*). *J. Insect Physiol.* **2015**, *72*, 70–78. [CrossRef] [PubMed]
21. Rodrigues, T.B.; Duan, J.J.; Palli, S.R.; Rieske, L.K. Identification of highly effective target genes for RNAi-mediated control of emerald ash borer, *Agrilus planipennis*. *Sci. Rep.* **2018**, *8*, 5020. [CrossRef]
22. Kyre, B.R.; Rodrigues, T.B.; Rieske, L.K. RNA interference and validation of reference genes for gene expression analyses using qPCR in southern pine beetle, *Dendroctonus frontalis*. *Sci. Rep.* **2019**, *9*, 5640. [CrossRef] [PubMed]
23. Kyre, B.R.; Bentz, B.J.; Rieske, L.K. Susceptibility of mountain pine beetle (*Dendroctonus ponderosae* Hopkins) to gene silencing through RNAi provides potential as a novel management tool. *For. Ecol. Manag.* **2020**, *473*, 118322. [CrossRef]
24. Kyre, B.R.; Rieske, L.K. Using RNAi to silence heat shock protein has congeneric effects in North America's *Dendroctonus* bark beetles. *For. Ecol. Manag.* **2022**, *520*, 120367. [CrossRef]
25. Rodrigues, T.B.; Dhandapani, R.K.; Duan, J.J.; Palli, S.R. RNA interference in the Asian longhorned beetle: Identification of key RNAi genes and reference genes for RT-qPCR. *Sci. Rep.* **2017**, *7*, 8913. [CrossRef] [PubMed]
26. Dhandapani, R.K.; Gurusamy, D.; Duan, J.J.; Palli, S.R. RNAi for management of Asian long-horned beetle, *Anoplophora glabripennis*: Identification of target genes. *J. Pest Sci.* **2020**, *93*, 823–832. [CrossRef]
27. Pampolini, F.; Rieske, L.K. Emerald ash borer specific gene silencing has no effect on non-target organisms. *Front. Agron.* **2020**, *2*, 608827. [CrossRef]
28. Pampolini, F.; Rodrigues, T.B.; Leelesh, R.S.; Kawashima, T.; Rieske, L.K. Confocal microscopy provides visual evidence and confirms the feasibility of dsRNA delivery to emerald ash borer through plant tissues. *J. Pest Sci.* **2020**, *93*, 1143–1153. [CrossRef]
29. Yu, N.; Christiaens, O.; Liu, J.; Niu, J.; Cappelle, K.; Caccia, S.; Huvenne, H.; Smagghe, G. Delivery of dsRNA for RNAi in insects: An overview and future directions. *Insect Sci.* **2013**, *20*, 4–14. [CrossRef]
30. Zhang, H.; Li, H.; Miao, X. Feasibility, limitation, and possible solutions of RNAi-based technology for insect pest control. *Insect Sci.* **2013**, *20*, 15–30. [CrossRef]
31. Lelito, J.P.; Watt, T.J.; Duan, J.J. Mass-rearing of emerald ash borer and its parasitoids. In *Biology and Control of Emerald Ash Borer*; Van Driesche, R.G., Reardon, R.C., Eds.; FHTET-2014-09; Department of Agriculture, Forest Service, Forest Health Technology Enterprise Team: Morgantown, WV, USA, 2015; Chapter 8; pp. 129–137. Available online: https://www.fs.fed.us/foresthealth/technology/pdfs/FHTET-2014-09_Biology_Control_EAB.pdf (accessed on 27 April 2015).
32. Rivera-Vega, L.; Mamidala, P.; Koch, J.L.; Mason, M.; Mittapalli, O. Evaluation of reference genes for expression studies in ash (*Fraxinus* spp.). *Plant Mol. Biol. Rep.* **2012**, *30*, 242–245. [CrossRef]
33. Madeira, F.; Park, Y.M.; Lee, J.; Buso, N.; Gur, T.; Madhusoodanan, N.; Basutkar, P.; Tivey, A.R.N.; Potter, S.C.; Finn, R.D.; et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res.* **2019**, *47*, W636–W641. [CrossRef]

34. Olson, D.; Rieske, L.K. Host range expansion may provide enemy free space for the highly invasive emerald ash borer. *Biol. Invasions.* **2018**, *21*, 625–635. [CrossRef]
35. Rajarapu, S.P.; Mamidala, P.; Mittapalli, O. Validation of reference genes for gene expression studies in the emerald ash borer (*Agrilus planipennis*). *Insect Sci.* **2012**, *19*, 41–46. [CrossRef]
36. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
37. Willow, J.; Cook, S.M.; Veromann, E.; Smagghe, G. Uniting RNAi technology and conservation biocontrol to promote global food security and agrobiodiversity. *Front. Bioeng. Biotechnol.* **2022**, *10*, 871651. [CrossRef]
38. 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. *Int. J. Mol. Sci.* **2022**, *23*, 6639. [CrossRef]
39. 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* **2021**, *10*, 264. [CrossRef] [PubMed]
40. Wang, M.; Dean, R. Movement of small RNAs in and between plants and fungi. *Mol. Plant Pathol.* **2020**, *21*, 589–601. [CrossRef]
41. de Andrade, E.C.; Hunter, W.B. RNA interference–natural gene-based technology for highly specific pest control (HiSPeC). In *RNA Interference*; InTech: Rijeka, Croatia, 2016. [CrossRef]
42. Biedenkopf, D.; Will, T.; Knauer, T.; Jelonek, L.; Furch, A.; Busche, T.; Koch, A. Systemic spreading of exogenous applied RNA biopesticides in the crop plant *Hordeum vulgare*. *ExRNA* **2020**, *2*, 12. [CrossRef]
43. Doering-Saad, C.; Newbury, H.J.; Bale, J.S.; Pritchard, J. Use of aphid stylectomy and RT-PCR for the detection of transporter mRNAs in sieve elements. *J. Exp. Bot.* **2002**, *53*, 631–637. [CrossRef]
44. Taning, C.N.T.; Andrade, E.C.; Hunter, W.B.; Christiaens, O.; Smagghe, G. Asian citrus psyllid RNAi pathway RNAi evidence. *Sci. Rep.* **2016**, *6*, 38082. [CrossRef]
45. Brosnan, C.A.; Sawyer, A.; de Felippes, F.F.; Carroll, B.J.; Waterhouse, P.M.; Mitter, N. Intact double stranded RNA is mobile and triggers RNAi against viral and fungal plant pathogens. *bioRxiv.* 2021, *preprint*. [CrossRef]
46. Koch, A.; Biedenkopf, D.; Furch, A.; Weber, L.; Rossbach, O.; Abdellatef, E.; Linicus, L.; Johannsmeier, J.; Jelonek, L.; Goesmann, A.; et al. An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. *PLoS Pathog.* **2016**, *12*, e1005901. [CrossRef]
47. Wang, M.; Weiberg, A.; Lin, F.M.; Thomma, B.; Huang, H.D.; Jin, H. Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* **2016**, *2*, 16151. [CrossRef]
48. 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. *Front. Plant Sci.* **2019**, *10*, 1319. [CrossRef]