Research Article

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Trunk injection delivery of dsRNA for RNAi-based pest control in apple trees

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Abstract

BACKGROUND: RNA interference (RNAi) is a promising new approach for controlling insect pests without the use of synthetic pesticides. Trunk injection is a delivery system for woody plants that harnesses the vascular system of the tree to transport materials to the tree canopy. Full size apple trees were injected with double-stranded RNA (dsRNA), and season-long leaf samples were taken to measure the vascular mobility and temporal persistence of dsRNA, using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

RESULTS: The qRT-PCR results revealed that the quantities of dsRNA in the apple leaves of treated trees were significantly greater than those in the leaves of untreated trees for both 2019 and 2020 studies. The peak dsRNA concentration in 2019 was 242 pg/30 mg of leaf tissue, and in 2020 was 16.4 pg/30 mg. The persistence of dsRNA in the apple tree canopy in 2019 was at least 84 days, and in 2020 was at least 141 days.

CONCLUSIONS: The highest mean measurement of dsRNA on a single date in 2019 was 242 pg, which is approximately equivalent to 8 ng/1 g leaf tissue. The projection using the highest replicate concentration from the same date is approximately equivalent to 27 ng/1 g leaf tissue, which may be sufficient to be considered biologically active.

Supporting information may be found in the online version of this article.

Keywords: vascular delivery; RNA interference; integrated pest management

1 INTRODUCTION

RNA interference (RNAi) technology has been increasingly exploited in the field of entomology and considered a potential tool for integrated pest management (IPM). RNAi is a biological process of silencing a gene's expression through sequence-specific translational inhibition or degradation of RNAs and which is guided by small RNAs.[1] In arthropods, three small RNA-directed silencing pathways exist: the small-interfering RNA (siRNA) pathway, microRNA (miRNA) pathway, and Piwi-interacting (piRNA) RNA pathway. RNAi-based insect pest control products that exploit the siRNA pathway have been registered with the US Environmental Protection Agency, with the first products expected on the market in 2022. These products engage the siRNA pathway through oral delivery of exogenous long double-stranded RNA (dsRNA). The orally delivered dsRNAs are taken up by the cells lining the insect midgut lumen and the siRNA pathway is initiated with recognition of the long dsRNA molecules in the cell cytoplasm by the ribonuclease III enzyme Dicer-2 (Dcr2) and processing into ~21-nt siRNAs.[2,3] The siRNA duplex is complexed with the Argonaute-2 (Ago2) protein of the RNA-induced silencing complex (RISC). After loading, the sense (passenger) strand is degraded leaving the guide strand to direct Ago2-mediated cleavage of specific target messenger RNAs (mRNAs) through Watson–Crick base pairing.[4,5] The siRNA-directed degradation of mRNA transcripts of essential genes leads to reduced fitness or death of the target arthropods.

Studies have shown positive results when dsRNA was administered orally to different species of various arthropod orders[6–8] including a number of economically important apple pests. For example, high mortality rates were observed when brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål), nymphs were fed with dsRNA designed against their three housekeeping genes.[9] Feeding codling moths, *Cydia pomonella* (L.), dsRNA targeting the cullin-1 gene impaired larval growth of this species.[10]

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The survivorship and fecundity of two spotted spider mites, *Tetranychus urticae* (Koch), were significantly decreased when fed with three dsRNAs targeting their cellular function genes (TuSNAPα, Turop, and TucoPB2).[11] These observations imply that RNAi is a promising alternative tool for controlling tree fruit pests.

To screen and identify suitable RNAi target genes for pest control, researchers employ microinjection, soaking, topical application, and ingestion as routes-of-entry to deliver the dsRNA molecules.[12] Oral ingestion methods used in the laboratory and glasshouse to assess candidate RNAi targets are most practically relevant to RNAi-based pest management products currently in development which include RNAi biopesticide sprays and plant-incorporated protectants that are delivered via transgenic plant expression to feeding arthropod pests. The success of oral dsRNA delivery to induce RNAi varies widely across arthropods due to the influence of a range of factors that limit arthropod RNAi sensitivity including insect gut pH, the activity of ribonucleases in the gut or hemolymph, insufficient uptake and release of dsRNA in the arthropod's cells, low expression of the RNAi machinery, and impaired or absent systemic spread of the RNAi signal.[13,14] Further, feeding adaptations and behaviors may also limit oral dsRNA delivery. For example, oral delivery of RNAi-based topical sprays, to piercing-sucking insects that feed by piercing cells or plant vascular tissues to access liquid nutrition from these locations is more challenging than to chewing insects.[15]

In plants, movement and transport of endogenous small RNAs is common and is involved in gene regulation for various functions such as plant defense, growth, adaptation to changing environment and many others.[16–19] These movements occur mainly through plasmodesmata for intercellular, cell-to-cell spread or through phloem for systemic, long-distance spread.[20,21] Evidence of uptake and mobility of exogenous long dsRNAs delivered via different methods have also been reported in various herbaceous and woody plant species.[15,22–25] Long dsRNAs applied by absorption through the roots, petiole absorption, and by trunk injection are transported intact through the xylem and apoplast where they can be ingested by feeding arthropod pests and subsequently processed via the pest's siRNA pathway.[15,22,23] In laboratory and glasshouse studies, Li *et al.*[15] and Hunter *et al.*[22] demonstrated that translocation of dsRNAs through the plant xylem could be used to overcome delivery barriers related to insect feeding type and feeding behavior. In separate experiments, root-absorbed dsRNAs targeting the piercing-sucking brown planthopper and the stem-boring Asian corn borer, respectively, were delivered to feeding insects and engaged the RNAi pathways in each. Movement of BMSB targeting-dsRNAs through vascular tissue was also demonstrated in green beans when immersed in a dsRNA solution, and the translocated dsRNA were found to elicit its adverse effects on this piercing-sucking pest species.[24] Hunter *et al.*[22] reported transport of exogenous long dsRNA, delivered through root drench or trunk injection, in small potted citrus trees and grape vines. These authors observed that dsRNA was translocated into the Asian citrus psyllid, a phloem-feeding insect, and the glassy winged sharpshooter, a xylem feeding insect, when exposed to dsRNA-treated plants that had been treated by root drench.

Trunk injection is a targeted delivery system for applying crop protection materials to tree crops that harnesses the vascular system of the tree to transport crop protection materials to the tree canopy. This technique has proven to be especially advantageous for systemic and biorational pesticides that are sensitive to ultraviolet (UV) and environmental degradation on canopy surfaces.[26,27] Trunk injection ensures maximum delivery of crop protection materials to the tree canopy, while minimizing environmental degradation and eliminating off-target spray drift.[28,29] This technique is efficient for controlling insects with various feeding habits such as foliage-feeding and sucking-piercing tree fruit pests.[30]

To date, demonstrations of trunk injection delivery of dsRNA in fruit trees has been limited to potted trees under semi-field conditions, and the measurement of dsRNA persistence in tree foliage limited to days or weeks of duration. The objective of this study was to determine the effectiveness of trunk injection in delivering dsRNA to the canopy of full-size apple trees under orchard growing conditions. In addition, temporal persistence of dsRNA in the foliage over multiple months was measured in order to project if sufficient titer can be maintained to expect RNAi effects on target pests.

2 MATERIALS AND METHODS

2.1 Field trials

This field study was conducted in a 20 year-old semi-dwarf 'Golden Delicious' apple (*Malus pumila* Miller) orchard at the Trevor Nichols Research Center (42° 35′ 42.4″ N, 86° 09′ 22.0″ W), Michigan State University, Fennville, MI, USA. Tree trunk diameter averaged 18–20 cm at 0.3 m above the ground, and tree canopies averaged 3 m high by 3 m wide. The experimental design included single tree plots in a randomized complete block design, with four replicates in 2019 and three replicates in 2020. Treatments in both years included a 500-bp *in vitro* transcribed intermolecular dsRNA molecule (%GC = 34%) (Genolution, Inc. AgroRNA, Seoul, South Korea) corresponding to a partial sequence of a *T. urticae* gene (TechAccel, LLC, St Louis, MO, USA) and untreated trees as controls with a minimum of one buffer tree on each side. Trees were injected one time on a single date, and different trees within the same orchard were injected in the second year. In 2019, injections were made on May 20 at tight cluster phenology stage of apples, with an approximate air temperature of 11 °C (www.enviroweather.msu.edu). In 2020, injections were made on April 8 at bud swell phenology stage of apples (adjusted to work around impending COVID-19 travel restrictions), with an approximate air temperature of 15 °C. Injections of 250-mL dsRNA treatment solutions (4 mg dsRNA/mL of solution) using the TREE I.V. system (Arborjet, Woburn, MA, USA). Four #4 Arborplugs® (Arborjet) per tree were tapped into a 0.95 cm hole drilled 5.1 cm deep, at 30–40 cm above the ground (Fig. 1). Injections were strategically placed under main scaffold branches of each tree. Each treated replicate tree received 1 g of dsRNA.

Leaf samples were collected from treated and untreated apple trees at predetermined dates throughout the growing season. Forty leaves were collected from each tree (approximately 20 g), ten leaves from each cardinal quadrant of the canopy. Collected samples were placed in tight sealing plastic bags, placed in separate coolers and transferred to the laboratory for processing. In 2019, leaf samples were collected 3 (May 23), 14 (June 3), 35 (June 24), 56 (July 15) and 84 (August 12) days after treatment (DAT). In 2020, leaf samples were collected 56 (3 June), 84 (1 July), 112 (29 July) and 141 (27 August) DAT. The original 2020 sampling regime was modified because of COVID-19 travel restrictions, thus missing early season sampling planned for April and May.

2.2 Processing of apple leaf samples

Leaf samples were transported in a cooler with ice packs to the Michigan State University laboratory on the same day of the collection from the field. Upon arrival at the laboratory, the leaf

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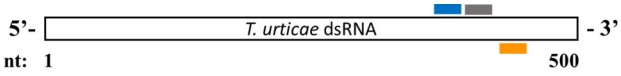
Fig. 1. Trunk injection of dsRNA into a 20 year-old semi-dwarf apple tree using the TREE I.V. system for delivering dsRNA treatment solutions, with injection ports placed under main scaffold branches of each tree.

samples were ground in liquid nitrogen, using separate autoclaved mortars and pestles for each of the dsRNA-treated plot and untreated plot samples. Then, 1 g of the leaf powder from each treatment and replicate were transferred into 1.7-mL RNase-free microcentrifuge tubes, and were stored at −80 °C until further processing and quantification.

2.3 Quantification of dsRNA concentration in leaf tissues

Frozen, ground leaf samples were packaged with dry ice and shipped to ARQ Genetics (Bastrop TX, USA) for dsRNA quantification. RNA was extracted with the Sigma Spectrum Plant Total RNA Kit (St Louis, MO, USA) according to the manufacturer's protocol from approximately 30 mg of plant tissue per sample and eluted in a final volume of 50 µL in RNAse-free water. The dsRNA-specific primers and probe were designed to detect a 69-bp fragment of the 500-bp dsRNA (Fig. 2) (Supporting Information Fig. S1). Absolute quantification of dsRNA was performed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) using a TaqMan MGB (minor groove binder) probe that is dual-labeled with a 5′-FAM fluorophore and a 3′ non-fluorescent quencher (NFQ) (ThermoFisher Scientific, Waltham, MA, USA). A one-step qRT-PCR was performed using the iTaq Universal Probes One-Step Kit (BioRad, Hercules, CA, USA), a total reaction volume of 10 µL, and 3.75 µL of RNA template per reaction. Consistent with Haddad *et al.*,[31] the following qRT-PCR cycling parameters were found to improve dsRNA assay sensitivity and specificity: RNA samples were heat-denatured at 95 °C for 5 min in the presence of 900 nmol/L forward and reverse primers and snap chilled on ice prior to the addition of the iTaq polymerase and buffer, the



Amplicon Length: 69

	Start	Stop	Tm (°C)	%GC
Forward:	370	391	60	50
Probe*:	393	411	70	32
Reverse:	419	438	60	50

Fig. 2. Schematic of the primer and probe locations on the 500 bp dsRNA sequence. *Dual labeled probe (5′ Modification: FAM; 3′ Modification: MGB-NFQ).

reverse transcriptase, and the probe (250 nmol/L). Cycling conditions were as follows: 50 °C for 10 min for reverse transcription with the iScript RNAse H+ reverse transcriptase, 95 °C for 3 min for polymerase activation, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The qRT-PCR was performed using the BioRad CFX384 Real Time System (BioRad). Sample reactions were run in triplicate, including a no-template control in triplicate.

Data analysis was performed using CFX Manager software from BioRad version 3.1. Each qRT-PCR run included a standard curve consisting of a six sample ten-fold dilution series ($R^2 > 0.98$ for each) with sample concentrations ranging from 2×10^7 to 2×10^2 dsRNA copies per PCR reaction. The experimental Cq (cycle quantification) was calibrated against this standard curve for quantification of each sample. Copy number of dsRNA per femtogram was calculated using the RNA Molecular Weight Calculator (AAT Bioquest: https://www.aatbio.com/tools/calculate-RNA-molecular-weight-mw), selecting 5′ hydroxyl modification, and entering the 500 bp sense strand RNA sequence, resulting in output molecular weight (MW) data for both sense (160.105 kDa) and anti-sense (160.022 kDa) strands. Copy number per 1 fg dsRNA was derived as follows:

1 mol 500 bp dsRNA = 320.127 kDa or kg/mol
Number of molecules in a mole = 6.0221×10^{23}
Estimated mass of one copy of the 500 bp dsRNA:
= (320 127 g/mol)/(6.0221×10^{23} molecule/mol)
= $5.316 \times 10^{-19} \times (1\ \text{fg}/1 \times 10^{-15})$
= 5×10^{-4} fg

Therefore, from these calculations, 1 fg was estimated to equal 2000 copies. The dsRNA copy numbers were converted to mass and graphically displayed to compare temporal delivery patterns across treatments.

2.4 Statistical analysis

The amount of dsRNA detected in apple tree leaves was analyzed over time. Data were transformed with $\log_{10}(x)$ for the 2020 trial and with $\log_{10}(x+1)$ for the 2019 trial prior to the analysis. Three measurements were missing in the untreated control for the 2019 data. A repeated measures analysis of variance (ANOVA) with mixed model approach was performed by using Proc MIXED procedure in SAS 9.4 (SAS Institute, Cary, NC, USA). Treatment, sampling day, and their interaction were entered as fixed effects in the model with block as a random effect. The selection of the covariance structure was carried out through visualization of the plot of covariance as a function of distance between pairs of sampling days, and also by applying Akaike's information criterion with small sample correction (AICC). When the values of the information criteria were similar, the simpler structure was opted to

increase the power of the analysis.[32] The compound symmetry covariance structure was then selected for both 2020 and 2019 data. The experimental unit tree was used as the subject of the repeated measures over time. The main effects of treatment and sampling day and their interaction were evaluated. With an occurrence of statistical difference in the main treatment effect, a separation of treatment means by sampling days was performed. Multiple comparisons of least square means for treatment by sampling day sliced by treatment was conducted using Proc GLIMMIX procedure in SAS 9.4 (SAS Institute). The Kenward–Roger degrees of freedom approximation was applied in all analyses and alpha at 0.05 was used for all tests. Non-transformed data were reported in the figures.

3 RESULTS

The analysis of dsRNA concentrations in the treated and untreated tree leaves over time indicates a lack of significant interaction between treatment and sampling day ($F_{4,14.8} = 0.59$, $P = 0.673$) for the 2019 data (Fig. 3(A)). However, a significant main effect of the sampling day ($F_{4,14.18} = 8.81$, $P < 0.001$) was detected, suggesting a variation in the quantity of dsRNA in leaves from dsRNA-treated trees over time. A statistical difference in the overall amount of the dsRNA between treated and untreated control trees was also observed ($F_{1,2.74} = 22.65$, $P = 0.021$). The follow-up test reveals that the quantities of dsRNA in the leaves of treated trees were significantly greater than those in the leaves of untreated trees for all five sampling days 3, 14, 35, 56 and 84 DAT ($F_{19.34} = 2.79$, $P = 0.011$; $F_{19.34} = 3.40$, $P = 0.003$; $F_{19.44} = 2.48$, $P = 0.022$; $F_{16.89} = 3.83$, $P = 0.001$; $F_{16.89} = 2.19$, $P = 0.042$; respectively) (Fig. 3(A)).

Similar results were observed in the 2020 data (Fig. 3(B)). There was no interaction effect between the treatment and sampling days ($F_{3,8.79} = 0.88$, $P = 0.487$) on the amount of dsRNA in the apple leaves, whereas the main effects of both the sampling day and the treatment were statistically significant ($F_{3,8.79} = 6.00$, $P = 0.016$; $F_{1,1.84} = 32.06$, $P = 0.035$, respectively). The follow-up test indicates that the amount of dsRNA in the leaves of treated trees was significantly higher than in the leaves of untreated trees for all four sampling days 56, 84, 112 and 141 DAT ($F_{8.32} = 3.17$,

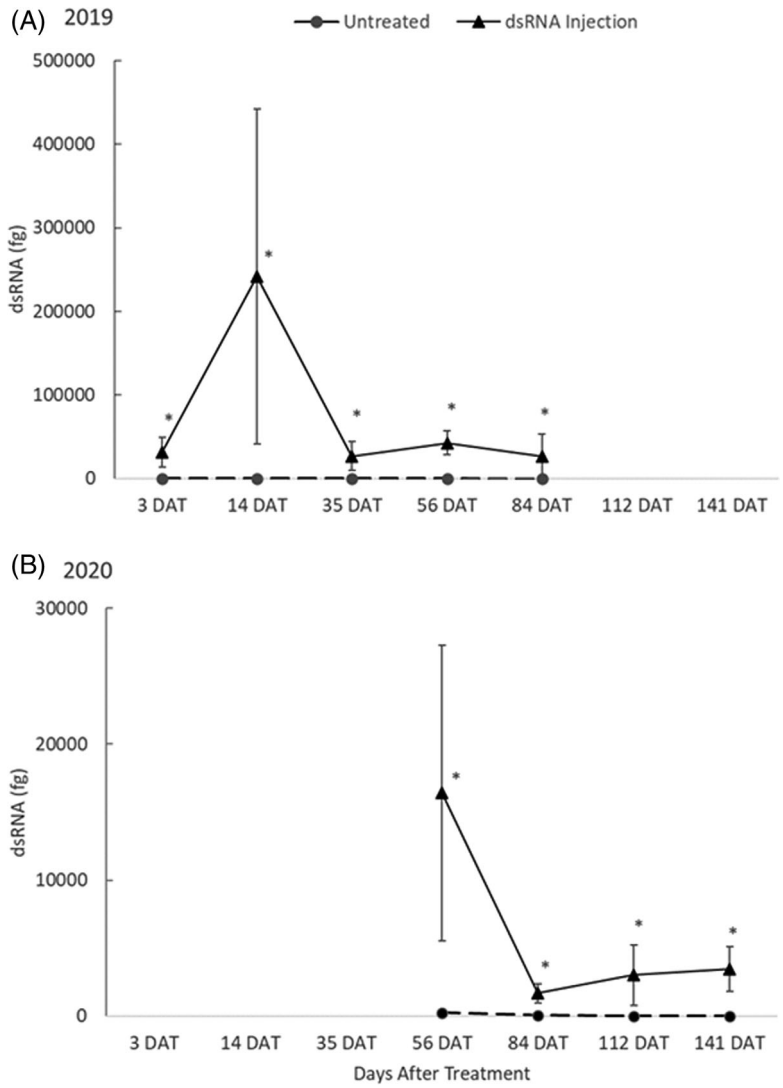


Fig. 3. Mean amount of dsRNA (femtogram) ± SE in the apple leaves for the 2019 (A) and 2020 (B) trials. 2019 days after treatment (DAT): 3 (23 May), 14 (3 June), 35 (24 June), 56 (15 July) and 84 (12 August). 2020 DAT: 56 (3 June), 84 (1 July), 112 (29 July) and 141 (27 August) days after treatment (DAT). Asterisk indicates significant difference between treated and untreated control (t test, $p < 0.05$) on the same day after treatment (DAT).

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$P = 0.012$; $F_{8.32} = 3.62$, $P = 0.006$; $F_{8.32} = 3.87$, $P = 0.004$; and $F_{8.32} = 5.01$, $P < 0.001$; respectively) (Fig. 3(B)).

4 DISCUSSION

This study provides important new knowledge as to the temporal distribution of dsRNA in the apple tree canopy, following trunk injection under orchard conditions. The temporal pattern of dsRNA mobility and persistence in the apple tree canopy was similar to what has been documented for small molecule insecticides and biopesticides. In comparison, the water-soluble systemic insecticide, imidacloprid, attains peak concentrations in apple leaves by 14 DAT (June 13, 2011) and persists in the apple tree canopy for approximately 3 months, following trunk injection.[33] The biopesticide, azadirachtin, attains peak concentrations in pear leaves at 7 DAT (June 1, 2017), and persists in the canopy for approximately 1 month, following trunk injection.[27]

There are several factors that may explain the significant change in dsRNA concentrations in the apple tree canopies over time. In 2019, the highest dsRNA concentrations were recorded on 14 DAT and then slowly declined over 70 days. Given that the dsRNA concentrations between the 2 years were similar at the 56 DAT timeline (2019: 26960 fg; 2020: 16409 fg), we expect that the peak delivery in 2020 was missed, as 3–35 DAT samples were not collected (because of COVID travel restrictions). The gradual decline over time may be caused in part by degradation of dsRNA in the leaf tissues. Another factor potentially contributing to the decline in detected dsRNA that this study does not address is cellular uptake of dsRNAs and subsequent processing into siRNAs. Future studies should address this possibility. Comparatively, when Dalakouras *et al.*[23] injected potted apple trees (1500 µg/tree) and potted grape vines (500 µg/tree) with hairpin RNA (hpRNA), detections in leaf tissues declined over 10 days. Similarly, Ghosh *et al.*[34] showed persistence of dsRNA was greatly diminished after 6 days, following delivery in beans (0.017 µg/µL). Hunter *et al.*[22] also documented persistence of dsRNA in citrus trees (2.5 m tall) (2 g dsRNA/tree) for up to 8 weeks. Moving from small-scale laboratory/glasshouse bioassays (Gosh *et al.* and Dalakouras *et al.* studies) to full size orchard trees (Hunter *et al.* and Wise *et al.* studies), along with the higher doses used in large tree studies, appears to result in longer duration of RNA persistence in tree canopies.

Tree phenology is likely another important factor influencing the distribution and persistence of dsRNA in orchard trees over the growing season. For both years of this dsRNA injection study, the peak concentrations that were measured in the canopy were in early June, shortly after petal fall stage in apples. Bloom to petal fall stages in apples represent a shift from the early season bud break and leaf tissue expansion period, fueled largely by stored carbohydrates in the tree, to rapid summer shoot expansion fueled by photosynthesis-driven energy.[35,36] The number of fully expanded leaves at petal fall stage will approximately quintuple over the following 2 months (Phil Schwallier, personal communication). Thus, if peak delivery concentration of dsRNA to the canopy is achieved within 2 weeks of injection, even if no dsRNA degradation were to occur, the canopy concentration would be expected to drop five-fold solely from the expansion of shoot and leaf tissue over the growing season.

PCR results showed that the 2019 trunk injection of dsRNA delivered the highest concentrations to the tree canopy, reaching a mean value of 241 923 fg (242 pg), whereas the 2020 trunk injection mean levels reached only 16 409 fg (16.4 pg) (although the true peak may have been missed). The 2019 recorded peak of 242 pg/30 mg of leaf tissue is approximately equivalent to 8 ng/1 g leaf tissue (average fully expanded apple leaf is 0.5 g). The projection using the highest replicate concentration from the same date (June 3, 2019) is approximately equivalent to 27 ng/1 g leaf tissue. Based on other RNAi studies, such as Armstrong *et al.*,[37] this concentration may be sufficient to be biologically active, depending on the pest and dsRNA being delivered. Further research is needed to demonstrate biological activity in arthropod pests of apple, following dsRNA delivery via trunk injection.

In addition to dsRNA persistence in the tree canopy, other variables are likely to have important implications for injection-based dsRNA delivery strategies for arthropod pest management. Building on the results of this study, future research should characterize routes of dsRNA translocation and distribution of dsRNAs in different plant tissues and assess amounts of plant cellular uptake and processing of injected dsRNAs, and directly measure RNAi effects on arthropod pests exposed to treated plant tissues.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Michigan State University.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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