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Double Strand RNA Absorption in Citrus Trees for Delivery to Asian Citrus Psyllids, *Diaphorina citri:* (Hemiptera: Liviidae) †

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Abstract: RNA interference, RNAi, continues to be a leading tool in functional genomics studies and in crop plants, for the management of viral pathogens, insect pests, and improving plant traits. However, there are still questions concerning consistent delivery into plants when using topically applied RNAi. RNAi products have the potential to reduce psyllid vector populations by disrupting nymph survival, which may provide an effective strategy in the disruption of *Candidatus* Liberibacter asiaticus transmission throughout citrus trees. This study examined factors that may affect dsRNA absorption and systemic movement in citrus trees and the subsequent delivery into the hemipteran, *Diaphorina citri*, the Asian citrus psyllid.

Keywords: RNAi; *Diaphorina citri*; dsRNA absorption

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1. Introduction

Since the discovery of RNA interference (RNAi) more than two decades ago [1], this natural method of RNA transcript modulation via messenger RNA (mRNA) degradation, has been used as a primary tool for functional genomics studies in many organisms including plants [2] and insects [3,4]. RNAi reportedly holds great promise as a management tool as it has become a widely used tool in developing species-specific pest management [5,6,7]. The Asian citrus psyllid, *Diaphorina citri*, Kuwayama (Hemiptera: Liviidae) is an economical and agriculturally important insect due to the capacity to transmit the *Candidatus* Liberibacter asiaticus (CLas) bacteria that leads to citrus greening disease, also known as, Huanglongbing. As the primary vector in its niche, effective management of the psyllid vector is of great concern.

Plant-feeding hemipteran insects require the dsRNA, which triggers the RNAi mechanism, to be in the tissues they feed upon. For *D. citri*, and other phloem-feeding insects, this means delivery of the dsRNA treatment into the plant's sap (inside vascular tissues, phloem). Psyllids have piercing- sucking mouthparts, as such, RNAi trials with hemipteran insects have utilized the in Planta System (iPS) by root drench, foliar sprays, trunk injections, or absorption by cuttings [8,9,10].

The iPS has become a frequently used method for screening a wide variety of dsRNA triggers across many insect species to evaluate the functions of various genes. In addition to simulating a natural feeding environment for hemipterans, the use of iPS has shown great results in the Asian citrus psyllid, *Diaphorina citri* [8,7,11]. Though iPS is a natural

approach to utilizing screening of dsRNA for development of RNAi technology, there are factors surrounding the use of iPS for both screening and in field studies. This study assessed three parameters that may impact the success of dsRNA-iPS screening process and potential success in field applications.

2. Materials and Methods

Citrus plants and the Asian citrus psyllid colony were maintained in a greenhouse under natural light (14D:10L) and temperature ~26 °C with *D. citri* colony being reared on *Citrus macrophylla*. In preparation for the various assays, branches of the respective citrus plants were clipped at a 45° angle under water with a sterile razor blade after soaking in 0.2% bleach for ~ 10 mins and rinsing twice with water.

2.1. Absorption of dsRNA by Citrus Cuttings

Thirty mature and young flush of Rootstock *Carrizo citrange* and *Citrus macrophylla* were used to assess the quantity of dsRNA absorbed in citrus cuttings. Mature plants were identified as plants whose leaves and thorns were hard, while flushes were identified as plants whose leaves and thorns were soft. The cuttings were placed in 500µL solution containing 0.5mg Chinese Sacbrood Virus (CSBV) dsRNA. CSBV-dsRNA is designed from a virus that targets the Western/European honey bee, *Apis mellifera*. In this study it is therefore used as a non-target gene for testing/observing method. Cuttings were left to absorb treatment solution for 2 days. The upper most leaf at the top of the stem was collected for RNA extraction.

2.2. Effects of Surfactant on dsRNA Absorption in Citrus Cuttings

Thirty Rootstock (*Carrizo citrange*) flush cuttings were placed in 500µL of treatment solution (0.5mg CSBV-dsRNA mixed with Silwet). Four treatment groups were used; 0.1%, 0.05%, 0.01% Silwet (Helena, TN), and control (0% Silwet). Cuttings were left to absorb treatment solution for 2 days. The upper most leaf at the top of the stem was collected for RNA extraction.

2.3. Soil Applied dsRNA and Absorption by Citrus Seedlings

Twenty potted Rootstock (*Carrizo citrange*) seedlings were used for this analysis. Two treatment groups were set up consisting of 10 plants treated with 50 mL solution containing 0.5mg CSBV-dsRNA and 10 plants treated with water (blank control) (50 mL). Each treatment had 5 pots with a single seedling and 5 pots with two seedlings each (Figure 1a). The top and bottom leaves were collected from each seedling after 2 days for RNA extraction. Leaves were collected from each plant for the pots with two seedlings.

2.4. Delivery and Ingestion of dsRNA by Asian Citrus Psyllid from Citrus Cuttings

2.4.1. Feeding Bioassay

The 'in planta system' was used to deliver dsRNA into citrus leaves and plants for psyllid feeding (Figure 1b and Figure 1c) [12]. Prepared citrus cuttings were transferred to a 1.5 mL centrifuge tube containing 500 µL of the respective treatment solution. Parafilm was wrapped around the mouth of tube to hold the plant stem in place and to cover the liquid solution. The tubes were placed under artificial lighting to stimulate uptake of solution for ~24 hours. Filtered water was then added to the tubes for sustaining cuttings. Tubes were then placed in a cage and *D. citri* added to cage after 24 hours. For adult *D. citri* Rootstock (*Carrizo citrange*) flush cuttings were used with two treatment groups; 500µL containing 0.5mg CSBV-dsRNA and 500µL control (water). Thirty cages were set up for each treatment and 4 adult *D. citri* were added to each cage. For *D. citri* nymphs, two treatment groups were used; 500µL containing 0.5mg CSBV-dsRNA and 500µL control (water). Thirty *D. citri* nymph infested *Citrus macrophylla* cuttings were placed in solutions for each treatment group respectively.

After 2-d feeding access, 1 adult and 1 nymph was collected per cage for RNA extraction and dsRNA quantification.

(a) **(b)** **(c)**

Figure 1 (a) Potted Rootstock *Carrizo citrange* seedlings with soil applied dsRNA treatments; **(b)** Inverted 50 mL Feeding Access Centrifuge Cage. The end of the centrifuge tube has been cut off, now being at the top. This open top is then capped with a screened lid insert (CELLTREAT, cell strainer, Product# 229486, 100 µm.), www.celltreat.com); **(c)** The screw on lid is the bottom of the cage and has a hole approximately 0.635 cm diameter, slightly larger than diameter to hold a 'cap-less' 2mL centrifuge vial. The lid can be unscrewed to remove the plant cutting or dead insects. The plastic vial was refilled with water using a sterile 10 mL syringe with a 26-gauge needle that is inserted through a hole made with the needle just below the blue cap. This permitted refilling without opening the cage.

2.5. RNA Extraction, dsRNA Quantification (qPCR)

To quantify the amount of dsRNA taken up by the plant cuttings/seedlings and *D. citri*, total RNA was extracted using the Direct-Zol™ RNA MicroPrep Plus kit (Zymo Research) according to manufacturer's instructions. The total RNA extracted was normalized to ~25ng/µL for all samples. A standard curve was created for quantification using the CSBV-dsRNA trigger used in experimental set up (1:10 dilution, 6-point dilution starting at 10ng). The master mix used was that of the One-Step SuperScript III Platinum SYBR Green qRT PCR kit (Invitrogen, CA) for a 25 µL 1X reaction using ~25ng/µL RNA template. Four technical replicates were conducted for both samples and standards when setting up qPCR plates with water used as a no template control.

2.6. Statistical analysis

Each biological replicate had four technical replicates. The quantities of CSBV (ng/µL) of each biological replicate represented the average of the quantities of CSBV (ng/µL) of the four technical replicates (as estimated from the standard curve). The data was analyzed using either a two way ANOVA (Type 3) for unbalanced designs, the Welch's t-test or the Two sample t-test. Mean separation *post-hoc* was conducted using Tukey HSD (α=0.05). Statistical analysis was conducted using R v3.4.4 and R studio software v1.1.442 (2009-2018 RStudio, Inc). If data was not normal it was log transformed. Graphs were constructed with back transformed data to facilitate interpretation.

3. Results and Discussion

No significant difference was observed in the amount of dsRNA absorbed by the two citrus varieties (P=0.32) (Figure 2a). The variety, cultivar, or species of citrus plant used appeared irrelevant to the absorption of dsRNA under these conditions. Though citrus plant variety did not affect dsRNA uptake, the age of the citrus leaves produced significant differences (Figure 2a). The new growth flushes of Rootstock absorbed significantly

more dsRNA than mature leaves of either plant ($P < 0.05$) (Figure 2a). Young leaves absorbed ~83% more dsRNA than the older leaves tested. Hall and Albrigo [13] highlighted that oviposition and development of D. citri is confined to the new growth, young flush of citrus trees. Since nymphs develop on the flush, based on these findings, they would potentially ingest greater concentrations of dsRNA if available. As such, systemic loading of dsRNA into the new growth tissues would be advantageous in citrus psyllid population suppression.

This study also compared the amount of dsRNA taken up by adult D. citri vs D. citri nymphs after feeding on treated cuttings. The nymphs absorbed significantly more dsRNA (~80% more) after a 2-d feeding access period as compared to the adults ($P < 0.001$) (Figure 2b). The nymphs may require more nutrients and water for growth as they develop through different instars and molting, requiring them to feeding more and thus ingesting more dsRNA in the natural feeding process. The greater concentration of dsRNA in nymphs may also be due to their more sessile life stage, while the adults tend to move about, stopping to feeding on both old and young tissues, moving around looking for mates. A synergistic approach to managing D. citri can be created, as observed, both the nymphs and the new growth flush they feed on absorb a greater concentration of the dsRNA. Research has highlighted that D. citri nymphs that develop on HLB infected trees are more likely to become infected and transmit the disease upon becoming adults, than adults that feed on infected trees and move on. This observation is thought to be as a result of the latency of HLB within the psyllid [14]. Therefore, with nymphs obtaining a greater concentration of the dsRNA treatment, it can have a positive impact on reducing not only the D. citri population but may reduce effective transmission of the HLB pathogen as well.

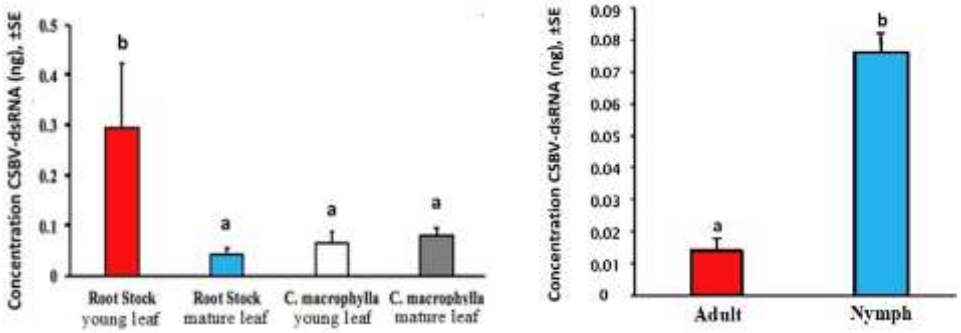
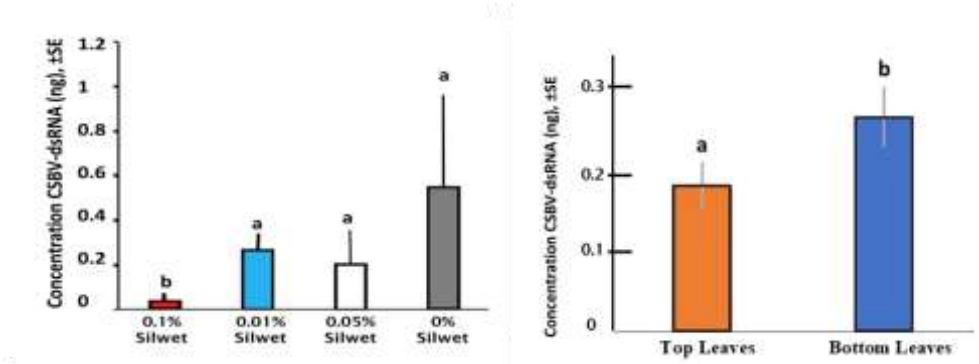


Figure 2 (a) Quantification of dsRNA concentration in young and mature leaves of treated Rootstock (*Carrizo citrange*) and alemow (*C. macrophylla*) citrus varieties. Mean values represent 30 individual leaves analyzed for each treatment. Leaf Age (Mature vs. young leaves, P=0.04) and plant variety (Rootstock *Carrizo citrange* vs. *C. macrophylla*, P=0.32); **(b)** Comparison of dsRNA concentration between single adult and 4th instar nymph after feeding for 48 hours. Mean values from 30 individuals processed from each treatment. Error bars are the ±SE of means. The dsRNA concentration between *D. citri* life stages was significantly different (adult vs nymphs, P<0.001).

Three concentrations of the surfactant (0.1%, 0.05% and 0.01%), were assessed for impact on the amount of dsRNA absorbed through plant stems. Though 0.1% worked optimally in sprayable solutions, it showed quite a contrast compared to absorption through stems of citrus cuttings. The concentration of Silwet L77 was inversely proportional to the amount of dsRNA quantified from the plant leaf. The results were shown to be highly significant showed that water alone moved the dsRNA through the stem more efficiently than with the addition of 0.1% Silwet L77, P<0.001 (Figure 3a). It should be noted that some surfactants degrade dsRNA, as such, they should be screened before being used on a large scale trial.

Soil applications of dsRNA showed that both the lowest, bottom mature leaves versus the upper, top leaves of treated citrus seedlings tested positive for dsRNA demonstrating systemic movement at 2 days post treatment (Figure 3b). These results support findings of dsRNA movement after soil application by Hunter et al. [10,16].

Analysis of pots with two seedlings and those with a single seedling, showed no significant difference in the absorbed dsRNA concentration in the leaves (P=0.24). This would suggest that root competition between plants did not negatively affect the absorption and distribution of the dsRNA in plants [15,16]. This is both helpful in the lab for the screening process as well as in the field, where complex rooting systems exist. In this study, the dsRNA was observed in the plants after 9 days at a lower concentration to that obtained after 2 days (data not shown) highlighting that though present, there is natural degradation of the dsRNA post treatment. As with any pest management applications, persistence is of great importance. The product should be effective within a time that does not cause a negative effect or accumulate in the environment [17].



(a) **(b)**

Figure 3. (a) Rootstock (*Carrizo citrange*) cuttings of flush treated with dsRNA (0.5mg) plus Silwet (surfactant) at different concentrations. Mean values represent 30 individual cuttings analyzed in each treatment. Error bars represent the ±SE of means. The dsRNA absorbed by plants treated with water only, (0% Silwet) had significantly greater concentration than those treated with 0.1% Silwet, ($P<0.001$); **(b)** The dsRNA was quantified in the young leaves at the top of potted rootstock *Carrizo citrange* seedlings, and in the older more mature leaves nearest the base of the citrus seedling. The mean concentration of dsRNA in Top and Bottom leaves was significantly different (Top vs. Bottom). Mean values shown are 15 individual samples per treatment replicated twice. $F(1, 20)=5.96$, $P=0.02$. Data was analyzed using Welch's t-test and Two sample t-test, $P<0.05$).

4. Conclusion

For quick efficient and reliable results when screening multiple targets for RNAi, this study demonstrated efficient delivery into cuttings using the in planta system, iPS, and soil applications [8]. Both mature leaves and new growth flushes acquired detectable amounts of dsRNA regardless of citrus variety when applied through soil or stem absorption. Future strategies might involve frequent pruning to maintain a constant supply of new growth flush to facilitate improved ingestion of the RNAi treatment in *D. citri* nymphs and adults. Nymphs were shown to ingest greater concentrations over adults, most likely due to being sessile and in a development growth stage. Based on these results, soil applied dsRNA treatments may provide a more cost-effective delivery of product over foliar spray applications [12,18,10,15,19]. Increasing concentration of dsRNA in trees results in a longer window of psyllid exposure, by ingestion, which may provide increased efficacy of RNAi psyllid suppression.

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J.Q.; project administration, W.H.; funding acquisition, W.H., J.Q. and S.B. All authors have read and agreed to the published version of the manuscript.

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