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Evaluation of RNA Interference (RNAi)-Based Biotechnological Strategies for the Control of Tomato Leaf Curl Disease Transmitted by Bemisia tabaci (Whitefly) in Tomato (Solanum lycopersicum) Plants

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Abstract

Bemisia tabaci whiteflies are the carrier of Tomato Leaf Curl Disease (ToLCD), which is a major issue for tomato production worldwide and seems to recur. In this study, we examined the potential efficacy of RNA interference (RNAi) biotechnology in treating ToLCD in tomatoes (Solanum lycopersicum). One hundred tomato plants were divided equally into control and RNAi-treated groups using a fully randomized method. Double-stranded RNA was sprayed on the skin to target the viral Rep gene as part of the RNA interference strategy. Following the plants' exposure to viruliferous whiteflies, a number of factors were monitored, including the number of whiteflies, the severity of the disease, the height of the plants, the leaf curl score, the viral load, and the yield per plant. The RNAi plants exhibited clear reductions in the number of whiteflies (approximately 53.3%), disease severity (57.5%), leaf curl score (from 4.46 to 1.84), and viral load (about 54.1%) as compared to the controls. In the meantime, the plants themselves appeared to grow bigger; their height went from 24.96 cm to 36.34 cm, and their output per plant increased by almost 90.5%, which is a significant gain. The differences were highly significant ($p < 0.001$) in each of these contrasts. Additionally, a Pearson correlation analysis revealed a significant negative relationship ($r = -0.908$) between viral load and yield. Even though the specifics still need to be worked out, RNAi appears to be a highly promising and possibly more sustainable route for controlling ToLCD in tomatoes.

Introduction

Tomato, (Solanum lycopersicum) is one of the most economically important vegetable crops worldwide, and it is an essential source of vitamins, antioxidants, also dietary nutrients for millions of people (1). However, global tomato production is kind of challenged by biotic stresses, and among them the Tomato Leaf Curl Disease, or ToLCD, really comes across as very destructive. ToLCD is caused by a complex of begomoviruses (family Geminiviridae) and it is transmitted in an exclusive manner by the whitefly, Bemisia tabaci (Gennadius), basically only by that vector (2). Infected tomato plants typically exhibit fairly common symptoms, such as excessive leaf bending, general stunting, and vein clearing. Yellowing and often smaller leaves follow, giving the impression that the plant is weaker or less resilient overall. Over time, this sort of issue can cause major problems and drastically lower the harvest, sometimes by as much as 50% or even 100%. It all depends on when the infection first appears, the local circumstances in the region, and the surrounding environment (3). Insecticide spraying to keep whitefly populations low, rogueing of plants that appear sick, and cultural practices like crop rotation or barrier cropping are the mainstays of conventional disease control. However, in reality, the entire strategy is becoming less successful since begomoviruses exhibit a high rate of mutation along with

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significant genetic variety, making things feel, well, less predictable, and whiteflies are rapidly gaining pesticide resistance (4). Consequently, there is this urgent need for novel sustainable and kind to the environment biotechnological approaches, that can hit both the viral pathogen and its insect vector at the same time. Lately, a few advances have shown that RNA interference can work for this dual objective quite well; by aiming at endogenous genes inside B. tabaci so it leads to mortality, and also at viral transcripts to dull or weaken disease symptoms. (5). This post transcriptional gene silencing mechanism basically causes the breakdown of like mRNAs, so protein production is effectively blocked and the total pathogenicity of the infecting viruses gets reduced (6).

RNA interference (RNAi) has emerged as a promising next-generation strategy for managing plant viral diseases, including those caused by geminiviruses (7). RNAi is like a naturally occurring gene silencing mechanism that is conserved in eukaryotes, in which double stranded RNA (dsRNA) molecules get processed by the Dicer enzyme into small interfering RNAs (siRNAs) which then guide the sequence specific breakdown of matching target messenger RNA (mRNA). In the plant protection context, RNAi can be used by engineering plants so they end up producing dsRNA that is homologous to essential viral genes. Then the plant's post transcriptional gene silencing (PTGS) machinery is triggered to munch through the viral transcripts and keep the virus from replicating and from spreading systemically (8). Moreover, recent advances have shown that RNAi can also be aimed at whitefly genes when the insect, takes in dsRNA while it is feeding, kind of like environmental RNAi or host-induced gene silencing (HIGS) (8,9). This sort of dual capability makes RNAi really attractive for handling circulative, persistently transmitted viruses like tomato leaf curl virus (ToLCV), because if you target either the virus itself or the vector's transmission related genes, it can disturb the whole disease cycle in one way or another (10).

A few studies have really managed to deploy RNAi mediated resistance against begomoviruses in model plants, however when it comes to translational work in commercially relevant tomato genotypes, under properly controlled inoculation conditions there is still some sort of gap. And especially limited are investigations that, at the same time, look at more than one disease signal along with additional plant health metrics, because otherwise you only get part of the picture (11). By integrating sequence-specific silencing with the targeting of key viral determinants or vector susceptibility genes, this approach offers a robust pathway toward durable crop protection (12,13).

So, this present study was designed to check whether an RNAi based biotechnological approach can actually control Tomato Leaf Curl Disease in tomato plants (Solanum lycopersicum) after they were challenged by inoculation using viruliferous Bemisia tabaci whiteflies. More specifically, we sort of thought that an RNAi treatment would meaningfully bring down the whitefly colonization, and also the viral load and overall disease severity, while at the same time it should help boost plant growth, and yield, relative to the untreated control plants.

Materials and Methods

❖ Experimental Location and Duration

A controlled greenhouse environment was used for the experiment. The greenhouse itself maintained its natural photoperiods, which are around 10 hours of darkness and 14 hours of light. Throughout the day and night, the temperature was maintained at 28 ± 3°C, and the relative humidity remained consistently between 60 and 75%. Since research was conducted over the course of a single growing season, it essentially followed the schedule for tomato plants from vegetative growth to the fruiting stage, which takes around 12 to 14 weeks, give or take a little.

❖ Plant Material and Growing Conditions

Directly from a commercial seed provider, seeds from a disease-prone tomato cultivar (Solanum lycopersicum) were gathered. The seeds were washed three times with distilled water after being surface sterilized for about two minutes in a 1% sodium hypochlorite solution. They were then planted in 15-cm plastic containers. A sterilized growth mix consisting of soil, sand, and farmyard manure was added to each pot in a volume ratio of 2:1:1 (v/v/v).

The potting material was autoclaved for 20 minutes at 121°C before to planting in order to eliminate any infections or bothersome little insects. Every day, tap water was used to water the plants, but only when it was necessary. Fertilized every week beginning 14 days after germination with a balanced NPK combination (20:20:20, 1 g/L). Additionally, no chemical pesticides or fungicides were applied during the study, only to avoid interfering with the establishment of whiteflies and the results of the RNAi therapy.

❖ Source and Maintenance of Whitefly Vector

Bemisia tabaci specimens from untreated tomato fields were used to establish a laboratory colony. In a different growing chamber, the colony was housed in insect-proof cages (60 × 60 × 60 cm, 0.15 mm mesh) under greenhouse-like circumstances (28 ± 2°C, 65% relative humidity, 14:10 h light: dark). In order to prevent viral infection, whiteflies were reared on healthy, uninfected cotton plants (Gossypium hirsutum L.). Yes, a stereomicroscope was used to verify the species identify morphologically. Only mature whiteflies (3–5 days old, both sexes combined) were utilized in each inoculation session.

❖ Source of Tomato Leaf Curl Virus Inoculum

The tomato leaf curl virus (ToLCV) inoculum was extracted from naturally infected tomato plants, which exhibited severe

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leaf curl, stunting, and yellowing. We used begomovirus-specific degenerate primers in PCR to determine that it was indeed ToLCV, and that procedure was successful. Then, in order to ensure that nothing else interfered, we retained a pure isolate undergoing serial whitefly transmission and transferred it to healthy tomato plants under insect-proof circumstances. To ensure the virus titer remained high, the original infected source plants were kept in their own cage and examined with PCR every two weeks or so. Following that, only plants with a high viral load (≥ 8.0 relative units) and consistent severe symptoms were selected as virus donors for the whitefly acquisition access period or periods.

❖ RNA Interference Treatment Application

For the RNAi-treated group (n = 50) plants got a foliar application of double stranded RNA dsRNA aimed at the ToLCV Rep/AC1 gene. The dsRNA itself was made with a commercial transcription kit, then it was diluted to 500 ng/µL using nuclease free water, with 0.02% Silwet L-77 mixed in. After that, each plant received 10 mL delivered with a hand-held atomizer. We did the treatments when the plants were at the four-leaf stage, so 14 days after sowing, then once more 7 days later at 21 days after sowing. The control plants (n = 50) were handled in the same way, except they received only nuclease-free water containing 0.02% Silwet L-77, and applied on the same timetable. After every application, all plants were left to air-dry for about 2 hours, before they were put back into the greenhouse.

❖ Whitefly Inoculation and Virus Transmission

Around 500 adult whiteflies were shifted from the healthy rearing colony onto ToLCV- infected source plants, for that 48-hour acquisition access period (AAP), kind of straight forward. After the AAP was done, the whiteflies were gathered up and later a small batch (n = 20) was PCR checked to see if the virus was there. Then at 28 days after sowing (which is 7 days after the second RNAi application), each untreated control and each RNAi-treated plant got challenged with 20 viruliferous whiteflies. Those insects were confined to the youngest fully expanded leaf, using clip-on cages during a 72-hour inoculation access period (IAP). After the IAP wrapped up, the whiteflies got taken off or removed somehow, and the plants were then sprayed with imidacloprid at 0.5 mL/L just to clear out whatever remaining insects might still be there. Next, they stayed in the greenhouse for symptom watch, day by day, like pretty much continuous observation.

❖ Experimental Design

For this experiment, a fairly randomized design (CRD) was used. In total, there were one hundred tomato plants that were broken into two equal groups, sort of an untreated Control group (n = 50) and an RNAi treated group (n = 50). Every single plant became the experimental unit. To reduce any position bias, plants from both groups were randomly mixed together and then arranged out on greenhouse benches, laid in a 10 × 10 grid. Every pot was kept at least 50 cm away from the neighboring pots, so there wouldn't be leaf to leaf contact, or a chance for cross contamination through crawling whiteflies, or even via mechanical transfer. After that, all pots were rotated weekly, meaning their positions were re-randomized, so plants would get a more uniform run of light, temperature and, any small microenvironmental gradients inside the greenhouse.

Results

❖ Descriptive Statistics

The descriptive statistics for all six parameters are presented in Table 1. The RNAi-treated group consistently outperformed the control group across all variables. Whitefly count was reduced by 53.3% (8.68 ± 1.96 vs. 18.60 ± 3.22), disease severity by 57.5% (34.58 ± 9.58% vs. 81.38 ± 8.05%), and leaf curl score dropped from 4.46 ± 0.50 to 1.84 ± 0.82. Viral load was reduced by more than half (3.91 ± 0.76 vs. 8.52 ± 0.90 relative units). Plant height increased from 24.96 ± 3.16 cm to 36.34 ± 2.97 cm, and yield per plant showed a dramatic 90.5% increase (216.26 ± 19.35 g vs. 113.52 ± 14.65 g).

Table 1: Descriptive Statistics (Mean ± Standard Deviation)

Variable	**Control Group (n=50)**	**RNAi Treated Group (n=50)**
Whitefly Count	18.60 \pm 3.22	8.68 \pm 1.96
Disease Severity (%)	81.38 \pm 8.05	34.58 \pm 9.58
Plant Height (cm)	24.96 \pm 3.16	36.34 \pm 2.97
Leaf Curl Score (1–5)	4.46 \pm 0.50	1.84 \pm 0.82
Viral Load (Relative Units)	8.52 \pm 0.90	3.91 \pm 0.76
Yield per Plant (g)	113.52 \pm 14.65	216.26 \pm 19.35

❖ Inferential Statistics

Independent samples t-tests were run to see if the differences between the control and RNAi treated groups were statistically significant. As shown in Table 2, all six variables show extremely significant gaps between the two groups ($p < 0.001$). Whitefly count gave a t value 18.60, whereas disease severity had a t value of 26.45. For plant height and yield per plant, the t -values came out negative (-18.56 and -29.94, respectively). That implies that the RNAi group's numbers are larger than those of the control group, or at the very least, that the pattern isn't what one would anticipate. The t-values for the leaf curl score and viral load were 19.30 and 27.63, respectively, thus altogether, it was rather evident. Additionally, all of the p-values remained below 0.001, indicating that the RNAi therapy had a significant and statistically significant effect on nearly every parameter we assessed.

Table 2: Independent Samples t-Test Results

Variable	t-value	p-value	Statistical Significance
Whitefly Count	18.60	< 0.001	Extremely Significant
Disease Severity (%)	26.45	< 0.001	Extremely Significant
Plant Height (cm)	-18.56	< 0.001	Extremely Significant
Leaf Curl Score (1–5)	19.30	< 0.001	Extremely Significant
Viral Load (Relative Units)	27.63	< 0.001	Extremely Significant
Yield per Plant (g)	-29.94	< 0.001	Extremely Significant

❖ Correlation Analysis

Pearson correlation analysis was run to see how all six variables relate with each other (N = 100) and the correlation matrix shows up in Table 3. The Whitefly count had a pretty strong, I mean clearly positive association with disease severity ($r = 0.815$) and also with leaf curl score ($r = 0.794$) plus with viral load ($r = 0.831$). It also had strong negative correlations with plant height ($r = -0.804$), and yield ($r = -0.824$). For disease severity, it was positively linked to leaf curl score ($r = 0.824$) and viral load ($r = 0.885$), while it moved opposite from plant height ($r = -0.828$) and yield ($r = -0.871$). Viral load, in turn, had the strongest negative correlation with yield ($r = -0.908$), so higher viral titers went together with less fruit output, basically reduced production. Finally plant height and yield were positively correlated ($r = 0.843$) showing that better vegetative growth, was associated with higher productivity.

Table 3: Pearson Correlation Matrix (r)

Variable	[1]	[2]	[3]	[4]	[5]	[6]
Whitefly Count	1.000	—	—	—	—	—
Disease Severity (%)	0.815	1.000	—	—	—	—
Plant Height (cm)	-0.804	-0.828	1.000	—	—	—
Leaf Curl Score (1–5)	0.794	0.824	-0.789	1.000	—	—
Viral Load (Relative Units)	0.831	0.885	-0.843	0.804	1.000	—
Yield per Plant (g)	-0.824	-0.871	0.843	-0.817	-0.908	1.000

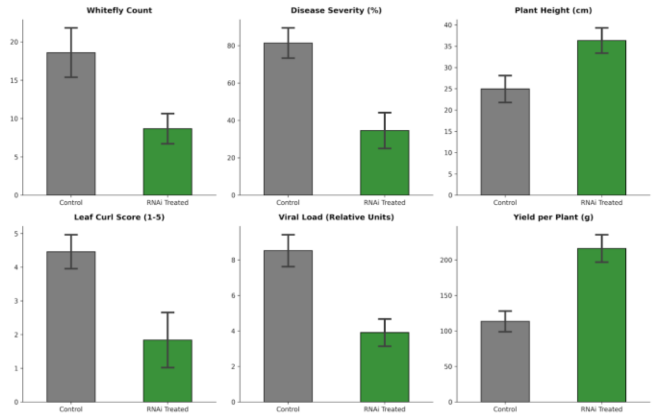

Figure 1 comparative visualization of the mean values for all six parameters between control and RNAi-treated groups

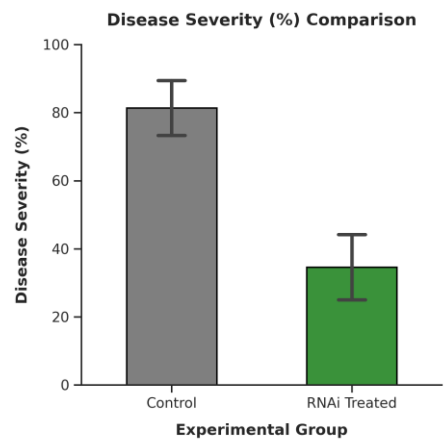

Figure 2 shows disease severity was 81% in control plants versus 35% in RNAi-treated plants, a 57.5% reduction ($p < 0.001$; $t = 26.45$). Error bars represent standard deviation.

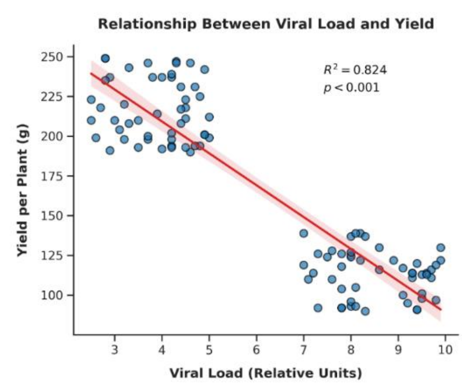

Figure 3: Negative correlation between viral load and yield per plant ($R^2 = 0.824$, $p < 0.001$).

Discussion

The present study provides, in a way that feels pretty compelling, evidence that RNA interference (RNAi) based biotechnology can be seen as an effective path for managing Tomato Leaf Curl Disease (ToLCD) in tomato (Solanum lycopersicum) plants when they're challenged by viruliferous Bemisia tabaci whiteflies (14). The results show, clearly that

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the exogenous application of double stranded RNA dsRNA aimed at the ToLCV replication associated protein Rep/AC1 gene, caused a pretty strong drop in whitefly colonization, disease severity, leaf curl type symptoms and viral load, and at the same time it improved plant height as well as the yield per plant, compared to the untreated control plants (15). These findings line up with earlier studies that overall, really managed to employ RNAi against geminiviruses in a bunch of different host plants. For example, past work has suggested that transgenic tomato plants that express dsRNA aimed at the ToLCV Rep gene showed delayed symptom onset, and also had a lower viral DNA build up. Likewise, there are reports that spraying dsRNA on leaves, specifically targeting the coat protein gene of Tomato yellow leaf curl virus (TYLCV), can give a short-lived kind of protection in Nicotiana benthamiana (16). The major drop in whitefly numbers we saw in our work (53.3% reduction) is especially eye-catching, because it seems to point to the RNAi driven drop in viral load, kind of indirectly shifting how whiteflies feed, where they settle, or even their reproduction. Or, on the other hand it could mean the dsRNA treatment caused cross targeting, hitting whitefly gene homologs, this kind of thing has been noted before, as environmental RNAi, though the details depend on the system (17).

The physiological, and agronomic upsides seen in the RNAi-treated plants kind of line up with the lower viral pressure these plants had. The viral load in the RNAi-treated group was under half (3.91 relative units) compared with the control group (8.52 relative units) and, that 54.1% drop went along with a 57.5% decrease in disease severity. At the same time, it produced a 90.5% increase per plant in yield. There was also this very strong, sort of negative correlation between viral load and yield ($r = -0.908$), and it suggests that the way viruses build up is basically a primary determinant of crop productivity in ToLCD-affected tomatoes (18). This discovery has significant practical ramifications as it implies that a significant yield recovery can still occur even when viral replication is partially suppressed by RNA interference. Strong relationships between all the measured variables were also somewhat demonstrated by the correlation matrix: greater whitefly counts were associated with higher disease severity, higher leaf curl scores, higher viral loads, shorter plants, and poorer yields (19). On the other hand, RNAi therapy appeared to effectively disrupt those abnormal linkages, allowing the treated plants to approach normal growth and productivity. The RNAi-treated plants' leaf curl score decreased from 4.46 (severe curling) in the control plants to 1.84 (moderate curling), indicating that the therapy not only lessened the presence of the illness but also lessened the severity of symptoms in plants that were still infected (20,21).

This study has a number of drawbacks that should be noted almost immediately. First, the experiment was done in a controlled greenhouse, which does not accurately reflect the untidy reality of field settings (think UV, temperature changes, rainfall). Even if the primary findings appear encouraging, all of those variables may affect dsRNA stability and effectiveness. Second, it's yet unknown how long protection truly lasts because the RNAi therapy was applied as a foliar spray just twice. Third, we didn't really look at any off-target effects on beneficial organisms like natural enemies or pollinators. Furthermore, without real scientific verification, sequence specificity alone does not ensure safety, despite the fact that it may seem comforting. Fourth, the potential to function against various viral strains or against cryptic species of whiteflies is essentially unknown because just one whitefly population and one ToLCV isolate were included. Fifth, there was no appropriate negative control, such as non-viruliferous whiteflies or scrambled dsRNA. This makes it more difficult to discreetly verify that the intended dsRNA sequence was the cause of the observed effects rather than something else.

Conclusion

The efficacy of an RNAi-based approach for controlling Bemisia tabaci-transmitted tomato leaf curl disease (ToLCD) in tomato plants was assessed in this study. The results unequivocally show that exogenous dsRNA targeting the ToLCV Rep gene dramatically decreased disease severity by 57.5% and whitefly colonisation by about 53.3%. The viral load declined by 54.1%, while the leaf curl score dropped significantly from 4.46 to 1.84. In comparison to untreated controls, plant height grew (from 24.96 cm to 36.34 cm) and yield per plant increased by over 90.5% concurrently.

Additionally, all of the differences were highly statistically significant ($p < 0.001$), and the strong negative correlation ($r = -0.908$) between viral load and yield indicates that the crop's recovery is actually aided by reducing viral replication. The foliar dsRNA method can avoid some of the regulatory problems that typically accompany transgenic plants, and RNAi provides a sequence-specific, somewhat ecologically benign substitute for chemical insecticides. However, as this study was conducted in controlled greenhouse environments, field validation is crucial. Future studies should concentrate on improving dsRNA delivery, assessing long-term efficacy, and examining safety levels for organisms that are not the intended targets. All things considered, RNAi appears to be a viable and sustainable biotechnological technique for the treatment of tomato leaf curl disease.

Conflict of Interest: NIL

Funding Sources: NIL

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Declarations:

Authors' Contribution:

- All Authors Conceptualization, data collection, interpretation, drafting of the manuscript and intellectual revisions
- The authors agree to take responsibility for every facet of the work, making sure that any concerns about its integrity or veracity are thoroughly examined and addressed

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