Field studies of a novel virus-like agent with commercial citrus varieties in California

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Introduction

Commercial citrus orchards continue to face the biological threat of phloem-limited pathogens such as citrus tristeza virus (CTV) and Huanglongbing (HLB). Currently, the primary mitigation strategies involve non-curative cultural methods of culling infected trees, using certified replant material, resistant/tolerant rootstocks, and vector control **(Moreno et al. 2007, Munir et al. 2018)**.

A new plant-associated virus-like RNA was recently reported from the Vidalakis lab **(Kwon et al. 2021)** and designated as citrus yellow vein-associated virus (CYVaV). CYVaV has properties suitable for potential applications in virus-induced gene silencing (VIGS) technologies targeting economically important pathogens/pests of citrus. These properties include its small genome size (~2.9 kilobases, Kb), phloem-limited colonization, and high titers. CYVaV-based VIGS systems potentially offer a robust and sustainable strategy for prevention and mitigation of several citrus viral/bacterial pathogens and insect pests. However, before it can be used as a VIGS expression vector in a commercial setting, it must be thoroughly evaluated under field conditions to be deemed citrus-safe.

This study was initiated to conduct field-based investigations into the effects of CYVaV on commercially popular citrus varieties and determine whether it is transmissible via natural modes, such as aphid vectors, pollen, or seed. Findings from this work will be crucial for leveraging the necessary federal approvals and subsequent comprehensive evaluations of CYVaV-based VIGS technologies in commercial citrus varieties.

Goals

A replicated field trial with 12 commercially popular citrus rootstock/scion (R/S) combinations has been established at UC Riverside (UCR) since October 2020 for investigating:

I. **CYVaV impacts** on tree health, and fruit yield and quality of commercially popular citrus varieties.

II. Potential **CYVaV transmissibility** via natural modes i.e., aphid vectors, pollen, or seed.

Methodology

I. Field trial establishment at UCR

- 12 commercially popular rootstock/scion (R/S) combinations **(Table 1)**.
- 15 trees planted per R/S combination.
- 6/15 trees per R/S combination graft-inoculated (GI) with CYVaV. Monitored for CYVaV symptoms.

Table 1. *Rootstock/Scion (R/S) combinations included in the UCR field trial.*

S. No.	Citrus variety name (Scion/Rootstock)	Abbreviated Name
1	Limoneira 8A Lisbon Lemon/Rubidoux Trifoliate	**L8A/RT**
2	Limoneira 8A Lisbon Lemon/Macrophylla	**L8A/Mac**
3	Parent Washington Navel/Carrizo Citrange	**PN/Czo**
4	Biogold Giulietta (Shiranui) Mandarin/Carrizo 5/6Citrange	**BGM/Czo56**
5	Limoneira 8A Lisbon Lemon/Carrizo Citrange	**L8A/Czo**
6	Cara Cara Navel/Carrizo Citrange	**CCN/Czo**
7	Biogold Giulietta (Shiranui) Mandarin/Rubidoux Trifoliate	**BGM/RT**
8	Cara Cara Navel/Rubidoux Trifoliate	**CCN/RT**
9	Tango Mandarin/Rubidoux Trifoliate	**TM/RT**
10	Miho Wase Satsuma/Carrizo Citrange	**MWS/Czo**
11	Tango Mandarin/Carrizo Citrange	**TM/Czo**
12	Parent Washington Navel/Rubidoux Trifoliate	**PN/RT**

II. CYVaV screening by RT-qPCR

- CYVaV infection status evaluated at 6m, 12m, and 24m timepoints post graft-inoculations.
- Leaf/budwood samples collected from each tree, high-throughput RNA extraction, followed by RT-qPCRs targeting CYVaV, citrus vein enation virus (CVEV), and citrus tristeza virus (CTV).

III. CYVaV pollen transmission assay

- Spring 2022: Pollen collected from CYVaV-infected and untreated trees. Tested for CYVaV by RT-qPCR.
- In-field hand pollinations (n=229) – Limoneira 8A Lisbon Lemon, Biogold Giulietta (Shiranui) Mandarin.
- Successful fruit set harvested and tested during winter 2023.

IV. Fruit harvest and qualitative analyses

- Winter 2023 – first harvest of field trial.
- Fruit qualitative analyses performed at Lindcove Research and Extension Center (LREC) Fruit Quality Lab.

Results

I. CYVaV screening by RT-qPCR

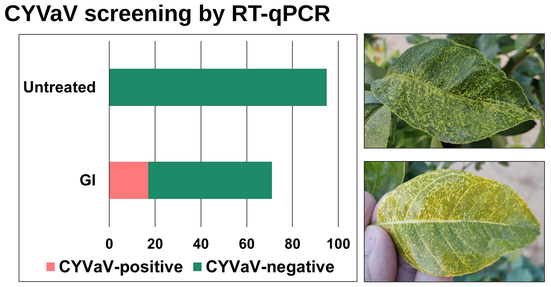

Figure 1. *Two-year (November 2022) post CYVaV graft-inoculation screening of the UCR field trial.* ***Left:*** *RT-qPCR testing summary. 17/71 (~24%) GI trees tested CYVaV-positive, and all untreated trees tested CYVaV-negative.* ***Right:*** *CYVaV foliar symptoms observed in infected Limoneira 8A Lisbon Lemon (top right) and Tango Mandarin (bottom right) trees.*

II. CYVaV pollen transmission assay. CYVaV was detected by RT-qPCR in pollen from CYVaV-infected GI trees and not in pollen from untreated trees. With this pollen, 229 flowers were hand-pollinated in the field trial in 2022 **(Figs. 2A and 2B)**. Treatments included:

a. CYVaV-positive pollen on untreated tree flowers.
b. CYVaV-negative pollen on CYVaV-positive tree flowers.
c. CYVaV-negative pollen on untreated tree flowers.

26 hand-pollinations resulted in successful fruit set by winter 2023, when they were harvested, dissected, **(Fig. 2C)**, and tested for CYVaV. All fruits from treatments 'a' and 'b' tested CYVaV-negative. Fruits from treatment 'b' tested CYVaV-positive.

III. Fruit harvest and qualitative analyses. In February 2023, all field trial trees were manually harvested. Overall, there were no significant differences in fruit quantity per tree between GI and untreated trees across all R/S combinations **(Fig. 3)**. Fruit qualitative analyses were performed with 24 samples of 10 fruits per treatment (2 samples per treatment; GI and untreated trees). Parameters studied included: rind colorimetry, firmness, rind texture and packline height/width, rind thickness, juice volume **(Fig. 4B)**, juice weight **(Fig. 4C)**, and juice acid content. Overall, fruits collected from GI and untreated trees across the 12 R/S combinations were morphologically similar **(Fig. 4A)** and did not have any significant qualitative differences.

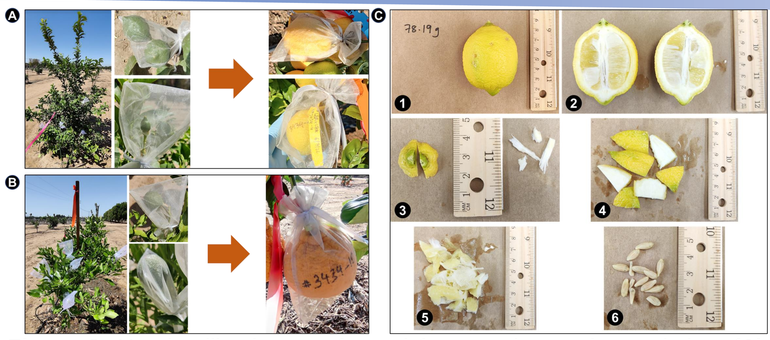

Figure 2. *Hand-pollinations performed for two citrus scion varieties,* ***(A)*** *Limoneira 8A Lisbon Lemon and* ***(B)*** *Biogold Giulietta (Shiranui) Mandarin.* ***(C)*** *Manual fruit dissection to separate the pedicel* ***(C3 left)****, central column* ***(C3 right)****, rind* ***(C4)****, pulp* ***(C5)****, and seed (****C6****, when produced).*

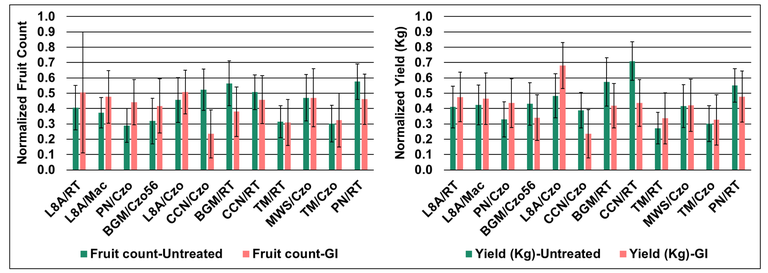

Figure 3. *Harvest data summary. Bar graph on the* ***left*** *shows normalized average fruit counts for GI and untreated trees across the 12 R/S treatments. Bar graph on the* ***right*** *shows normalized yield (Kg) for the same. Vertical lines on top of colored bars indicate standard error.*

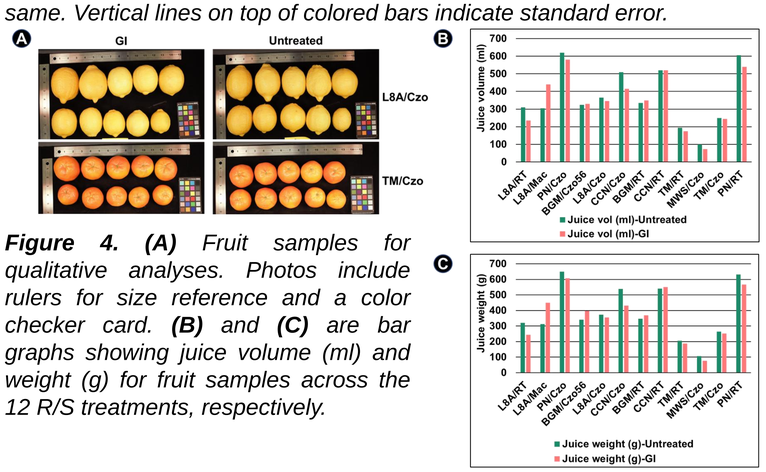

Figure 4. ***(A)*** *Fruit samples for qualitative analyses. Photos include rulers for size reference and a color checker card.* ***(B)*** *and* ***(C)*** *are bar graphs showing juice volume (ml) and weight (g) for fruit samples across the 12 R/S treatments, respectively.*

Conclusions

- A field trial was established at UCR with **12 commercially popular citrus R/S combinations**. CYVaV graft-inoculations performed, and trees being monitored for infection and symptom development.
- **Limoneira 8A Lisbon Lemon** and **Tango Mandarin** currently the most compatible citrus varieties for CYVaV colonization.
- **CYVaV detected in pollen** collected from infected field trees and used for hand-pollinations. Based on our results, there was no evidence of pollen transmission of CYVaV from infected pollen to untreated flowers (and corresponding tree).
- Harvested fruit showed **no significant differences in yield or qualitative parameters** between GI and untreated trees for all 12 R/S treatments.

References and Funding

- Moreno et al., 2008. *Molecular plant pathology*, *9*(2), pp.251-268.
- Munir et al., 2018. *Microbial Ecology*, *76*, pp.192-204.
- Kwon et al., 2021. *Frontiers in microbiology*, *12*, p.683130.

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