The Root Cause of HLB Initiation and Progression and Its Implications in Disease Management

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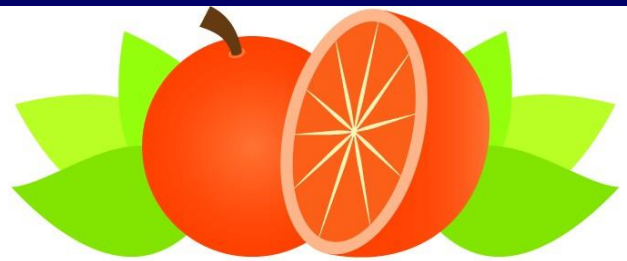

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Outline

- **Citrus Huanglongbing is a pathogen-triggered immune disease and its implications in HLB management**
- **Non-transgenic CRISPR-edited disease resistant sweet orange (canker and HLB)**

nature COMMUNICATIONS

ARTICLE

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Citrus Huanglongbing is a pathogen-triggered immune disease that can be mitigated with antioxidants and gibberellin

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Huanglongbing (HLB) is a devastating disease of citrus, caused by the phloem-colonizing bacterium *Candidatus* Liberibacter asiaticus (CLas). Here, we present evidence that HLB is an immune-mediated disease. We show that CLas infection of *Citrus sinensis* stimulates systemic and chronic immune responses in phloem tissue, including callose deposition, production of reactive oxygen species (ROS) such as H_2O_2, and induction of immunity-related genes. The infection also upregulates genes encoding ROS-producing NADPH oxidases, and downregulates antioxidant enzyme genes, supporting that CLas causes oxidative stress. CLas-triggered ROS production localizes in phloem-enriched bark tissue and is followed by systemic cell death of companion and sieve element cells. Inhibition of ROS levels in CLas-

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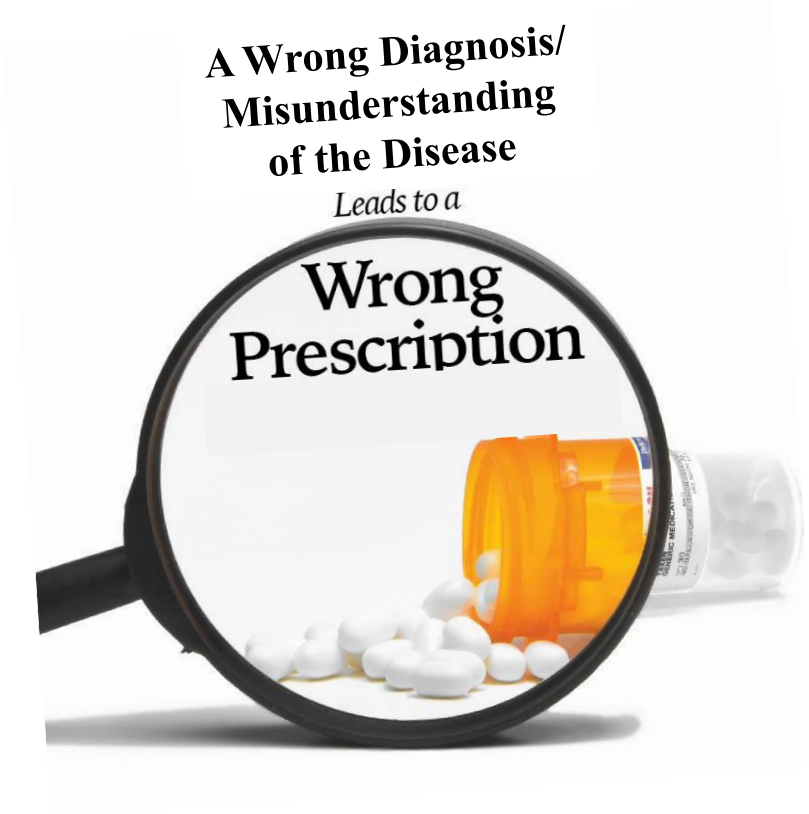
Take-home messages (How does CLas cause damage to citrus?)

- **Citrus HLB is a pathogen-triggered immune disease (Ma et al., 2022)**
- **HLB pathogenicity mechanism mimics that of human sepsis diseases induced by various microbes, in which overwhelming immune response is the main cause of disease damage.**
- **CLas, the HLB pathogen, stimulates systemic and chronic immune response in phloem tissues.**
- **HLB disease symptoms are caused by systemic cell death of phloem tissues. This response is instigated primarily through excessive and chronic *reactive oxygen species (ROS)* production.**

- **Simply: CLas → ROS (excessive and chronic) → cell death of phloem tissues → HLB symptoms or damages to the tree**

Take-home messages (How does CLas cause damage to citrus?)

- Prior studies regarding how CLas causes HLB symptoms
  - ❖ root decay
  - ❖ chloroplast disruption due to excessive starch accumulation in plastids
  - ❖ phloem blockage resulting from deposition of callose and phloem proteins
  - ❖ differential transportation of sugars.
- Our study demonstrated that these observations are the consequence of CLas infection, rather than the root cause of HLB disease.
- That means if we try to control HLB by manipulating root decay, starch accumulation, callose deposition, it probably will not work.



Take-home messages (What can you do now?)

- **Ways to reduce ROS damages or increase tolerance to ROS damages:**
  - ✓ **Balanced nutrition (nutrition deficiency causes more ROS production)**
  - ✓ **Application of micronutrients**
  - ❖ **Micronutrients (B, Fe, Zn, Mo, Ni (<u>not to be mixed with Cu</u>) increase the activity of antioxidant enzyme activities and promote plant growth;**
  - ❖ **Mixture of B (2 μM), Fe (3 μM), Mo (2 μM), Ni (6 μM), and Zn (12 μM)) are effective for suppressing H_2O_2-triggered cell death of citrus cells.**
  - ❖ **Foliar spray of micronutrients is suggested (the narrow window of concentrations in help and damages.)**
  - ✓ **GA (GA protects cells against ROS damages, inhibits ROS production, promotes plant growth hormone and phloem cell regeneration, reverses ROS induced plant growth inhibition)**
- **Factors that increase ROS damages in addition to that caused by CLas**
  - ✓ **Heat stress causes excessive ROS production**
  - ✓ **Under salinity stress, the level of ROS production increases.**
  - ✓ **Drought increases ROS production**

Take-home messages (What can you test by yourself? Ed Leotti)

- **Antioxidants, immunoregulators, and nutrients are commonly used to treat human immune-mediated diseases by halting or reducing ROS-mediated cell death.**
- **For citrus, you can try different combinations and optimization of application timing of antioxidants (such as uric acid), immunoregulators (plant growth hormones, such as GA), and nutrients (macro- and micro-nutrients)**

- **We are working on such an optimized combinations and timing of application!**

Take-home messages (HLB tolerant/resistant citrus cultivars)

- **We have successfully developed non-transgenic CRISPR genome editing technology for citrus.**
- **We have generated non-transgenic canker resistant Hamlin sweet orange using the CRISPR technology.**
- **Non-transgenic HLB tolerant/resistant citrus cultivars are being generated.**

- **What phenotypes can we explain using this model?**
- **How did we reach the conclusion that HLB is a pathogen-triggered immune disease**
- **What horticultural approaches are working to mitigate HLB damages?**

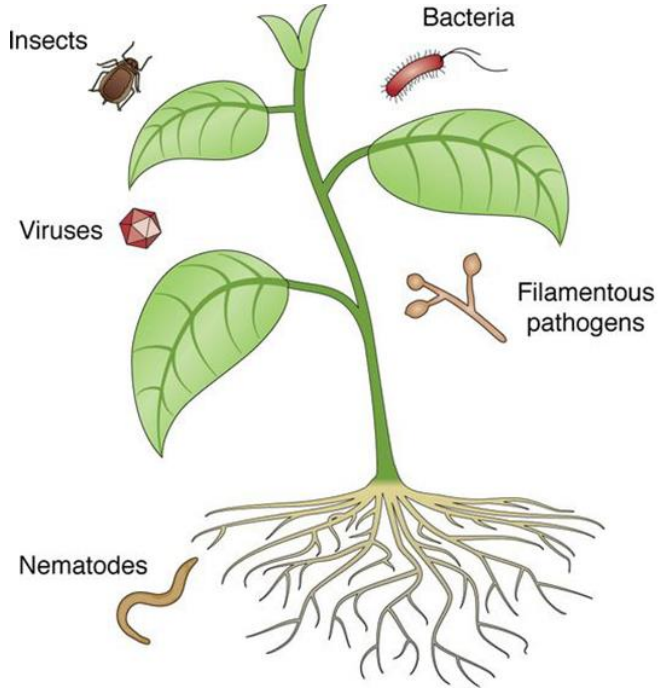
What phenotypes can we explain using this model?

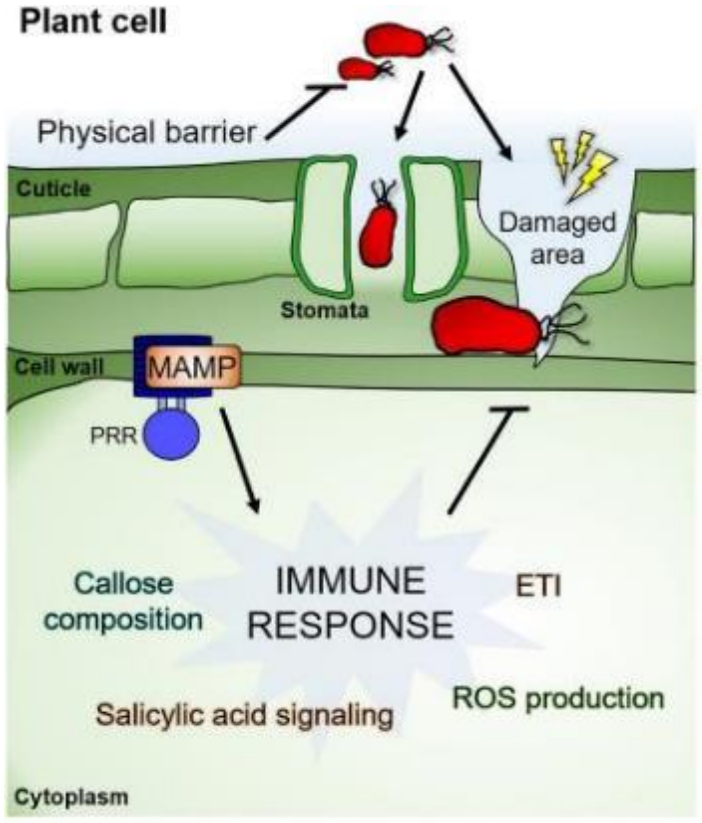
- **Simply: CLas → ROS (excessive and chronic) → cell death of phloem tissues → HLB symptoms or damages to the tree**

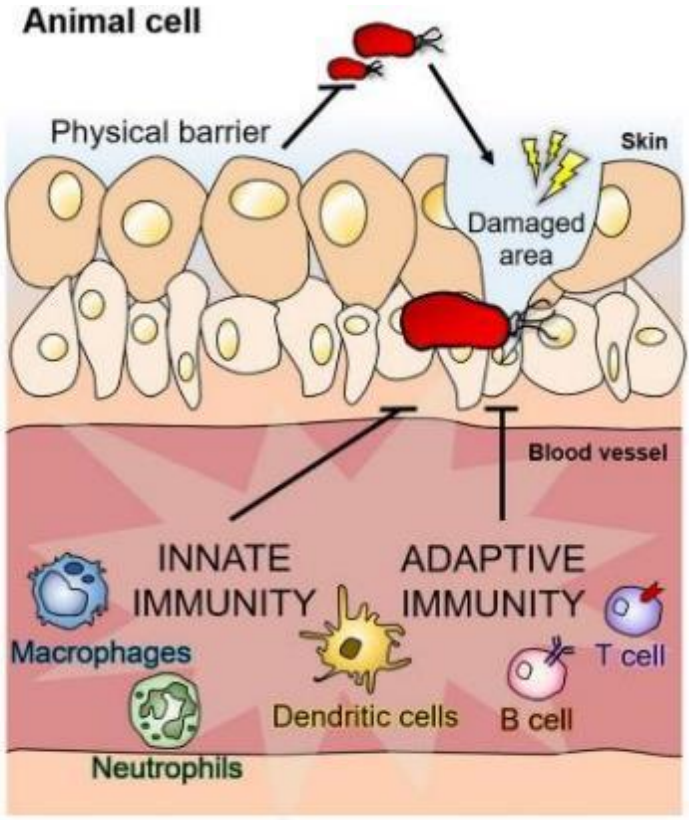
- **Stunted growth: high concentration of ROS inhibit plant growth, phloem cell death reduces transport of carbohydrates and hormones.**
- **Root decay: cell death of the phloem tissue, reduced transport of photosynthates, and ROS inhibition of root growth.**
- **Starch accumulation and blotchy mottle: death of companion and sieve element cells.**
- **Hardened leaves: ROS cause strengthening of host cell walls.**
- **During active growth (such as in the later spring and summer), HLB symptoms are not obvious (potential reasons: a tradeoff relationship between growth and immunity in plants (Growth usually suppresses immunity, and vice versa); CLas takes time to buildup in the young shoots and newly generated phloem tissues).**

How did we reach the conclusion that HLB is a pathogen-triggered immune disease?

Plant and animal immune systems







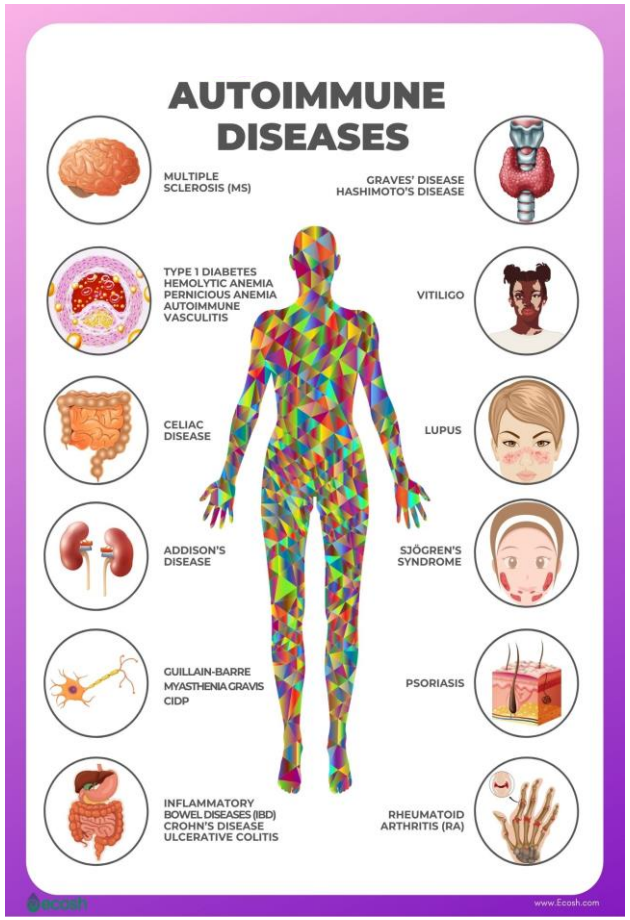
Bentham et al. 2020

Kim et al. 2020

- Both plants and animals have innate immunity. Plant immunity is also called plant defense.
- Animals have adaptive immunity, while plants do not have the adaptive immunity
- Immunity protects plants and animals against most microbes
- When you call plant immunity as plant defense, you might not think it has any negative effect!

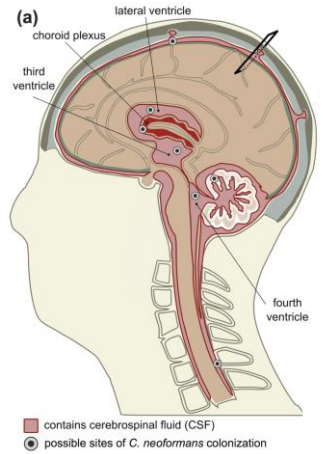
Immune-mediated diseases

Autoimmune diseases



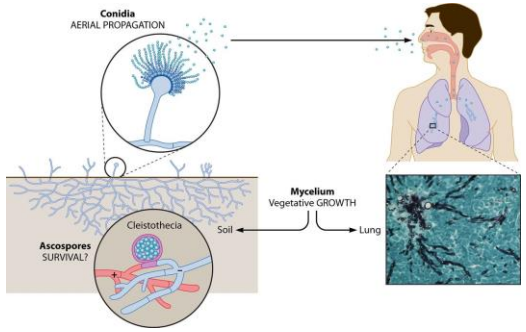
Non-autoimmune diseases

Asthma



- *Cryptococcus neoformans* Meningoencephalitis
- CD4+ T cell-mediated response to *C. neoformans* is a major contributor to tissue damage in cryptococcal meningitis in mice even though it also mediates fungal clearance
  Neal et al. 2017

Sepsis



- Allergic aspergillosis: dysregulated inflammatory response to *Aspergillus* antigens, affects the respiratory tract

Host immune responses have been known to be an important factor in addition to microbial pathogenicity factors for human diseases caused by microbial pathogens

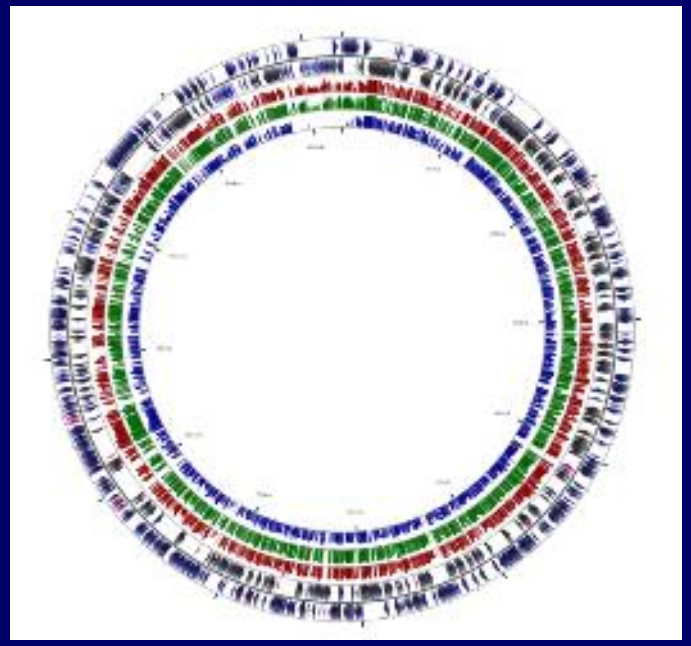
Evidence that demonstrate citrus HLB is a pathogen-triggered immune disease

- **CLas does not contain known pathogenicity factors that are directly responsible for causing plant disease symptoms.**

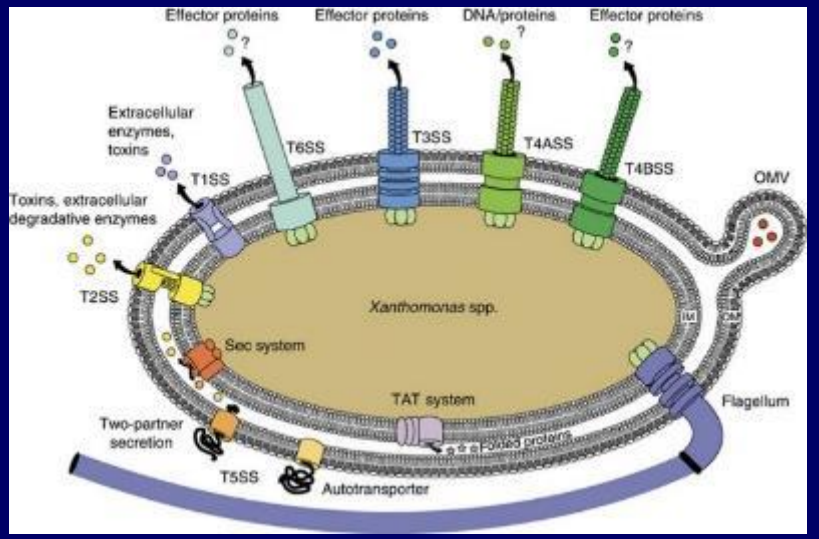
This is different from what we know about plant pathogens! All known plant pathogens have pathogenicity factors to cause disease.

- **CLas infection triggers immune response and cell death in the phloem tissue**
- **CLas-triggered cell death is caused by ROS**
- **Suppressing CLas-triggered ROS production mitigates phloem cell death and HLB symptoms.**

CLas does not contain pathogenicity factors that are directly responsible for the HLB symptoms



Duan et al. 2009

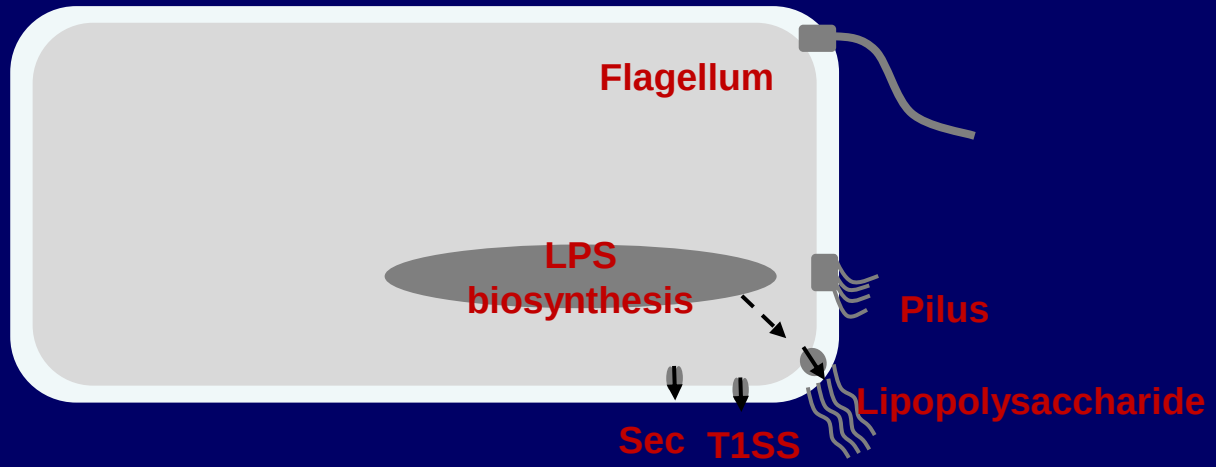


Büttner & Bonas 2010

Most known pathogens are like pirate ships with many weapons ready to do damages!

CLas is like a yacht without weapons!

1.23 Mb for *Las*, 1.26 Mb for *Lso*
3.4 Mb for *Agrobacterium* sp. H13-3
7.3 Mb for *A. radiobacter* K84.
***A. tumefaciens:* tumor-inducing (Ti) plasmids, vir genes for the formation of tumors**
***Rhizobium rhizogenes:* vir genes for formation of hairy roots**
Bioinformatic analyses indicate that CLas does not contain homologs of known pathogenicity factors.



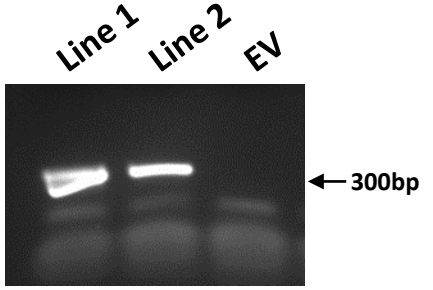
Overexpression of four putative CLas virulence genes in *Citrus* does not cause HLB symptoms

CLIBASIA_04405 transgenic sweet orange

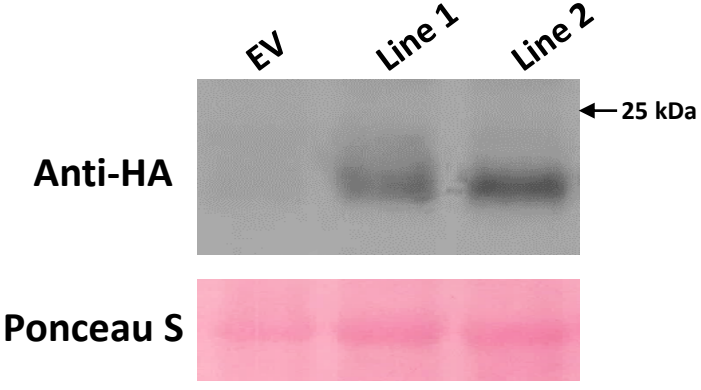
EV Line 1 Line 2

CLIBASIA_05315 (SDE1),
CLIBASIA_02845,
CLIBASIA_04405,
CLIBASIA_04025 (SDE15)

PCR validation



WB validation



CLas does not contain pathogenicity factors that directly cause HLB symptoms

Stable Overexpression	Overexpressed genes	Phenotypes
Overexpressed in *Arabidopsis thaliana* alone (30)	CLIBASIA_00460, CLIBASIA_00530, CLIBASIA_02145, CLIBASIA_02215, CLIBASIA_02470, CLIBASIA_03695, CLIBASIA_03975, CLIBASIA_04320, CLIBASIA_04580, CLIBASIA_04735, CLIBASIA_05115, CLIBASIA_05320, CLIBASIA_05330, CLIBASIA_04260, CLIBASIA_03315, CLIBASIA_03105, CLIBASIA_05640, CLIBASIA_02305, CLIBASIA_03085, CLIBASIA_00420, CLIBASIA_01300, CLIBASIA_02425, CLIBASIA_03295, CLIBASIA_04055, CLIBASIA_04410, CLIBASIA_05150, CLIBASIA_01640, CLIBASIA_04865, CLIBASIA_05160, CLIBASIA_05475	Same as wild type
Overexpressed in *Nicotiana tabacum* only (10)	CLIBASIA_00255 (SahA), CLIBASIA_00830, CLIBASIA_04330, CLIBASIA_00470, CLIBASIA_02160 (metalloprotease), CLIBASIA_04030, CLIBASIA_00520, CLIBASIA_02395, CLIBASIA_04040, CLIBASIA_01555 (hemolysin)	Same as wild type *A. thaliana* and *N. tabacum*
Overexpressed in both *A. thaliana* and *N. tabacum* (3)	CLIBASIA_02935, CLIBASIA_01345 (serralysin), CLIBASIA_04520	Same as wild type *A. thaliana* and *N. tabacum*
Overexpressed in both *Citrus paradisi* and *N. tabacum* (2)	CLIBASIA_05315 (SDE1), CLIBASIA_02845	Same as wild type *C. paradisi* and *N. tabacum*
Overexpressed in *A. thaliana*, *C. paradisi*, and *N. tabacum* (2)	CLIBASIA_04405, CLIBASIA_04025 (SDE15)	Same as wild type *A. thaliana*, *C. paradisi* and *N. tabacum*

Evidence that demonstrate citrus HLB is a pathogen-triggered immune disease

- CLas does not contain known pathogenicity factors that are directly responsible for causing plant disease symptoms
- **CLas infection triggers immune response and cell death in the phloem tissue**
- CLas-triggered cell death is caused by ROS
- Suppressing CLas-triggered ROS production mitigates phloem cell death and HLB symptoms

CLas infection triggers immune response and cell death in the phloem tissue

- ✓ **CLas infection causes systemic and chronic immune responses including reactive oxygen species (ROS) production, callose deposition, and induction of immune related genes such PR genes.**
- ✓ **CLas infection causes phloem cell death. Cell death was more severe in leaves with severe symptoms.**

CLas induces systemic immune response in the phloem tissues following systemic CLas infection

CLas titers Log10
(CLas cells/g tissue)

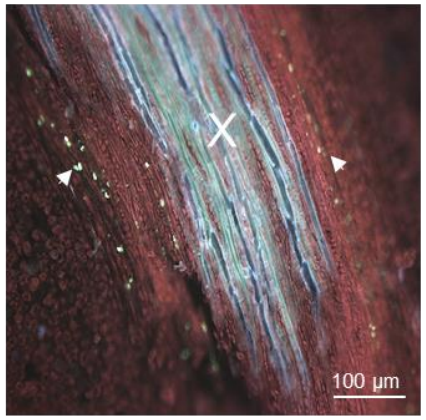
4.30

5.60

ND

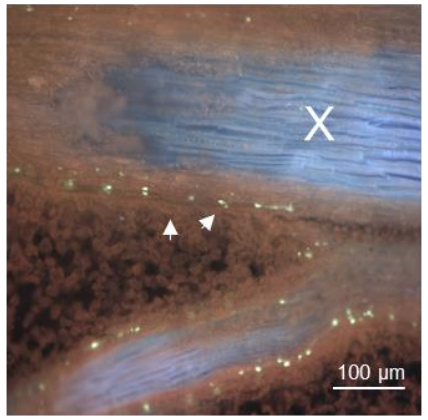
Asymptomatic

A



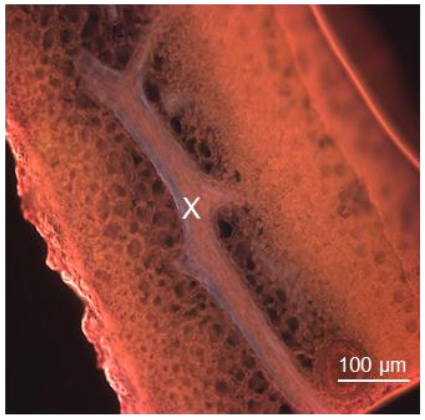
Petiole (1)

B



Midrib (2)

C



Lamina (3)

CLas titers Log10
(CLas cells/g tissue)

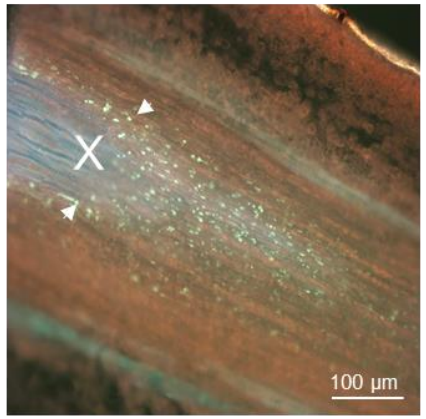
6.34

6.70

6.55

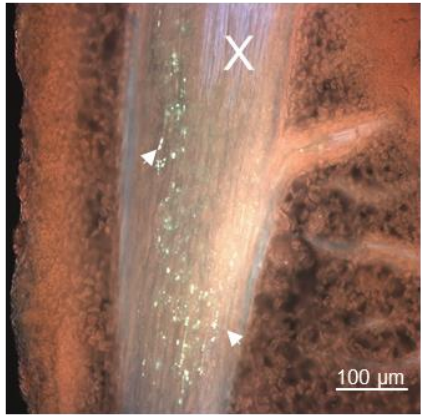
Symptomatic

D



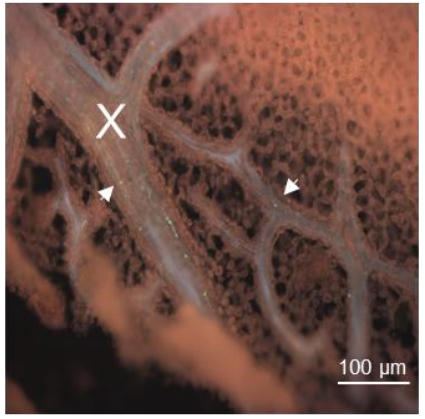
Petiole (4)

E



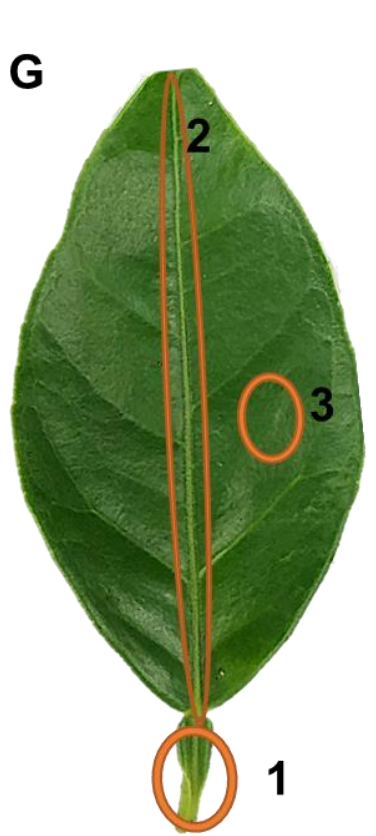
Midrib (5)

F

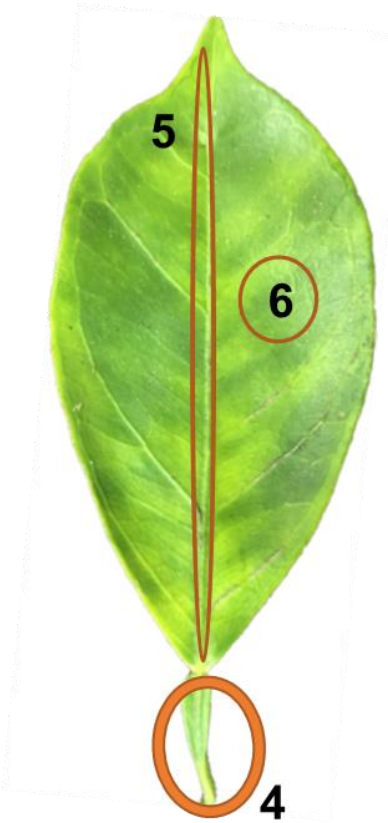


Lamina (6)

G



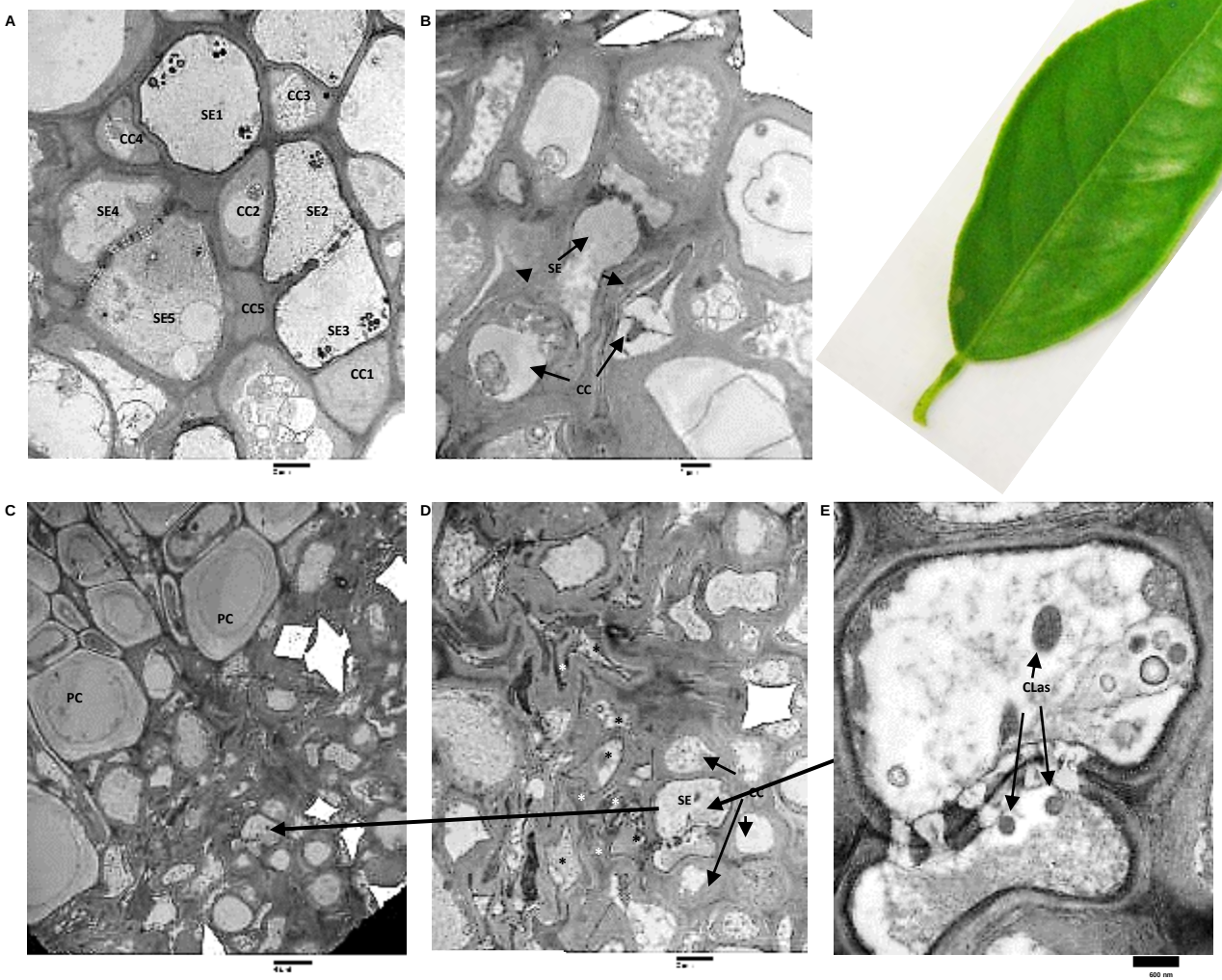
Asymptomatic



Symptomatic

stained with 0.005% aniline blue

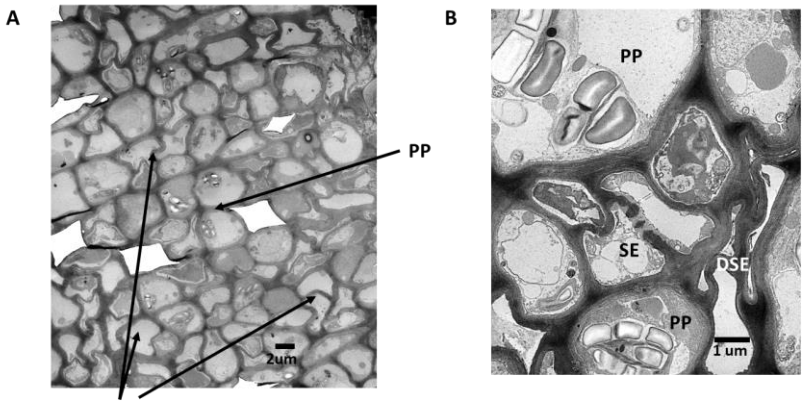
Cell death of sieve element and companion cells was observed in asymptomatic young leaves of HLB positive *C. sinensis* ‘Valencia’ trees



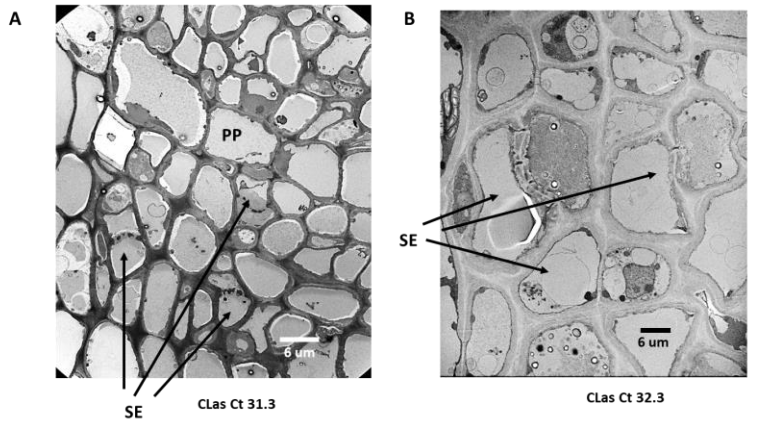
Key observations:

- Cell death of phloem tissues can occur prior to the appearance of HLB symptoms.
- Cell death was more severe in leaves with severe symptoms.
- Some sieve element and companion cells undergoing cell death while others remained intact in the same field.
- Cell death was limited to sieve element and companion cells, but not occurring in surrounding parenchyma cells (C).

Cell death of sieve element and companion cells positively correlate with CLas titers and HLB symptoms



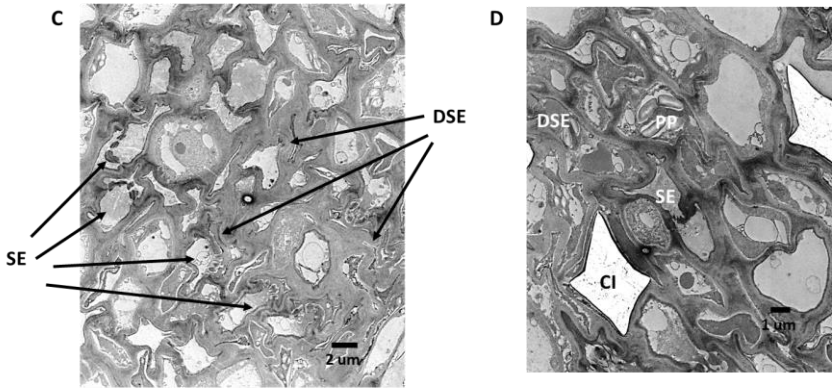
Midvein of asymptomatic mature leaf (CLas Ct 31.7)



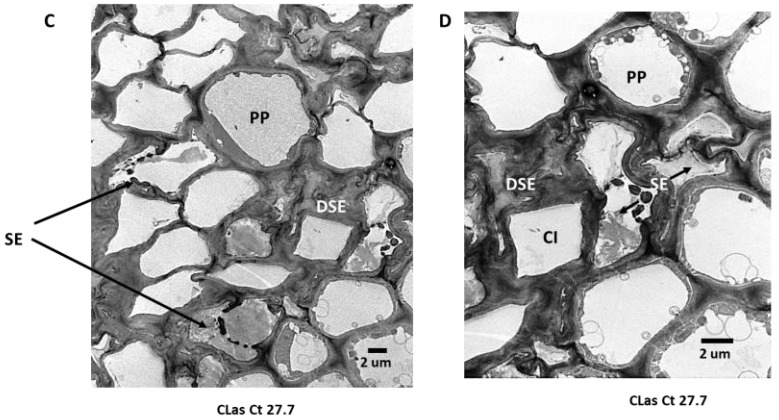
CLas Ct 31.3

CLas Ct 32.3

Stem of asymptomatic branches



Midvein of symptomatic mature leaf (CLas Ct 28)



CLas Ct 27.7

CLas Ct 27.7

Stem of symptomatic branches

- Cell death of phloem tissues seems to be the key.
- How does CLas cause cell death of phloem tissues?

Evidence that demonstrate citrus HLB is a pathogen-triggered immune disease

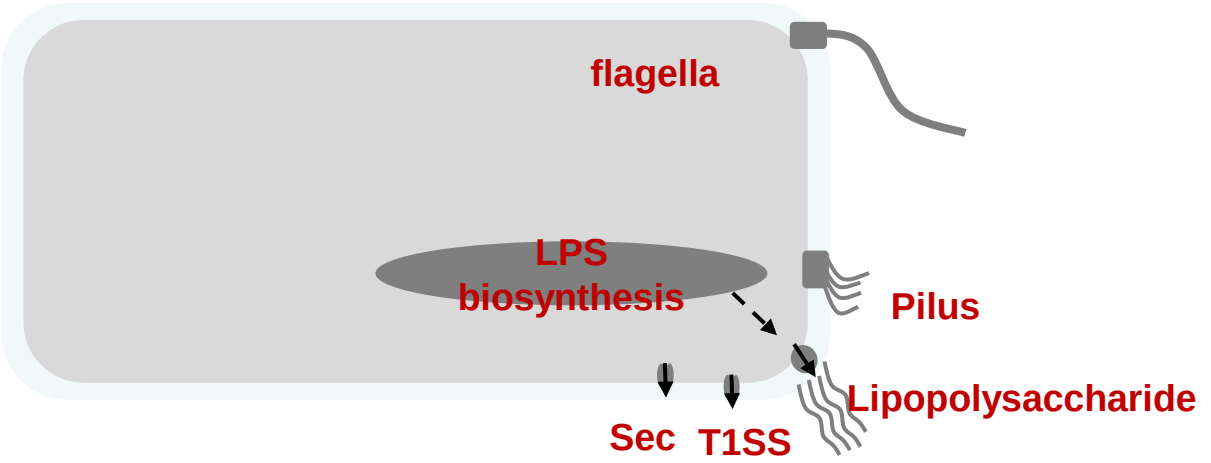
- CLas does not contain known pathogenicity factors that are directly responsible for causing plant disease symptoms
- CLas infection triggers immune response and cell death in the phloem tissue
- **CLas-triggered cell death is caused by ROS**
- Suppressing CLas-triggered ROS production mitigates phloem cell death and HLB symptoms

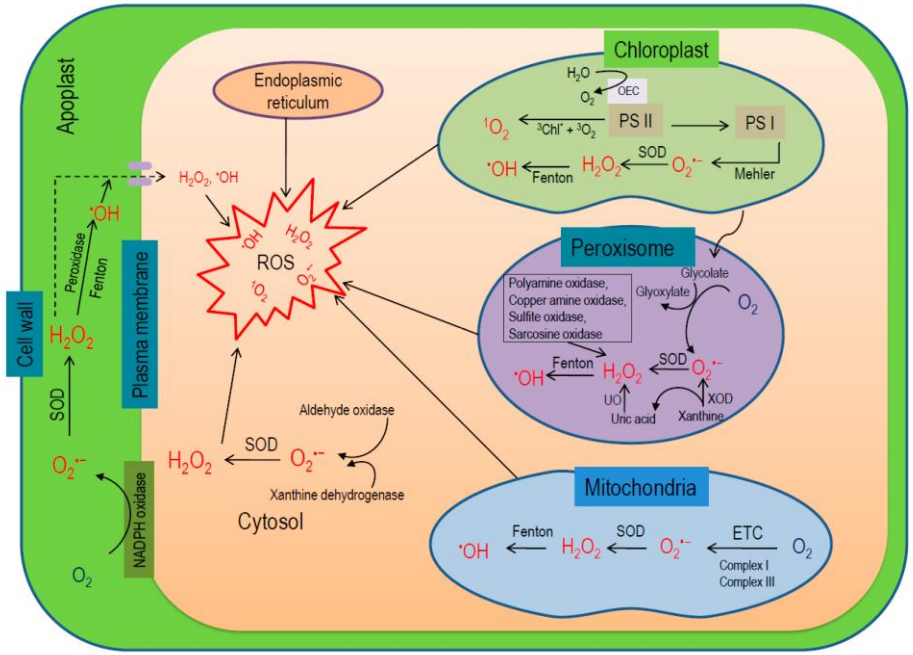
How does CLas cause cell death of phloem tissues?

Toxin

Effector

ROS: PTI, ETI





At high concentrations, ROS triggers necrotic cell death, but induces programmed cell death below the ROS threshold.

Hasanuzzaman et al. 2020

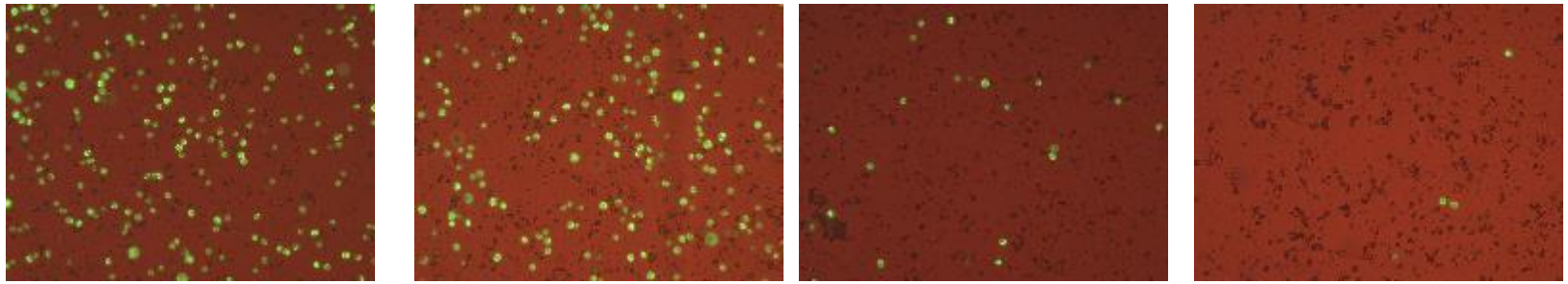
CLas infection triggers ROS production

In phloem sap, H_2O_2 concentrations from symptomatic (1.80 ± 0.13 mmol/L) branches were much higher than that (0.59 ± 0.01 mmol/L) of healthy trees.

H_2O_2 induces necrosis of immortalized rat embryo fibroblasts at a concentration of 0.7 mmol/L (Guénal et a. 1997).

ROS reach threshold to kill citrus cells

Viability assay: fluorescein diacetate (FDA) staining: Green spots indicate live citrus cells



H_2O_2 0 mM **0.6 mM** **1.5 mM** **1.8 mM**

CLas induces chronic and systemic ROS production

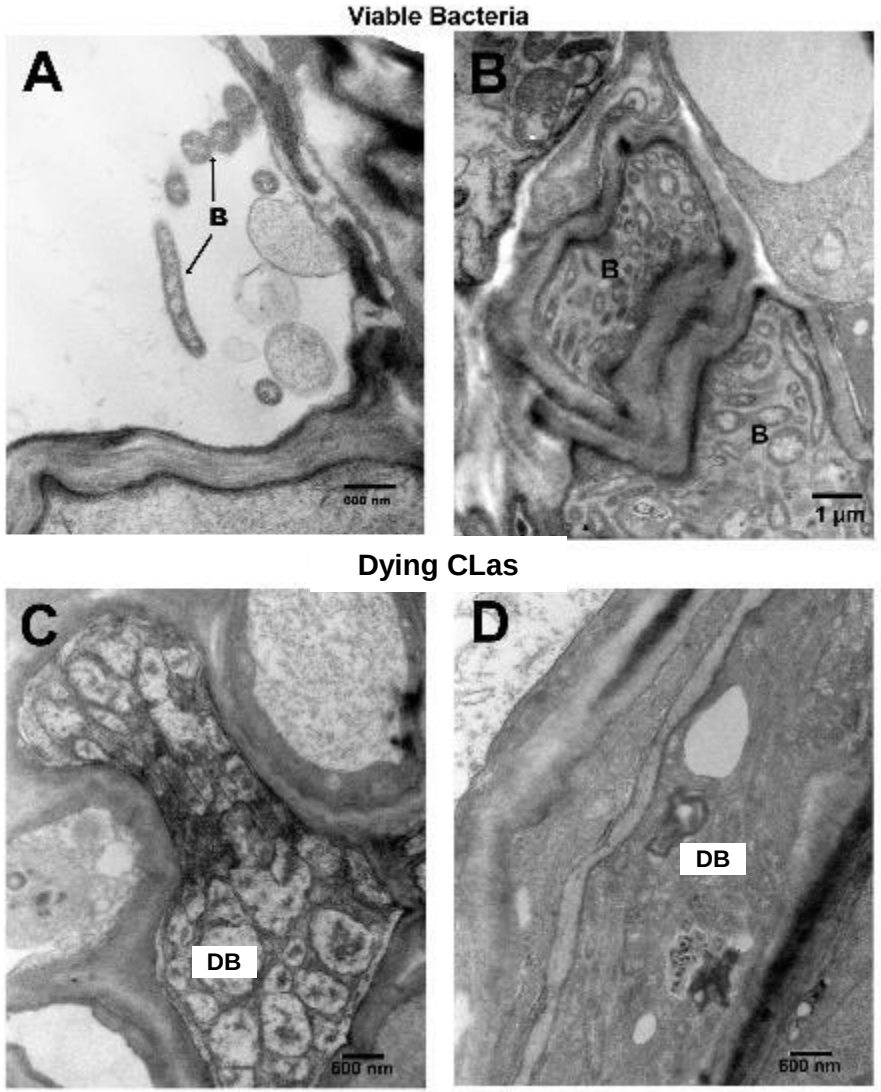
- **CLas induces ROS production in phloem tissues**
- **In defense response of most cases, ROS production induced by pathogen is temporary.**
- **CLas induces chronic and systemic ROS production, in young flushes, in mature leaves, in stems…Because CLas keeps infecting new phloem tissues and the phloem tissues are connected.**

HLB is a pathogen (CLas)-triggered immune disease

- **Simply: CLas → ROS (excessive and chronic) → cell death of phloem tissues → HLB symptoms or damages to the tree**

- **ROS are the executor of phloem cell death.**
- **Immune responses (chronic and systemic) in addition to ROS also have negative effect on plants.**
- **Continuous activation of immune system harms both plants and human.**
- **Activation of immune responses suppress plant growth!**

Do ROS Kill CLas?



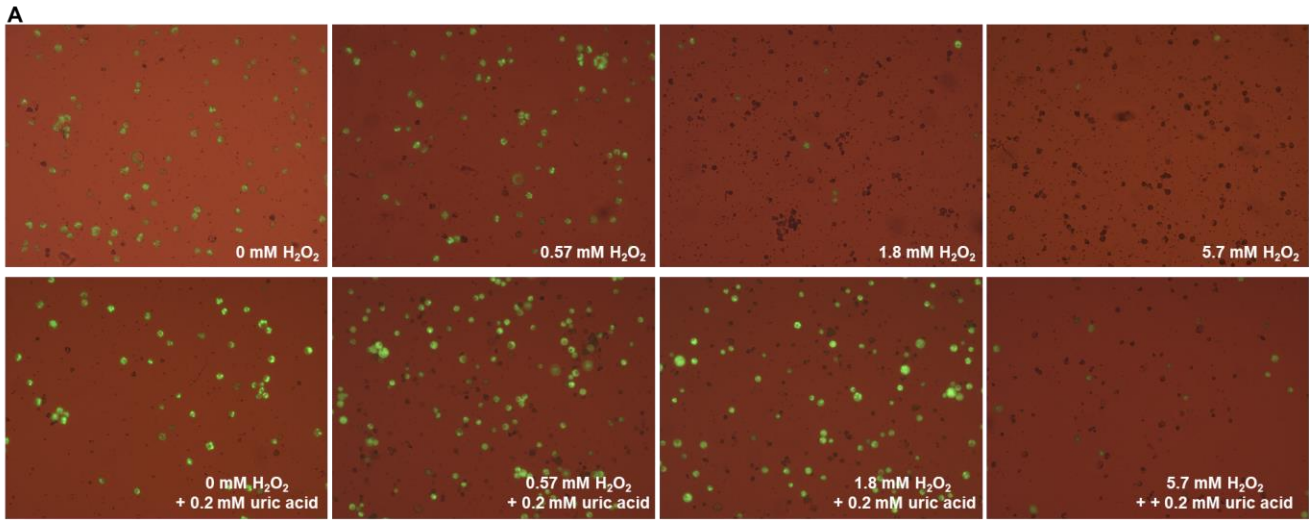
- **Do ROS kill CLas?**
  ROS concentration is high enough to kill CLas
  Dying CLas is observed infected tissues.

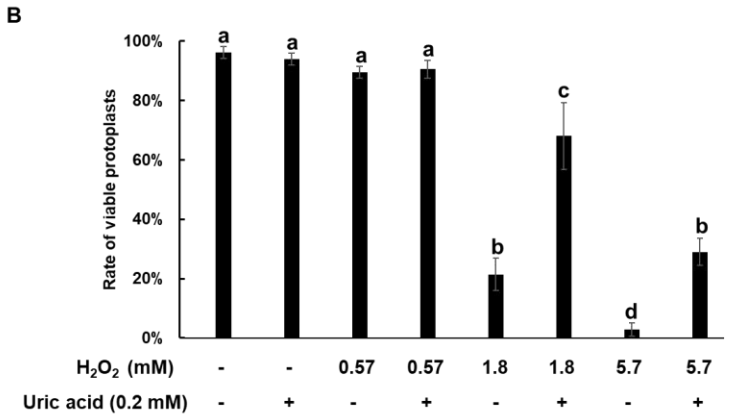
- **Can we rely on ROS to kill CLas?**
  No. We could not use ROS to kill CLas, but not citrus phloem cells.

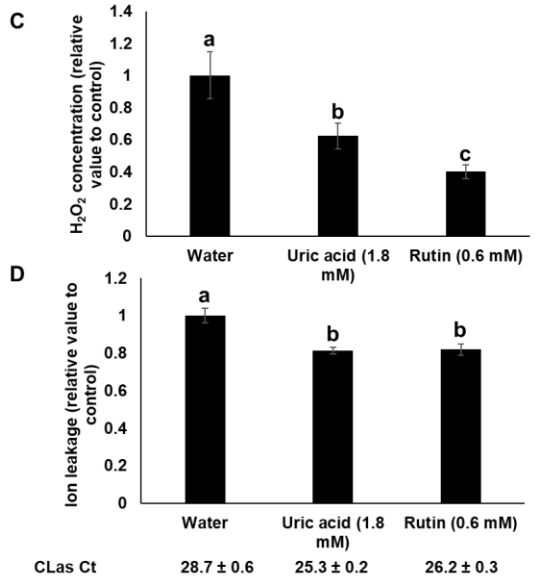
Evidence that demonstrate citrus HLB is a pathogen-triggered immune disease

- CLas does not contain known pathogenicity factors that are directly responsible for causing plant disease symptoms
- CLas infection triggers immune response and cell death in the phloem tissue
- CLas-triggered cell death is caused by ROS
- **Suppressing CLas-triggered ROS production mitigates phloem cell death and HLB symptoms---implications in HLB management**
  - **Antioxidants, immunoregulators, and nutrients are commonly used to treat human immune-mediated diseases by halting or reducing ROS-mediated cell death.**

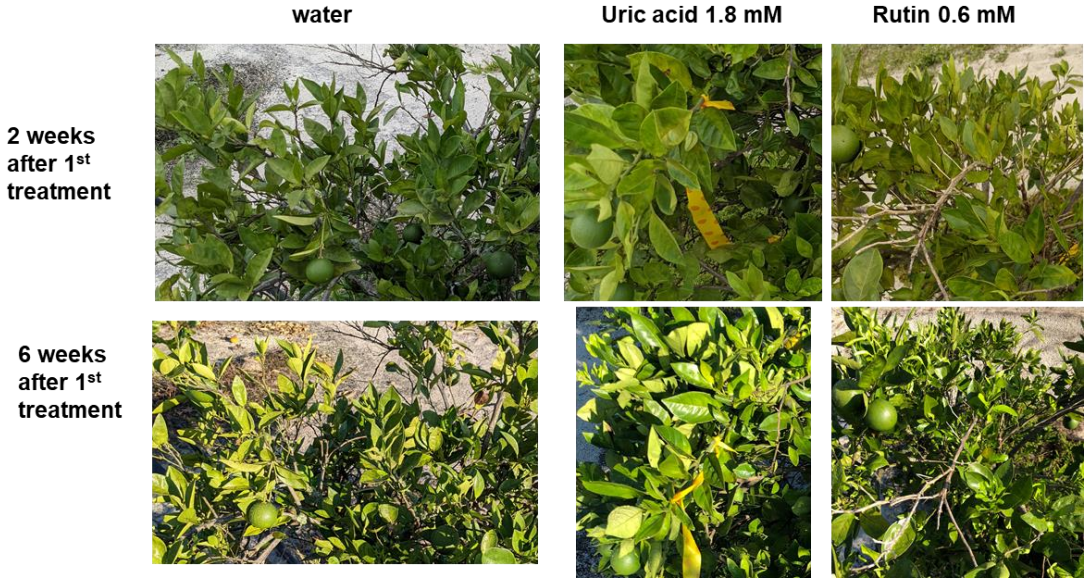
Antioxidants mitigate ROS-triggered cell death and HLB symptoms





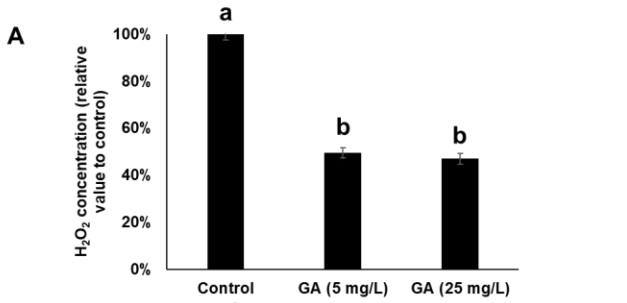


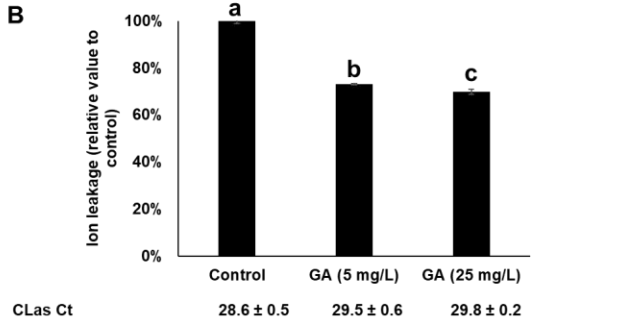
foliar spay weekly for six weeks

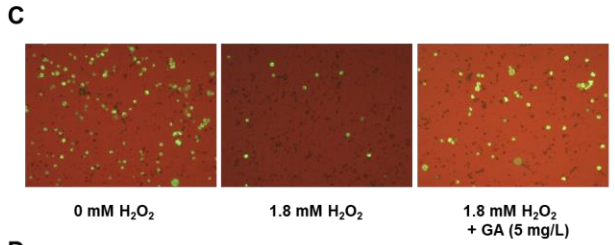


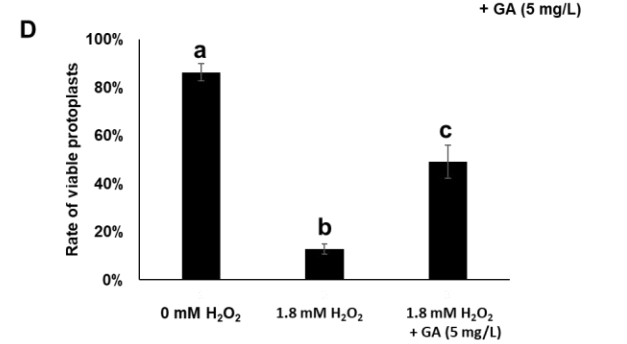
- Antioxidants can prevent or reduce damage to cells caused by ROS
- ROS are responsible for cell death of the phloem tissues of CLas infected citrus
- Cell death of phloem tissues is responsible for HLB symptoms
- Mitigating ROS damages reduces HLB damages
- Antioxidants such as uric acid have not been registered for agriculture.
- Can antioxidants be used to control HLB economically?

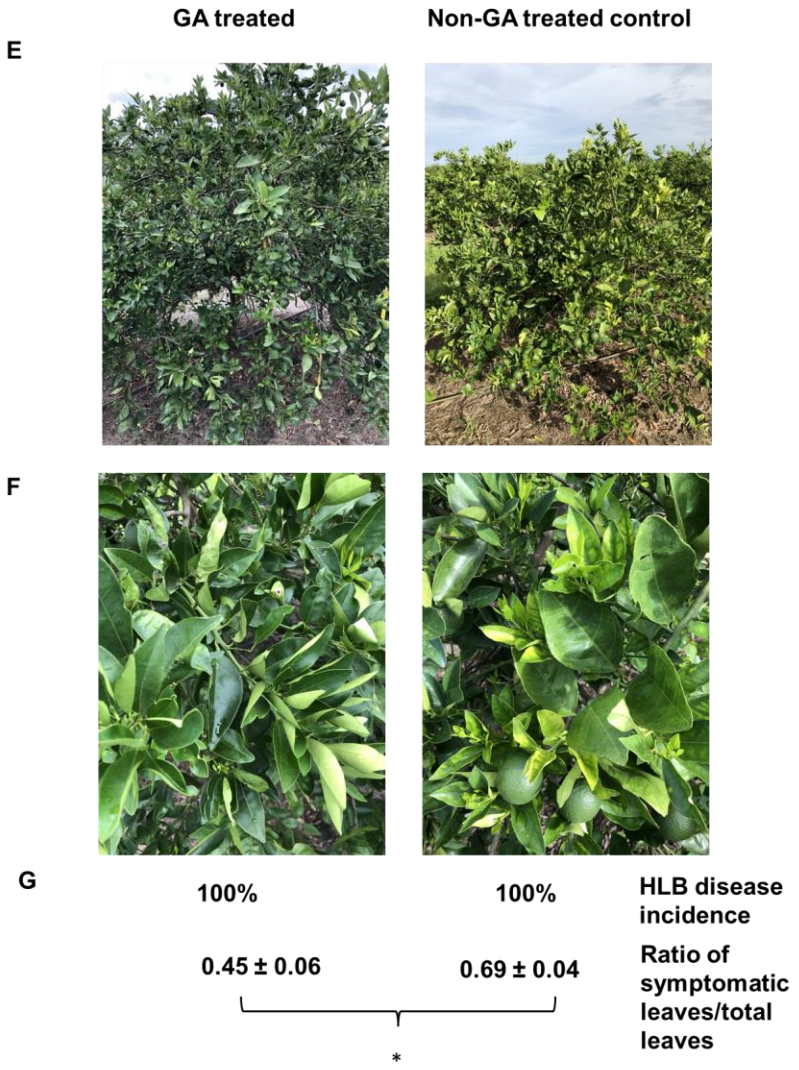
Immunoregulator gibberellin (GA) suppresses cell death and HLB development







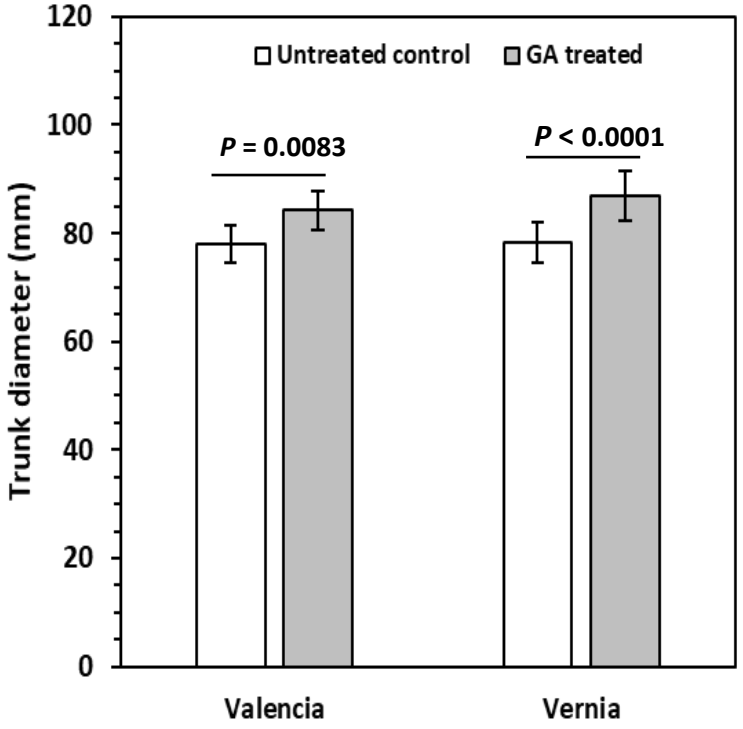




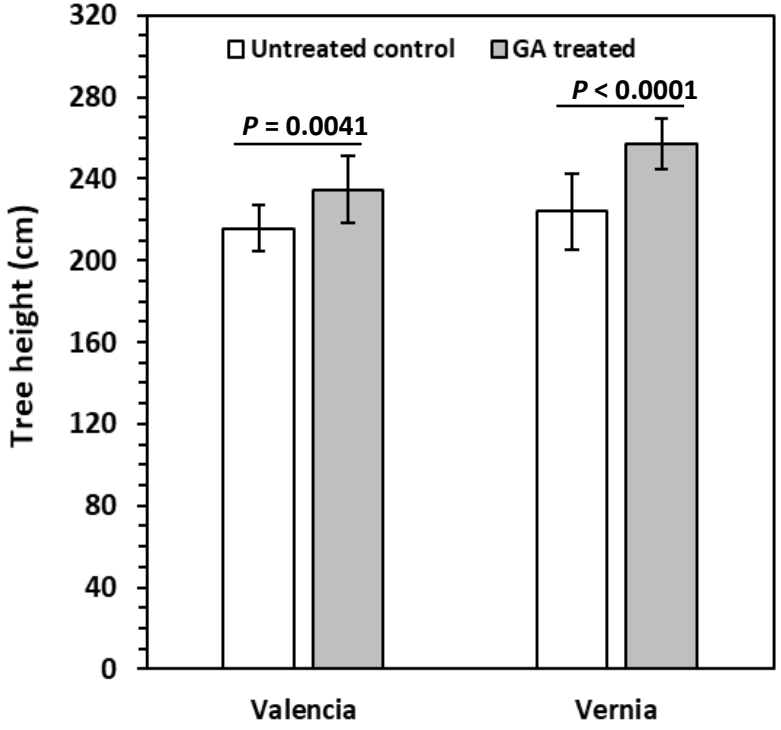
- **GA is a known plant growth hormone and <u>modulates PAMP-triggered immunity and PAMP-induced plant growth inhibition.</u>**
- **Foliar sprays of HLB-positive *C. sinensis* trees with GA at both 5 mg/L and 25 mg/L reduced tissue H_2O_2 levels and cell death.**
- **GA induced the expression of genes encoding ROS scavenging enzymes catalases, ascorbate peroxidases, and glutathione peroxidases. GA also inhibited the expression of *RBOHD, the gene primarily responsible for CLas-triggered ROS production.***
- **Foliar spray of GA (1247 ppm) suppresses HLB symptoms, improves tree growth.**
- **30% average increase in yield in GA_3-treated trees was observed over a period of 4 years (Tripti Vashisth (Singh et al. 2022))**

Gibberellin (GA) suppresses the growth inhibition caused by HLB

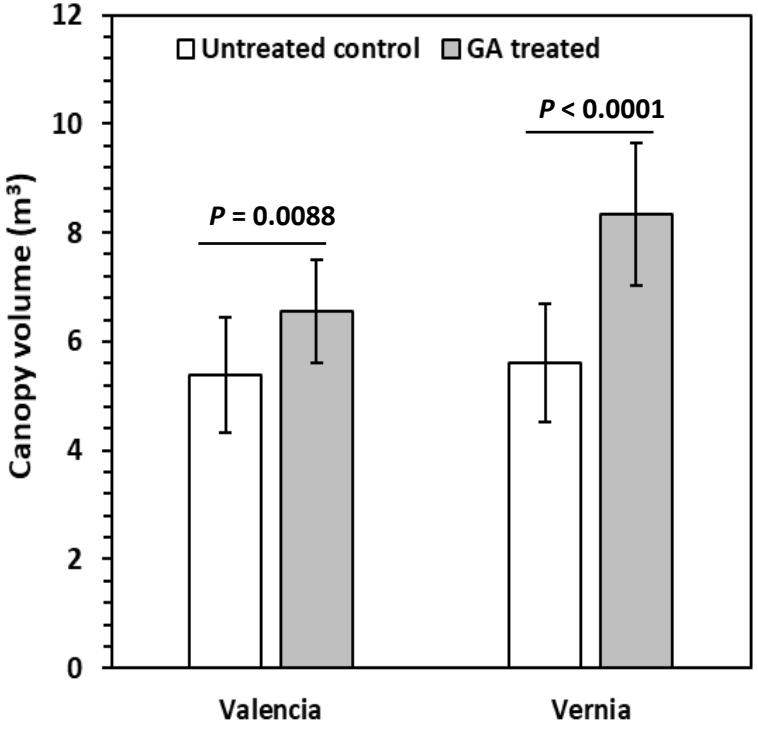
(A) Trunk diameter



(B) Tree height



(C) Canopy volume



Evidence from other colleagues' study supporting that HLB is a pathogen-triggered immune disease

	Las-		Las+AS		Las+S	
	Mexican lime	Persian lime	Mexican lime	Persian lime	Mexican lime	Persian lime
MDA (Arb. unit)	1 ± 0.15 a	1.15 ± 0.32 a	2.44 ± 0.06 c	1.37 ± 0.50 ac	1.98 ± 0.06 c	3.74 ± 0.36 d
H_2O_2 (Arb. unit)	1 ± 0.03 a	0.96 ± 0.04 a	2.15 ± 0.08 c	2.04 ± 0.11 c	1.77 ± 0.15 b	2.08 ± 0.04 c
AsA (Arb. unit)	1 ± 0.31 a	2.34 ± 0.08 b	3.69 ± 1.59 b	7.85 ± 0.72 c	1.16 ± 0.20 a	2.60 ± 0.38 b
APX (Arb. unit)	1 ± 0.69 ab	0.08 ± 0.08 a	1.05 ± 0.38 ab	1.76 ± 0.82 b	4.08 ± 0.10 c	4.74 ± 0.05 c
CAT (Arb. Unit)	1 ± 0.6 a	2.9 ± 0.2 b	1.6 ± 0.4 a	15.0 ± 5.7 d	2.4 ± 0.9 b	4.8 ± 0.1 c

Values of Mexican lime prior infection were used as baseline to adjust the values of Las+AS and Las+S of Mexican lime and Persian lime. Results are expressed as mean + SE (n = 4–6). ANOVA tests were performed to determine if HLB led to significant differences. For each given datum, different letters indicate a statistical difference between genotypes (one-way ANOVA followed by Tukey's post hoc test, $P \leq 0.05$).

- **Sivager et al. 2021 Front. Plant Sci.**
- The Persian triploid lime (*Citrus latifolia*) is one of the most HLB-tolerant citrus varieties, Mexican lime (*Citrus aurantiifolia*) is susceptible to HLB.
- CLas triggers significant ROS production in both Persian lime and Mexican lime.
- CLas triggers significant higher activities of antioxidant enzymes and antioxidant (ascorbate (AsA)) to scavenge ROS in Persian lime than in Mexican lime.

Outline

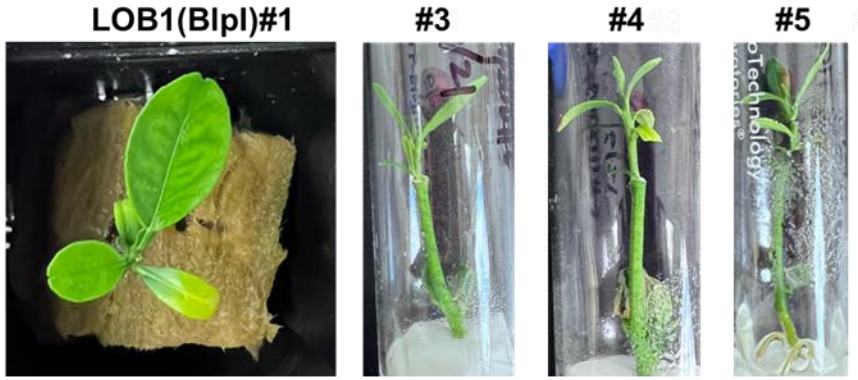
- Citrus Huanglongbing is an immune-mediated plant disease and its implications in HLB management
- **Non-transgenic CRISPR-edited disease resistant sweet orange (canker and HLB)**

Generating non-transgenic HLB tolerant/resistant citrus varieties

- **0 → 1** and **1 → N**

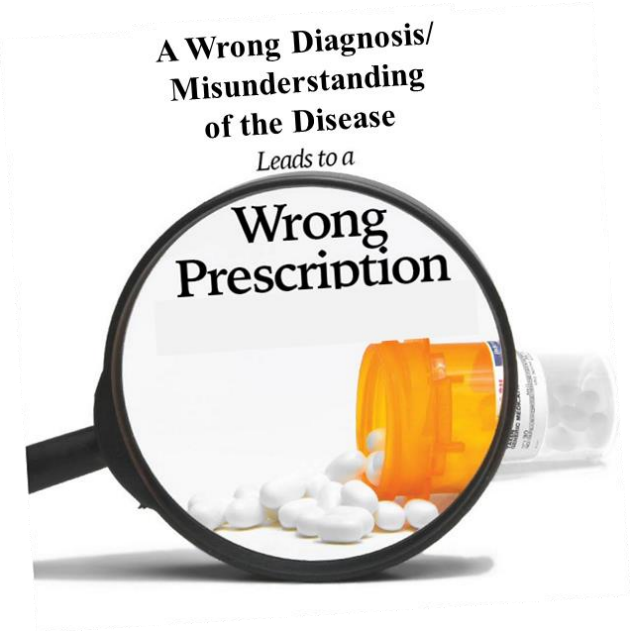
completed/ongoing **ongoing**

- **We have successfully developed non-transgenic CRISPR genome editing technology for citrus.**
- **On July 20, 2022, I showed one non-transgenic canker resistant sweet orange line, we now have eight different non-transgenic canker resistant sweet orange lines (7 biallelic and 1 homozygous lines).**
- **Multiple target genes needed for HLB resistance/tolerance were identified.**
- **Non-transgenic HLB resistant/tolerant sweet orange cultivars will be under testing later this year.**



Non-transgenic biallelic/homozygous canker resistant Hamlin generated using CRISPR genome editing. (The first non-transgenic CRISPR-edited canker resistant Hamlin sweet orange!!!)
Contacted John Beuttenmuller, Florida Foundation Seed Producers, regarding data needed for Citrus Cultivar Release.

Take-home messages



- **Citrus HLB is a pathogen-triggered immune disease, in which the HLB pathogen initiates a systemic and chronic immune response including reactive oxygen species (ROS) production, subsequent cell death of phloem tissues, and eventual HLB symptom development.**
- **Optimized nutrients (macronutrients and micronutrients (B, Fe, Zn, Mo, Ni (not to be mixed with Cu), antioxidants (such as uric acid), immunoregulators (such as GA) have potential to treat HLB disease by halting or reducing ROS-mediated cell death.**
- **Non-transgenic CRISPR genome editing technology is ready for citrus and non-transgenic canker resistant Hamlin has been generated using CRISPR genome editing technology.**
- **Non-transgenic CRISPR edited HLB tolerant/resistant varieties**
- **<u>The right prescription comes from a better understanding of the disease, the pathogen, citrus, and psyllids.</u>**

Acknowledgements

Collaborators

Dr. Jude Grosser, UF
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Citrus Initiative

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