Citrus Huanglongbing is an immune-mediated plant disease and its implications in HLB management

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Outline

- **Citrus Huanglongbing is an immune-mediated plant disease and its implications in HLB management**
- **The first non-transgenic CRISPR-edited canker resistant Hamlin sweet orange**
- **Trunk injection**

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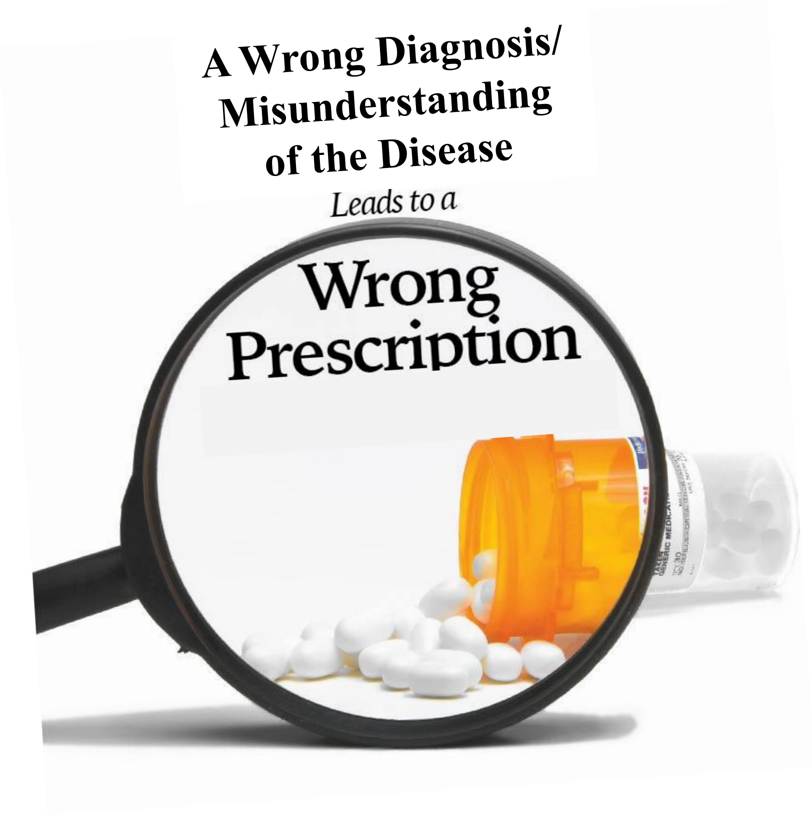
Take-home messages (How does CLas cause damage to citrus?)

- **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 <u>systemic cell death</u> of phloem tissues. This response is instigated primarily through <u>excessive and chronic *reactive oxygen species (ROS)*</u> 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 callose deposition, root decay, starch accumulation, it probably will not work.



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

- Reducing ROS damages caused by HLB increases plant growth and productivity.
- Promoting tolerance to ROS damages increases plant growth and productivity.
- 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 (not to be mixed with Cu) increase the activity of antioxidant enzyme activities and promote plant growth)
  - ✓ 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)

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

- Horticultural approaches reducing ROS damages will help.
- Horticultural approaches increasing citrus tolerance to ROS damages will help.
- Horticultural approaches promoting plant growth will help.

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.**
- **HLB tolerant/resistant citrus cultivars are being generated by editing genes responsible for CLas-triggered ROS production and transgenic expression of antioxidant enzyme genes.**

- **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**
- **Known HLB symptoms: root decay, blotchy mottle, stunt growth, starch accumulation, hardened leaves…**

➢ **Stunted growth probably results from the direct effect of ROS and reduced transport of carbohydrates and hormones.**

➢ **Cell death of the phloem tissue, reduced transport of photosynthates, and ROS inhibition of root growth may be responsible for root decay.**

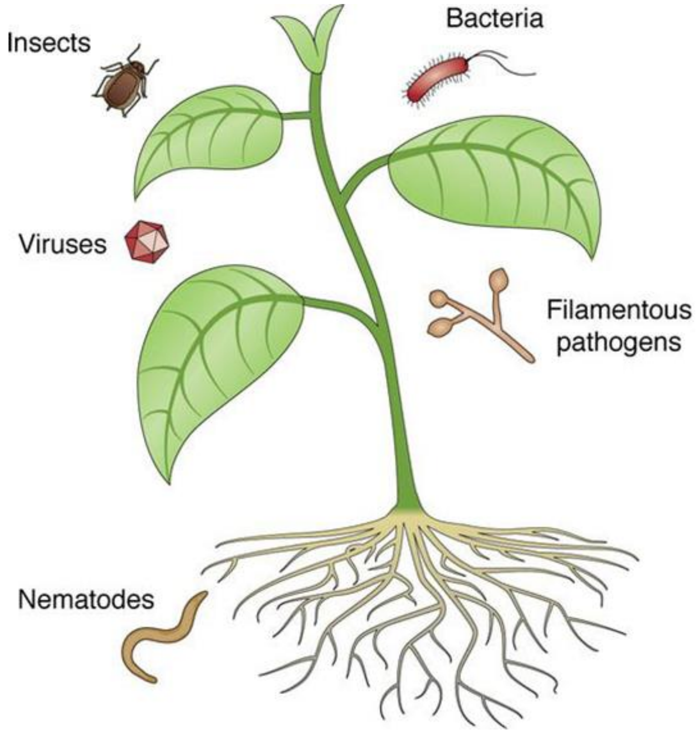
➢ **Death of companion and sieve element cells can lead to starch accumulation, and the resultant blotchy mottle symptoms.**

➢ **Hardened leaves likely result from the action of ROS which are known to directly cause strengthening of host cell walls.**

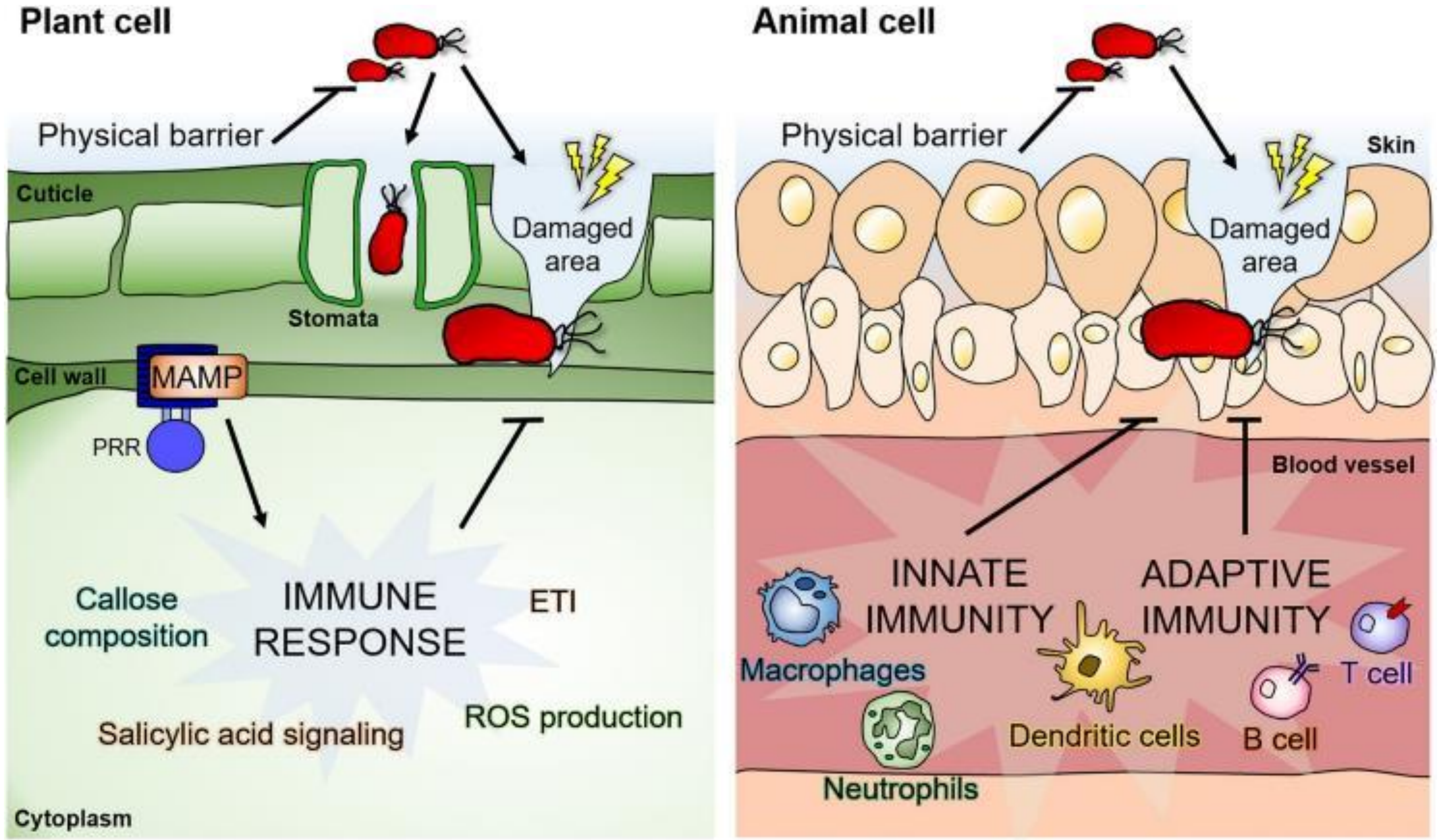
➢ **During active growth (such as in the later spring and summer), HLB symptoms are not obvious (potential reasons: a tradeoff relationship between growth and immunity in plants (Growth usually suppresses immunity, and vice versa); CLas takes time to buildup in the young shoots and newly generated phloem tissues).**

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

Plant and animal immune systems



Bentham et al. 2020

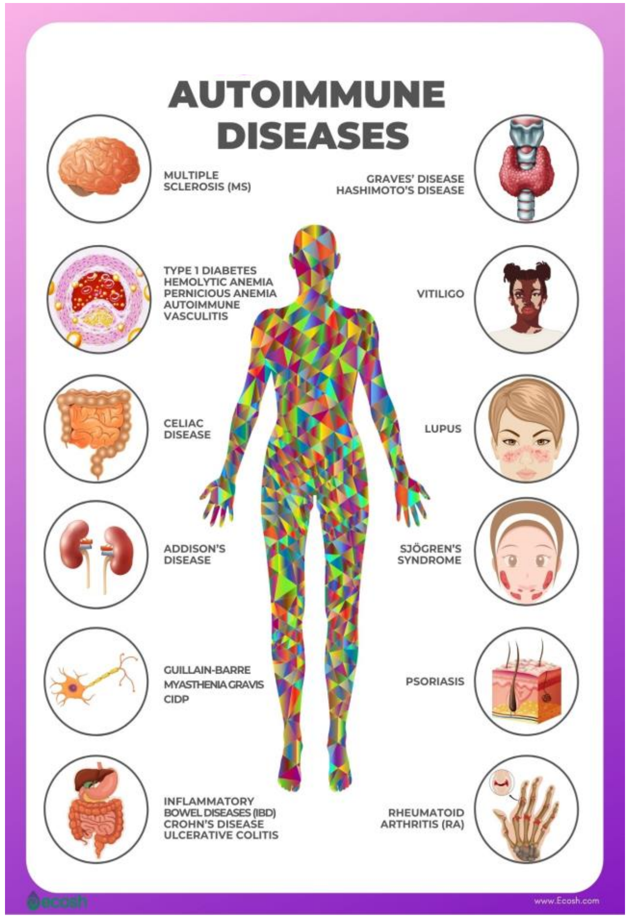


Kim et al. 2020

- Both plants and animals have innate immunity. In the plant world, 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!

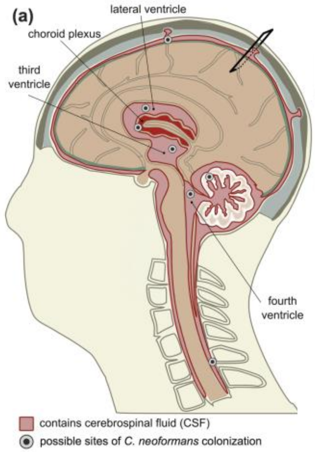
Immune-mediated diseases

Autoimmune diseases



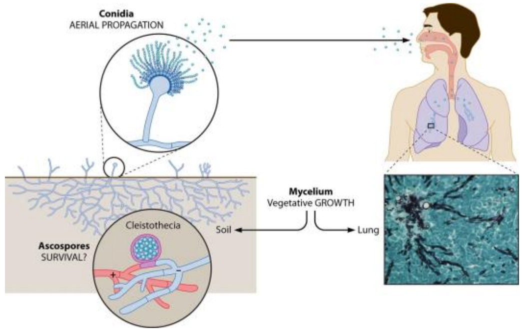
Non-autoimmune diseases

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

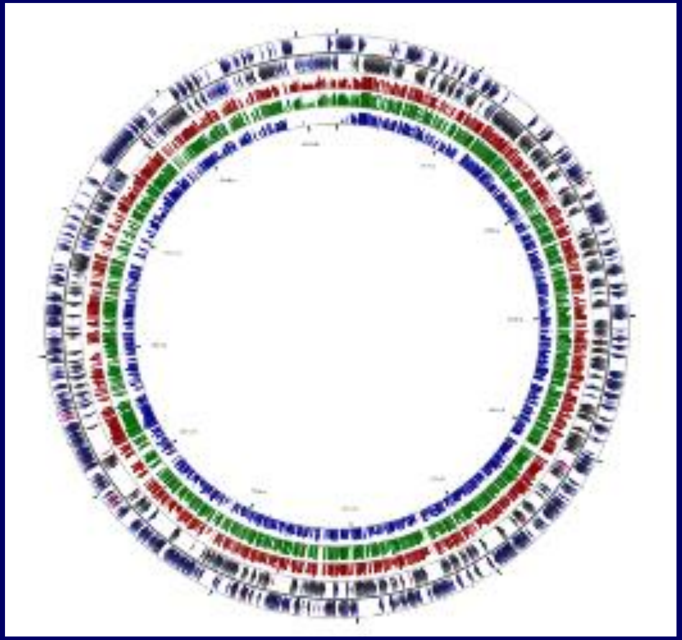
Evidence that demonstrate citrus HLB is a pathogen-triggered immune disease

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

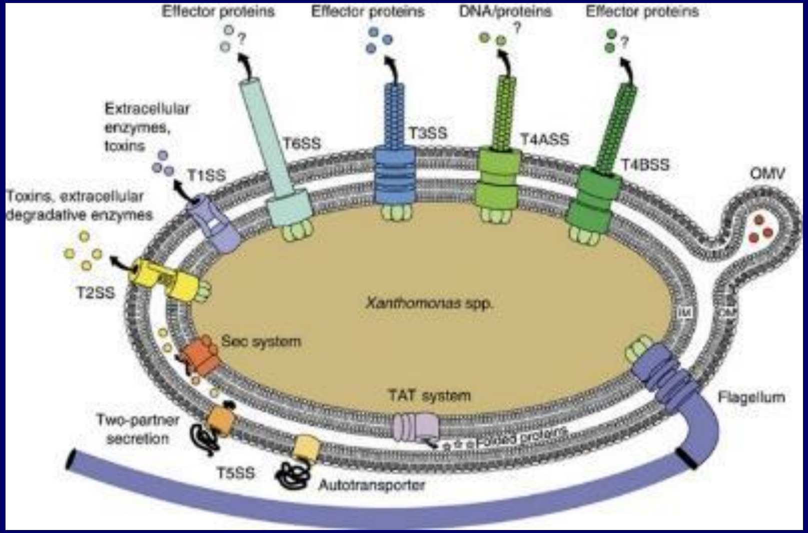
This is different from what we know about plant pathogens! All known pathogens have pathogenicity factors to cause disease.

- 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

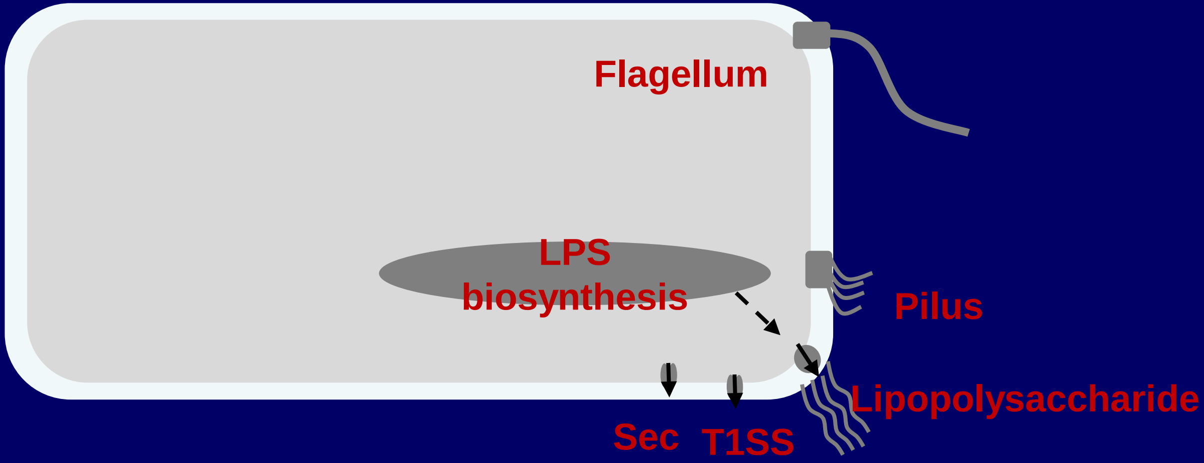


Büttner & Bonas 2010

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.



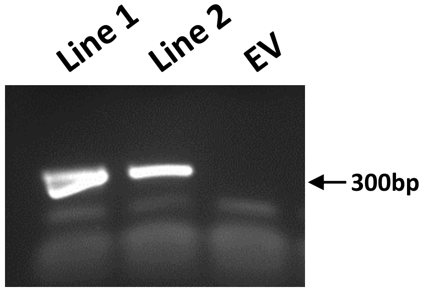
Overexpression of four putative CLas virulence genes in *Citrus* does not cause HLB symptoms

CLIBASIA_04405 transgenic sweet orange

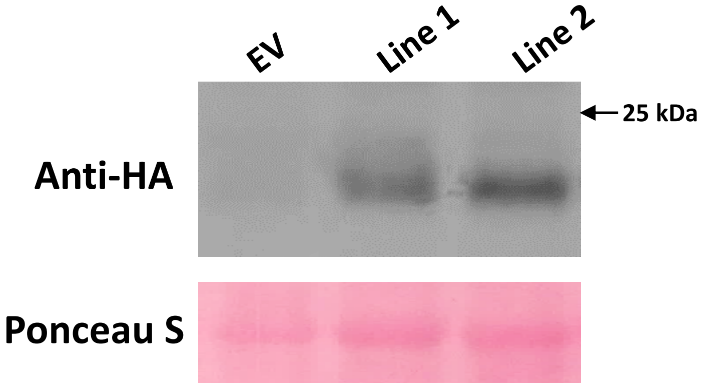
EV Line 1 Line 2

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*

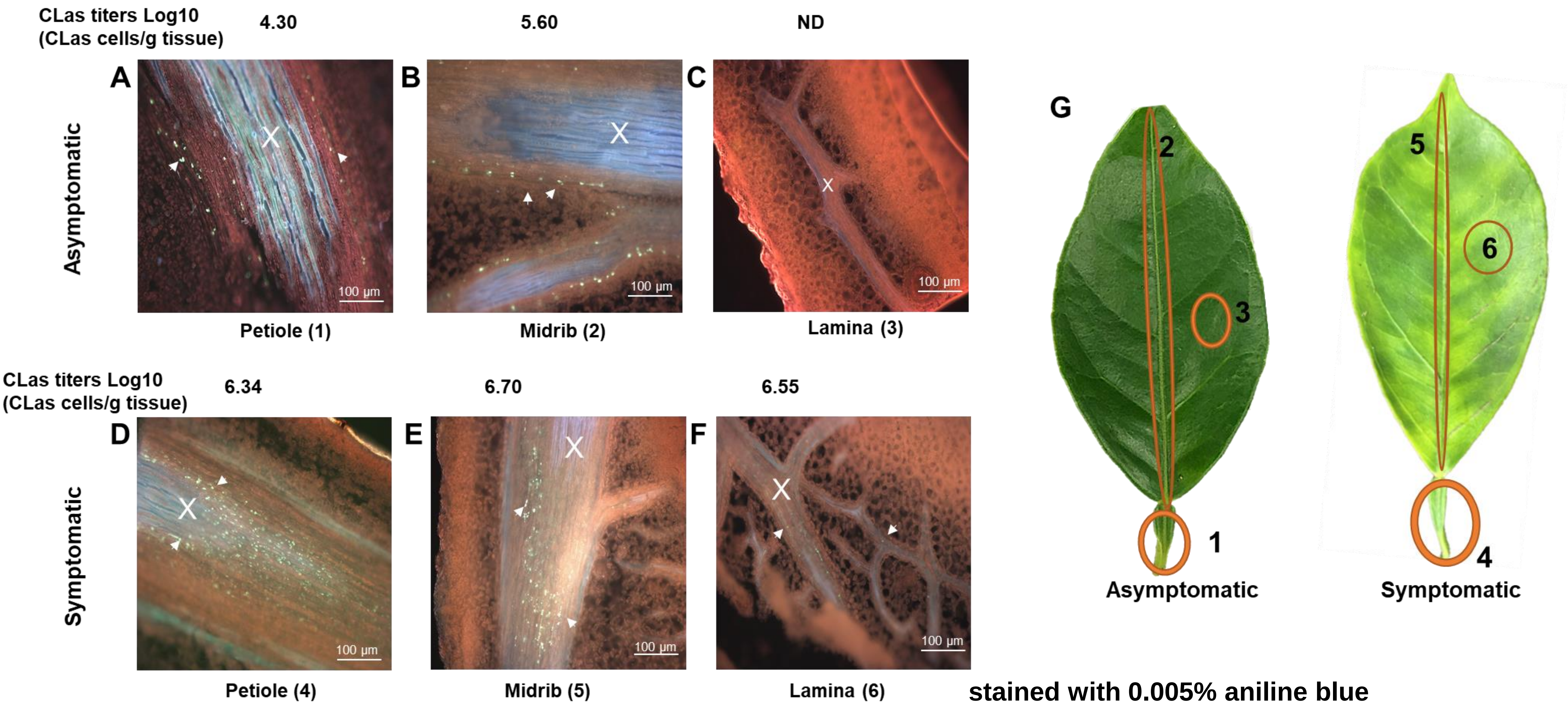
Evidence that demonstrate citrus HLB is a pathogen-triggered immune disease

- 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

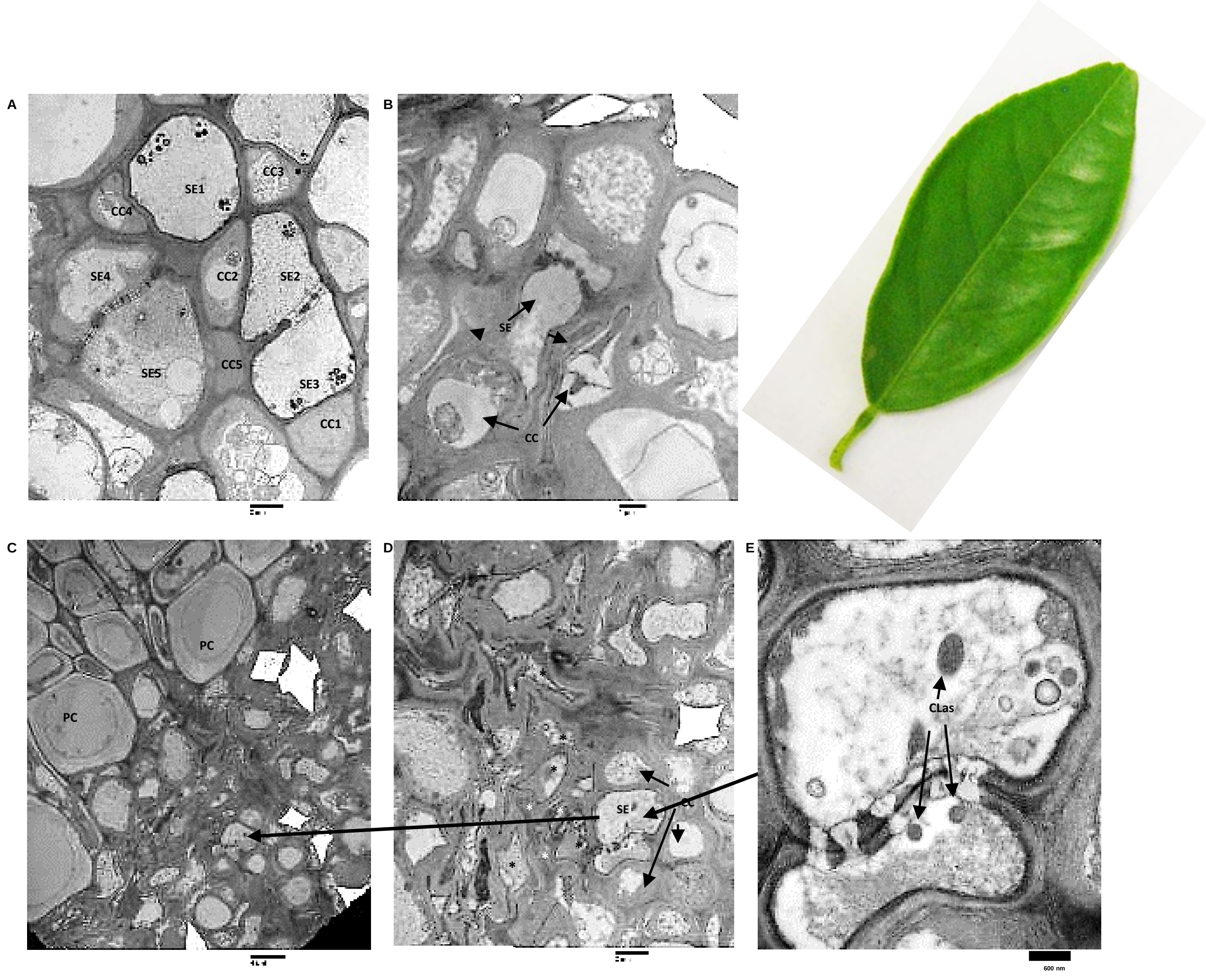
- ✓ **CLas infection causes reactive oxygen species (ROS) production, callose deposition, and starch accumulation in young citrus leaves**
- ✓ **CLas infection induces immune related genes such PR genes.**
- ✓ **CLas infection causes phloem cell death. Cell death was more severe in leaves with severe symptoms.**

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



stained with 0.005% aniline blue

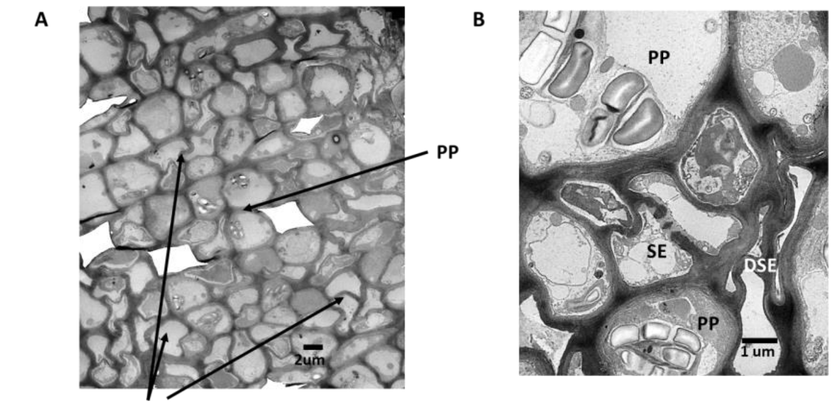
Cell death of sieve element and companion cells was observed in asymptomatic young leaves of HLB positive *C. sinensis* ‘Valencia’ trees



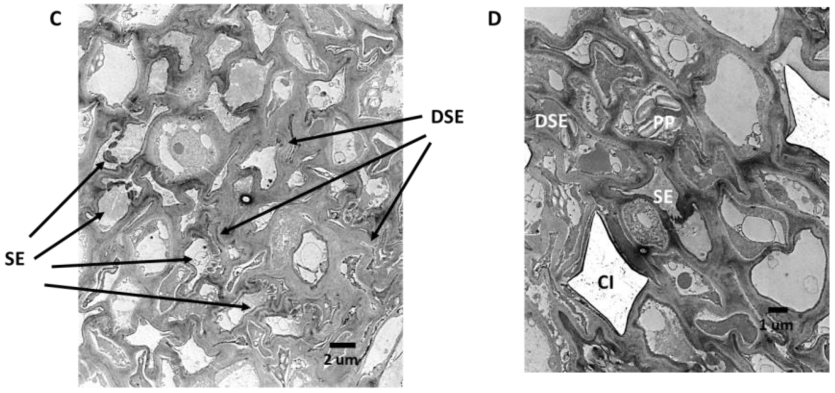
Key observations:

- **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).**

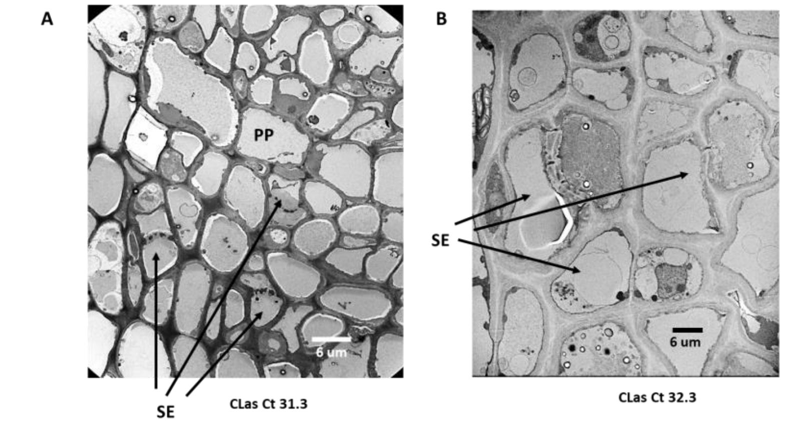
Cell death of sieve element and companion cells positively correlate with CLas titers and HLB symptoms



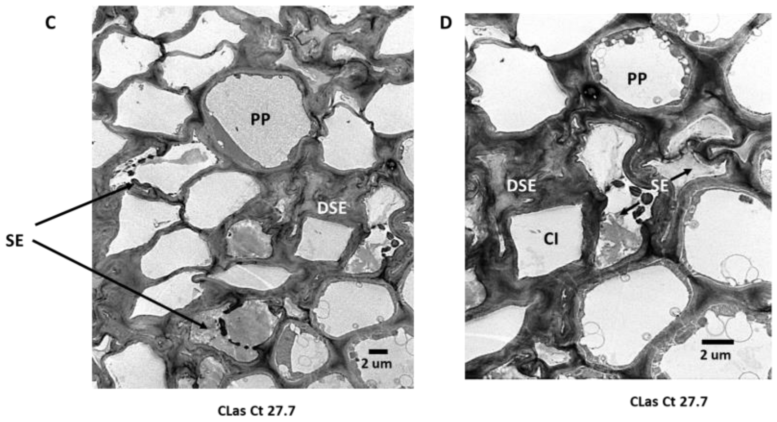
Midvein of asymptomatic mature leaf (CLas Ct 31.7)



Midvein of symptomatic mature leaf (CLas Ct 28)



Stem of asymptomatic branches



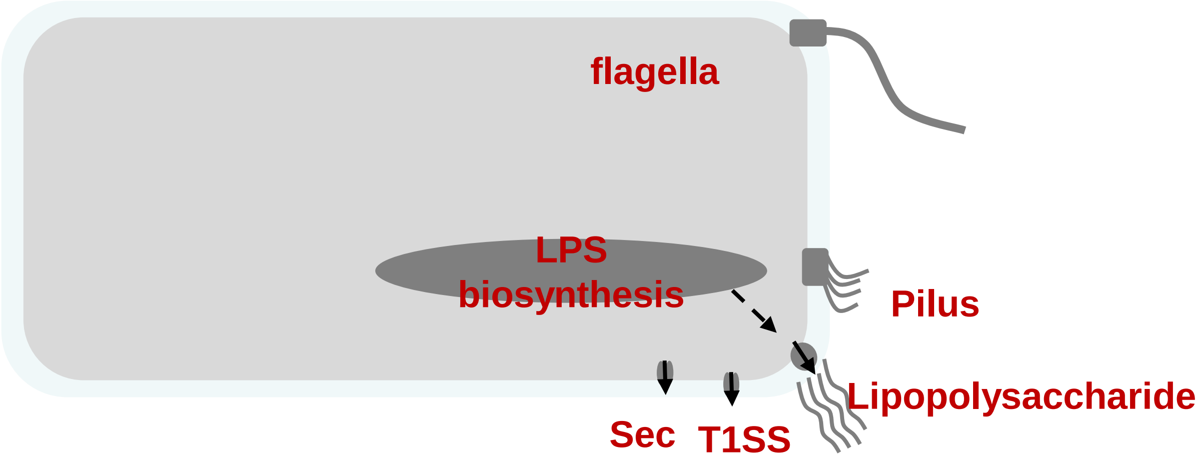
Stem of symptomatic branches

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

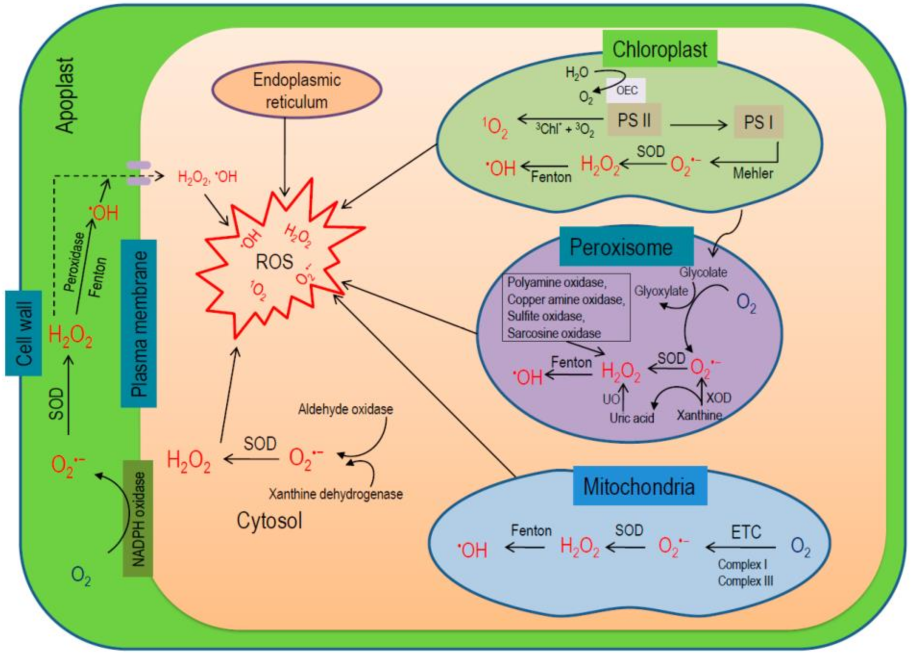
Evidence that demonstrate citrus HLB is a pathogen-triggered immune disease

- 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

The H_2O_2 concentration is **10–15 μmol g^{-1} FW** in mature symptomatic leaves, but is **2 μmol g^{-1}** FW in healthy mature leaves.

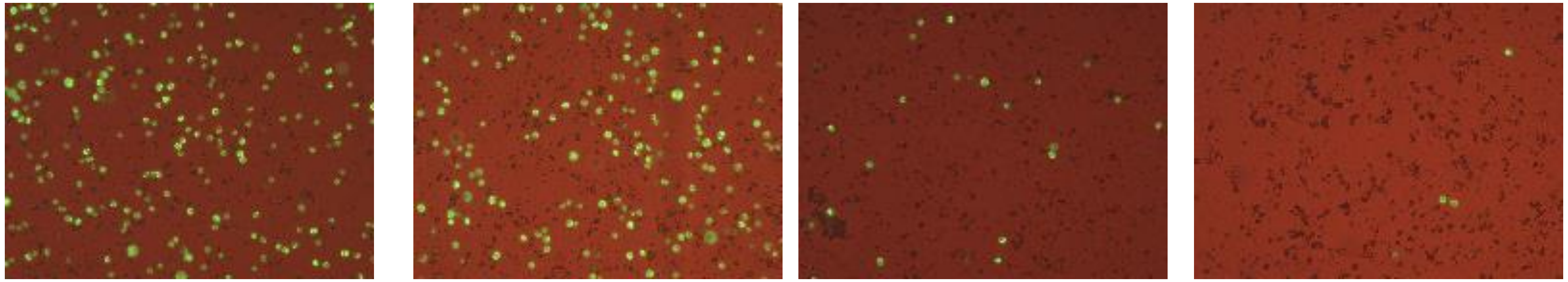
Xanthomonas citri subsp. citri infection of kumquat triggers H_2O_2 production (9.86 μmol g^{-1} FW) at 8 days after inoculation and causes **cell death/hypersensitive response** (Kumar et al. 2011).

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.

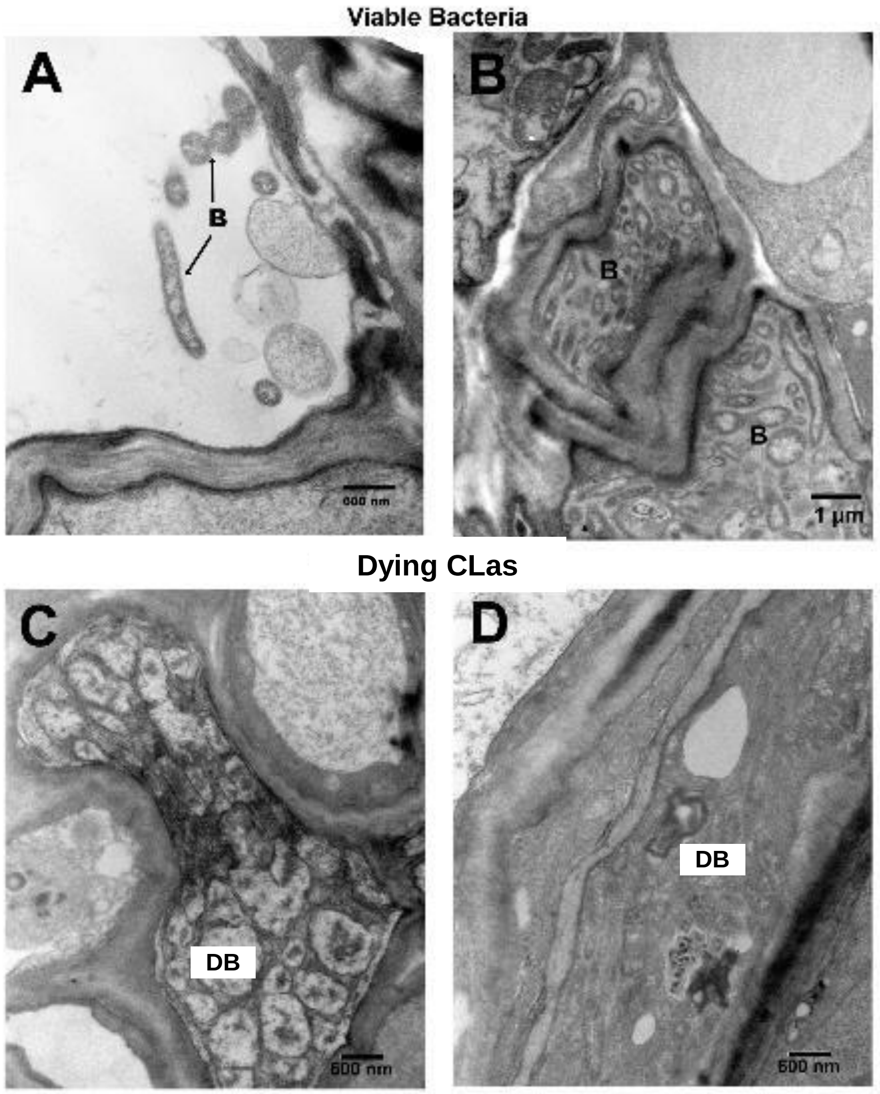
HLB is a pathogen (CLas)-triggered immune disease

- **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?

Dying CLas was observed in infected tissues with increasing CLas titers.



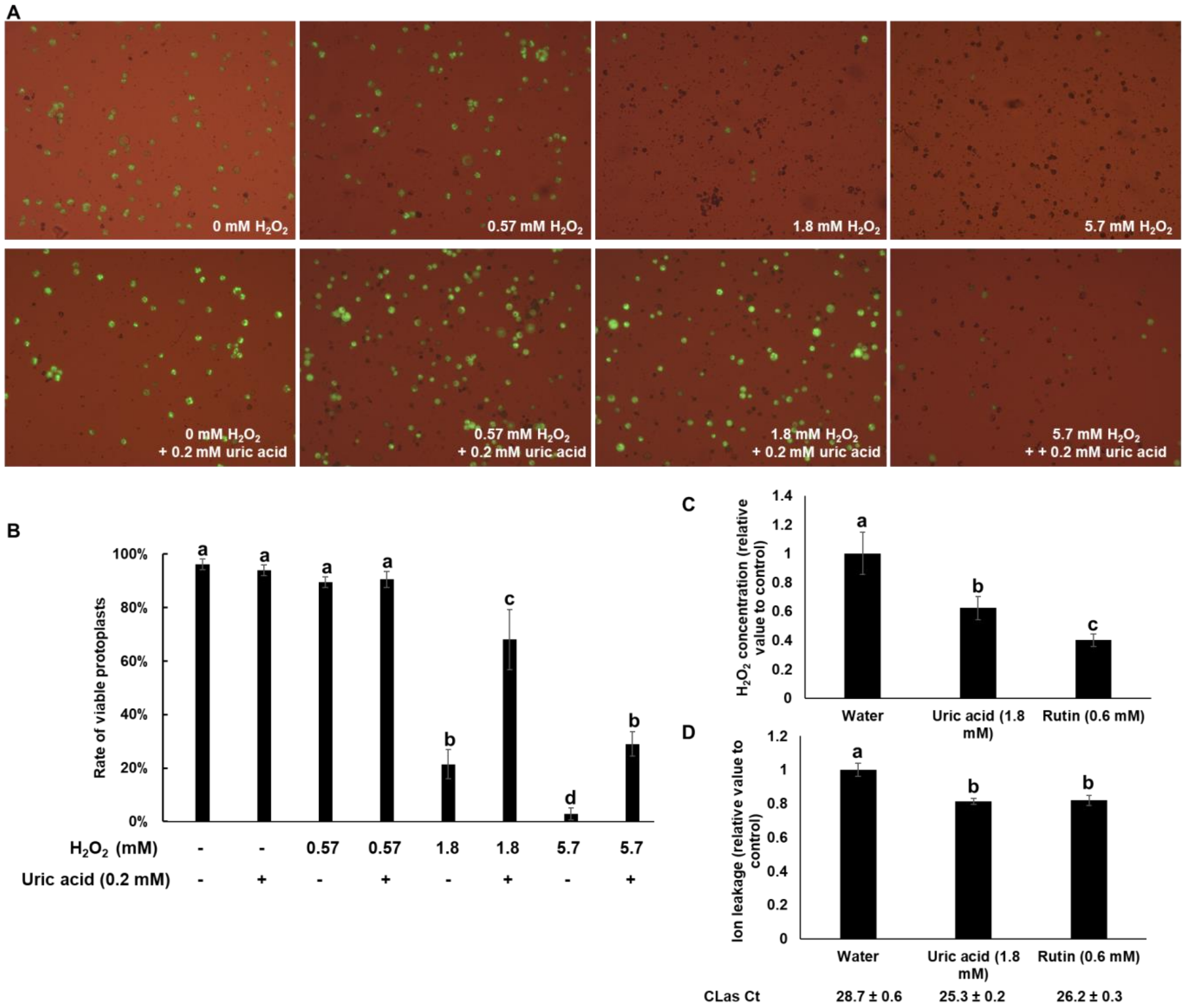
- **Do ROS kill CLas?**
  ROS concentration is high enough to kill CLas
  Dying CLas is observed infected tissues.

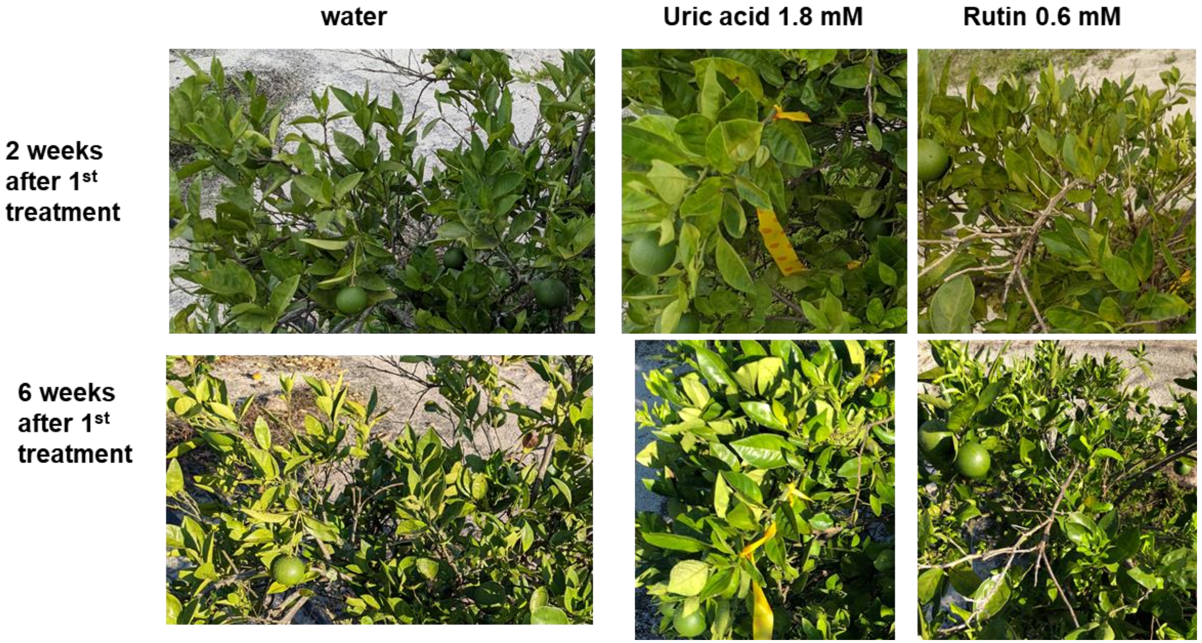
- **Can we rely on ROS to kill CLas?**
  No. We could not use ROS to kill CLas, but citrus phloem cells.

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

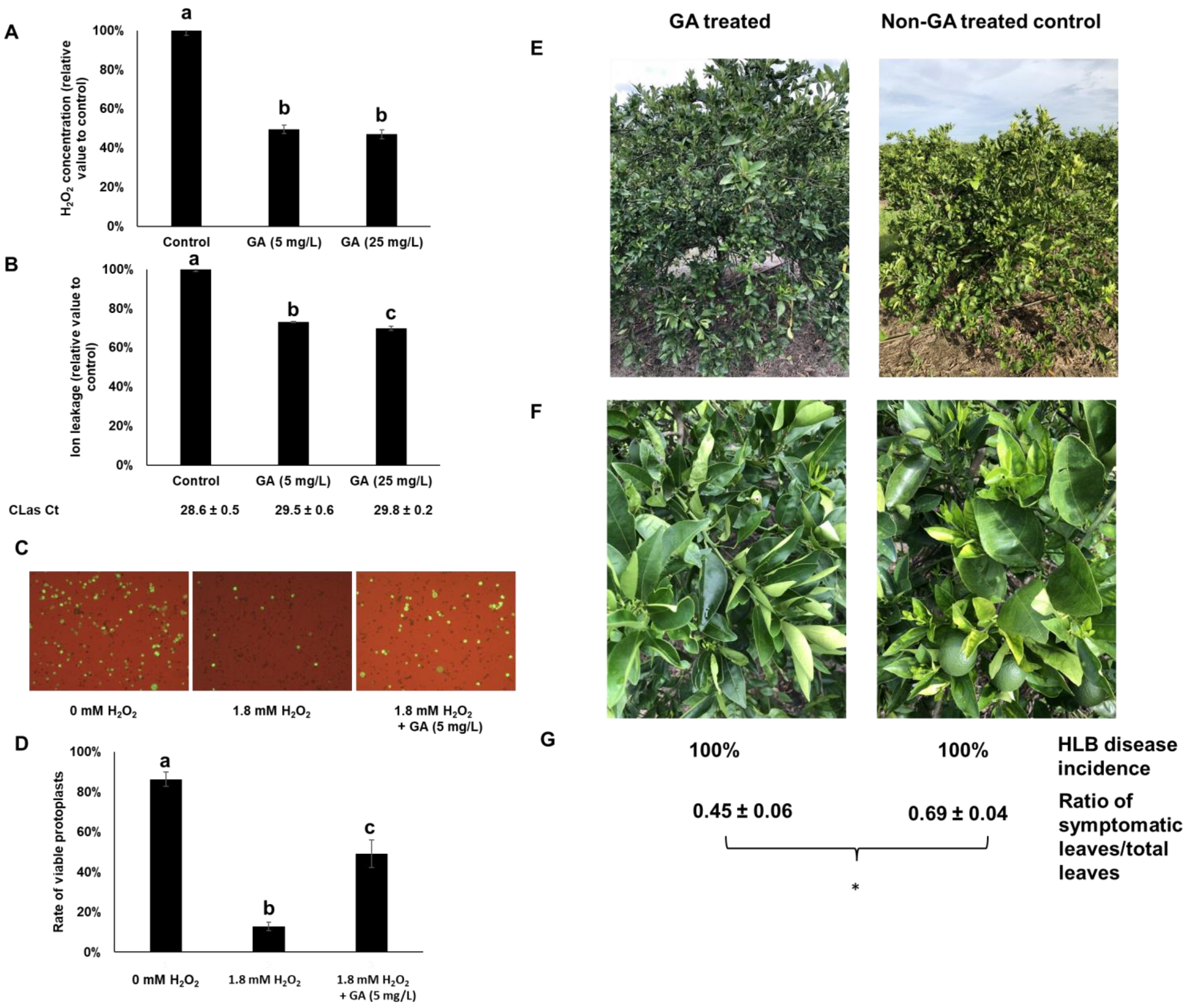




foliar spay weekly for six weeks

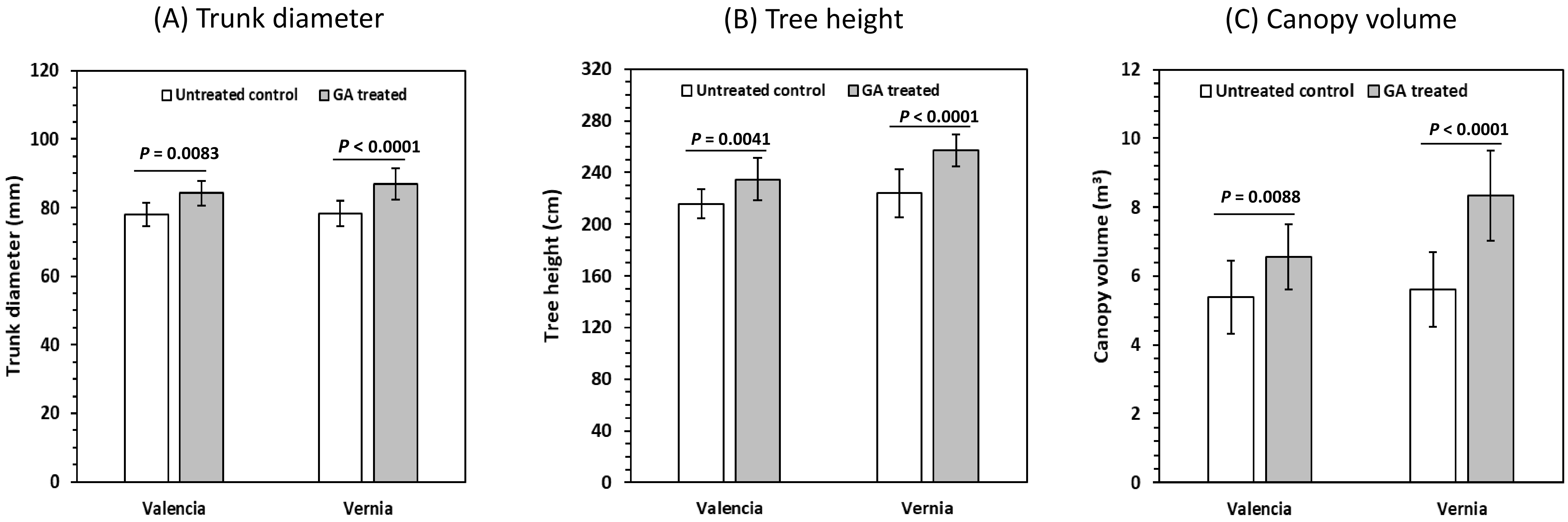
- ❖ Antioxidants can prevent or reduce damage to cells caused by ROS
- ❖ ROS are responsible for cell death of the phloem tissues of CLas infected citrus
- ❖ Cell death of phloem tissues is responsible for HLB symptoms
- ❖ Mitigating ROS damages reduces HLB damages
- ❖ Antioxidants such as uric acid 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



GA effect on HLB control needs to be combined with optimized nutrient program

- ✓ Balanced nutrition (nutrition deficiency causes more ROS production)
- ✓ Application of micronutrients
- Cu, Fe, B, Mo, Ni, and Zn at suitable concentrations suppressed protoplast cell death caused by ROS, whereas no effect was observed for Mn by increasing the activity of antioxidant enzyme activities and promoting plant growth
- The effect of B, Fe, Zn, Mo, and Ni application was additive, whereas Cu reduced the level of suppression of ROS-triggered protoplast cell death when combined with other micronutrients. Suggestions: do not mix Cu with other micronutrients and leave a 2-week winder between Cu and other micronutrient application.

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.

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

- Ute Albrecht and Kim D.Bowman 2012 Plant Science
- HLB tolerant US-897 (*Citrus reticulata* × *Poncirus trifoliata*) and susceptible 'Cleopatra' mandarin (*C. reticulata*)
- 326 genes (including many plant defense/immune genes) were significantly upregulated by CLas at least 4-fold in the susceptible genotype, compared with only 17 genes in US-897.

Outline

- Citrus Huanglongbing is an immune-mediated plant disease and its implications in HLB management
- **The first non-transgenic CRISPR-edited canker resistant Hamlin sweet orange**
- Trunk injection

Generating HLB tolerant/resistant citrus varieties

- **We have successfully developed non-transgenic CRISPR genome editing technology for citrus.**
- **Prevent overproduction of ROS via editing the promoter or coding regions of respiratory burst oxidative homolog D (RbohD, the main producer of ROS in response to CLas infection) gene to reduce the induction by CLas.**
- **Evade recognition of CLas by editing the promoter or coding regions of receptor genes and immune signaling genes.**
- **Enhance plant tolerance of ROS via phloem-specific overexpression of antioxidant enzymes (such as superoxide dismutase, catalases, glutathione peroxidases, ascorbate peroxidase, and glutathione reductase) using CRISPR gene editing, transgenic, or cisgenic approaches or citrus tristeza virus vectors).**
- **Non-transgenic biallelic/homozygous HLB resistant/tolerant citrus cultivars are being generated using CRISPR genome editing.**

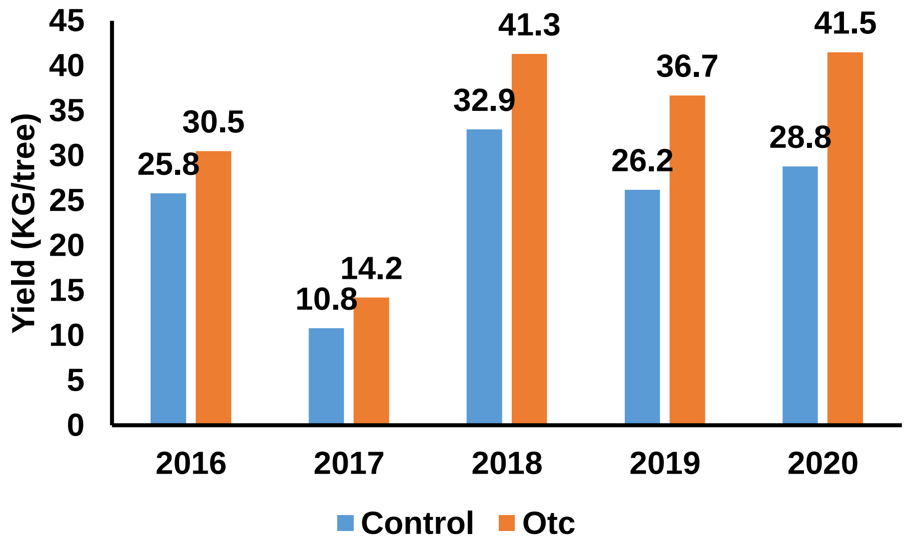
Non-transgenic biallelic/homozygous canker resistant Hamlin generated using CRISPR genome editing. (The first non-transgenic CRISPR-edited canker resistant Hamlin sweet orange!!!)
Contacted John Beuttenmuller, Florida Foundation Seed Producers, regarding data needed for Citrus Cultivar Release.

Outline

- Citrus Huanglongbing is an immune-mediated plant disease and its implications in HLB management
- The first non-transgenic CRISPR-edited canker resistant Hamlin sweet orange
- **Trunk injection**

Trunk injection with oxytetracycline for five years

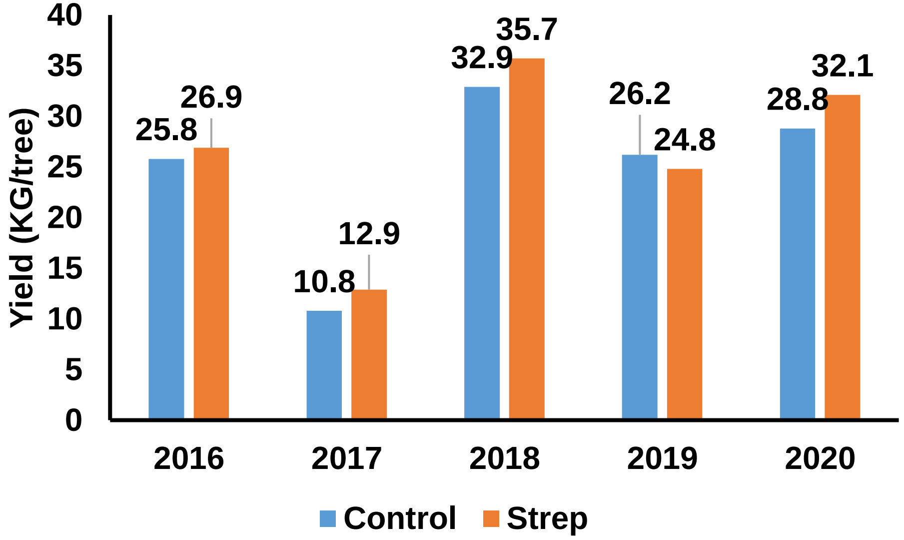
Days post injection	Ct value									
	2016		2017		2018		2019		2020	
	CK	OTC (0.25g/tree)	CK	OTC (0.25g/tree)	CK	OTC (0.5g/tree)	CK	OTC (3.0g/tree)	CK	OTC (4.0g/tree)
0	32.7a	32.1a	29.6a	30.7a	28.2a	28.9b	26.3a	25.6f	26.2a	28.5c
7	N/A	N/A	N/A	N/A	28.7a	29.4ab	25.7a	28.1e	N/A	N/A
14	N/A	N/A	N/A	N/A	27.9a	29.9ab	26.4a	31.9c	N/A	N/A
28	31.5a	32.8a	28.9a	31.2a	28.3a	30.5a	25.5a	34.1a	N/A	N/A
60	N/A	N/A	N/A	N/A	27.7a	29.8ab	26.3a	33.4ab	26.4a	32.3a
120	30.6a	31.9ab	29.2a	30.8a	28.2a	29.1ab	25.6a	32.5bc	25.7a	31.5ab
180	N/A	N/A	N/A	N/A	27.4a	28.7b	25.9a	32.7bc	25.9a	31.8ab
240	30.1ab	31.5ab	28.3a	30.1a	28.1a	27.9bc	26.6a	32.2bc	27.3a	31.1ab
300	N/A	N/A	N/A	N/A	27.3a	27.4bc	25.9a	31.7c	26.8a	30.7b
360	29.2b	30.4b	28.7a	29.7a	27.1a	26.3c	27.2a	30.1d	26.1a	30.2b



Hamlin sweet orange on Swingle citrumelo rootstock, planted in 2013.
At lower doses, Otc did not reduce CLas titers in 2016, 2017, and 2018.
At higher doses, Otc reduced CLas titers.
In all five years, Otc significantly increased yield ($p<0.05$)

Trunk injection with streptomycin for five years

2016			2017		2018		2019		2020	
Days post injection	CK	STR (0.25g/tree)	CK	STR (0.5g/tree)	CK	STR (0.5g/tree)	CK	STR (3g/tree)	CK	STR (4g/tree)
0	32.7a	33.1a	29.6a	30.9a	28.2a	29.3a	26.3a A	26.6e	26.2a	27.9c
7	N/A	N/A	N/A	N/A	28.7a	29.1a	25.7a	27.9d	N/A	N/A
14	N/A	N/A	N/A	N/A	27.9a	30.2a	26.4a	30.4bc	N/A	N/A
28	31.5a	33.5a	28.9a	31.6a	28.3a	30.6a	25.5a	32.9a	N/A	N/A
60	N/A	N/A	N/A	N/A	27.7a	29.7a	26.3a	32.3a	26.4a	31.1a
120	30.6a	32.7ab	29.2a	30.8a	28.2a	28.9a	25.6a	31.9ab	25.7a	30.6ab
180	N/A	N/A	N/A	N/A	27.4a	28.1ab	25.9a	31.5ab	25.9a	30.4ab
240	30.1ab	32.3ab	28.3a	30.4a	28.1a	27.4b	26.6a	30.8b	27.3a	30.8ab
300	N/A	N/A	N/A	N/A	27.3a	27.7b	25.9a	30.4bc	26.8a	30.2ab
360	29.2b	31.1b	28.7a	29.6a	27.1a	26.9b	27.2a	29.3c	26.1a	29.5b



Hamlin sweet orange on Swingle citrumelo rootstock, planted in 2013.
At lower doses, Strep did not reduce CLas titers in 2016, 2017, and 2018.
At higher doses, Otc reduced CLas titers.
In all five years, Strep did not significantly increase yield ($p<0.05$)

Does trunk injection of Otc and Strep cause tree collapse?

Not observed.

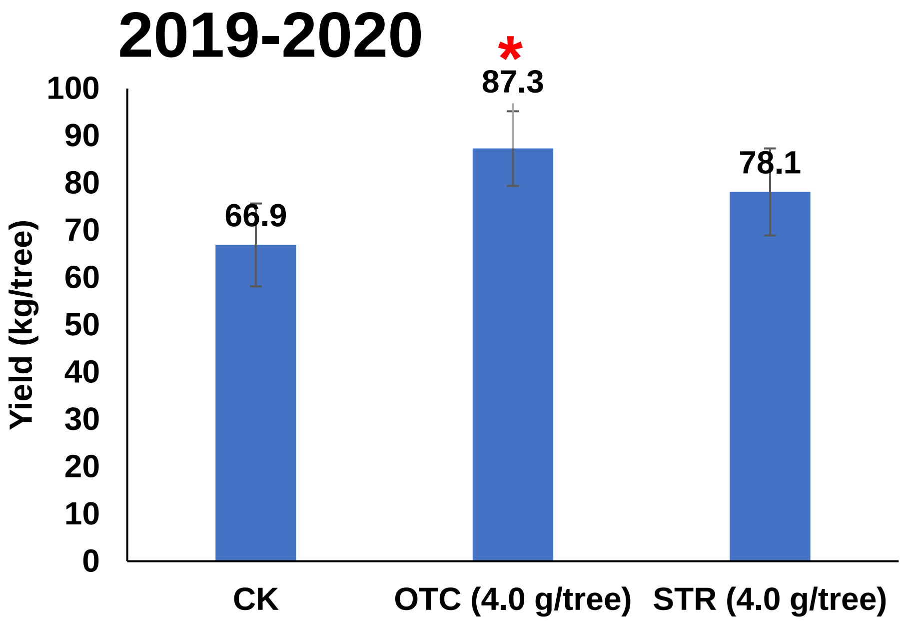
For both Otc and Strep trunk injection, some trees have thicker canopy than control trees, but not always.

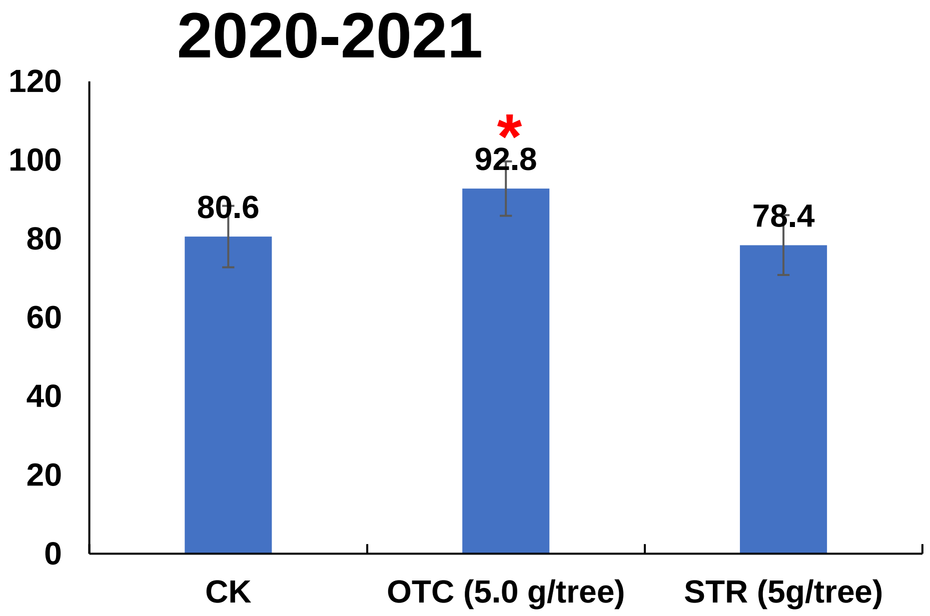
Control Otc g/tree 2016 0.25
2017 0.25 2018 0.5
2019 3.0 2020 4.0

Control Otc g/tree 2016 0.25,
2017 0.25 2018 0.5
2019 3.0 2020 4.0

Control Strep g/tree 2016 0.5
2017 0. 5 2018 0.5
2019 3.0 2020 4.0

Control Strep g/tree 2016 0.25
2017 0.25 2018 0.25
2019 1.5 2020 4.0





Trunk injection Trial 2

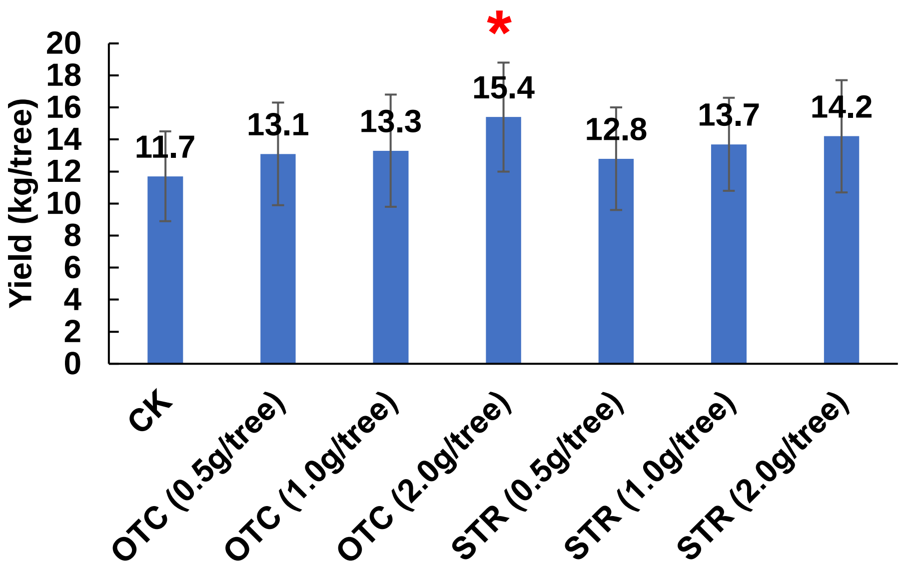
- Hamlin on Swingle planted in March 1999
- Otc at 4g/tree (2019) and 5g/tree (2020) significantly increased yield in both 2019-20 and 2020-21 seasons.
- Strep at 4g/tree (2019) and 5g/tree (2020) did not significantly increase yield in both 2019-20 and 2020-21 seasons.
- Otc fruit residues (ppm):

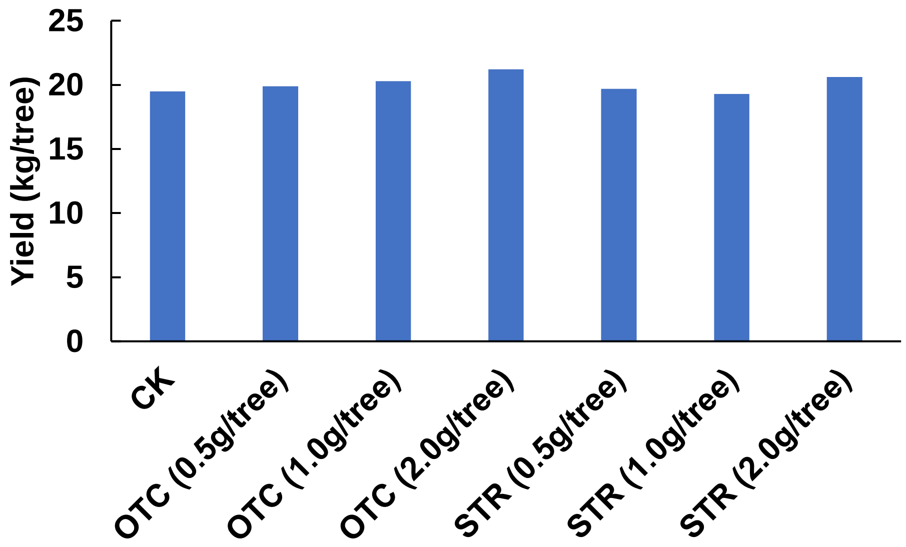
0.19 ± 0.09 in 2019-2020
0.25 ± 0.11 in 2020-2021

OTC: The U.S. maximum residue limits of 0.01 µg/g for OTC in or on citrus fruit (EPA 2018)

Strep residues (ppm):
0.17 ± 0.08 in 2019-2020
0.22 ± 0.09 in 2020-2021
Strep: the maximal residue limit of 2.0 µg/g for STR in or on citrus (EPA 2017)

Trunk injection Trial 3





- Valencia orange trees, planted in 2016
- Injection was conducted in February-March 2019, but not in 2020.
- Otc (2 g/g, but not less) significantly increased yield in the year of injection (2019-2020 season), but not in the following year without injection (2020-2021 season).
- Strep had no effect on yield.

Residue levels in fruits

OTC or STR residue (ppm)														
2016			2017			2018			2019			2020		
CK	OTC (0.25g/tree)	STR (0.5g/tree)	CK	OTC (0.25g/tree)	STR (0.5g/tree)	CK	OTC (0.5g/tree)	STR (0.5g/tree)	CK	OTC (3.0g/tree)	STR (3.0g/tree)	CK	OTC (4.0g/tree)	STR (4.0g/tree)
N/A	N/A	N/A	ND	0.018	0.029	ND	0.025	0.021	ND	0.19	0.21	ND	0.24	0.28

OTC: The U.S. maximum residue limits of 0.01 μg/g for OTC in or on citrus fruit (EPA 2018)

Strep: the maximal residue limit of 2.0 μg/g for STR in or on citrus fruit permitted by government agencies (EPA 2017)

Take-home messages

- **Citrus HLB is a pathogen-triggered immune disease (Ma et al., 2022)**
- **Simply: CLas → ROS (excessive and chronic) → cell death of phloem tissues → HLB symptoms or damages to the tree**

Take-home messages (What you can do now)

- Reducing ROS damages caused by HLB increases plant growth and productivity.
- Promoting tolerance to ROS damages increases plant growth and productivity.
- 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 (not to be mixed with Cu) increase the activity of antioxidant enzyme activities and promote plant growth)
  - ✓ 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 you can test by yourself)

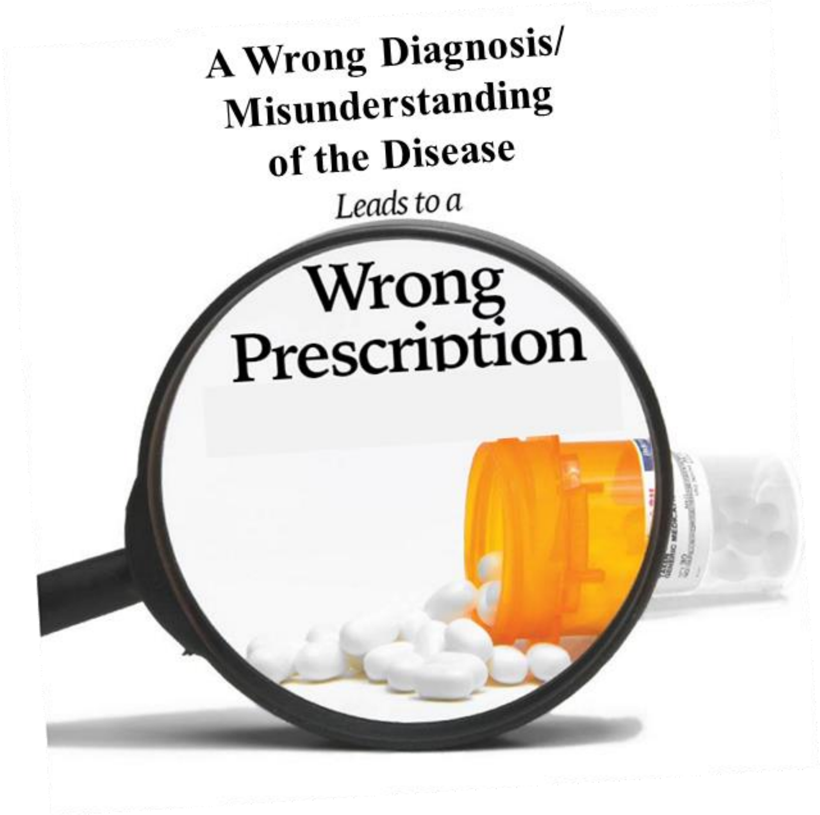
- Simply: CLas → ROS (excessive and chronic) → cell death of phloem tissues → HLB symptoms or damages to the tree

- Horticultural approaches reducing ROS damages will help.
- Horticultural approaches increasing citrus tolerance to ROS damages will help.
- Horticultural approaches promoting phloem growth will help.

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

- Non-transgenic CRISPR genome editing technology is ready for citrus.
- HLB tolerant/resistant varieties:

  Not quite ready, but getting there.
- The right prescription comes from a better understanding of the disease, the pathogen, citrus, and psyllids.



Acknowledgements

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