Citrus Tree Defensin Project

September 20, 2016

This is the normal research process…..

Step 01: Topic → Step 02: Research Plan → Step 03: Research

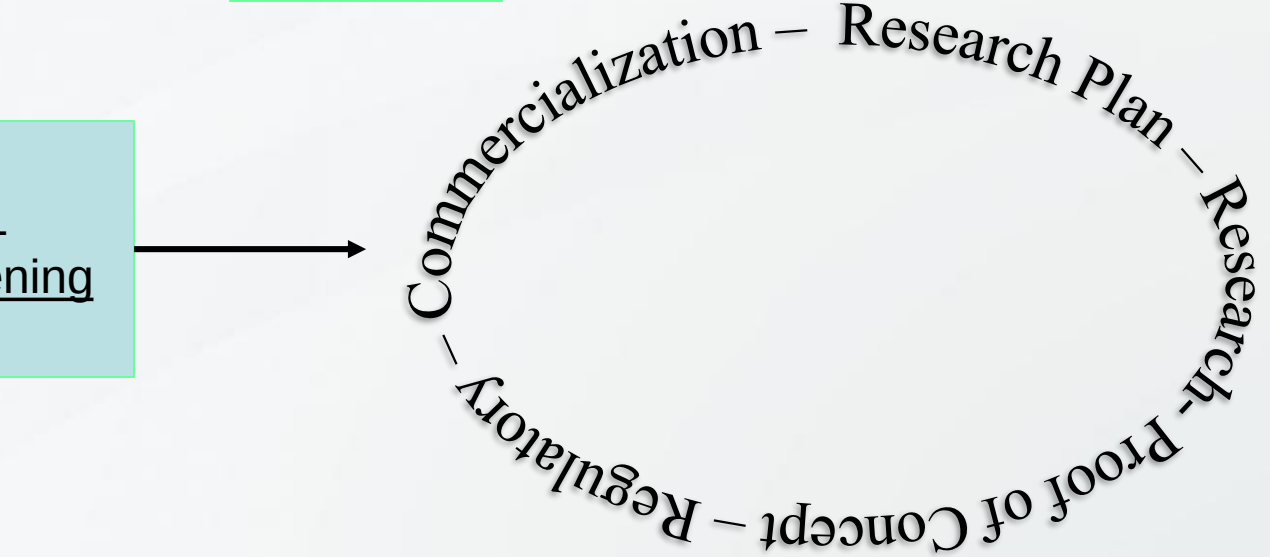
↓

Step 04: Proof of Concept → Step 05: Regulatory → Step 06: Commercialization

This is the research process with HLB

Next Step:

Step 01
Topic: Greening



Any Questions?

A little perspective:

Who is doing this?

and

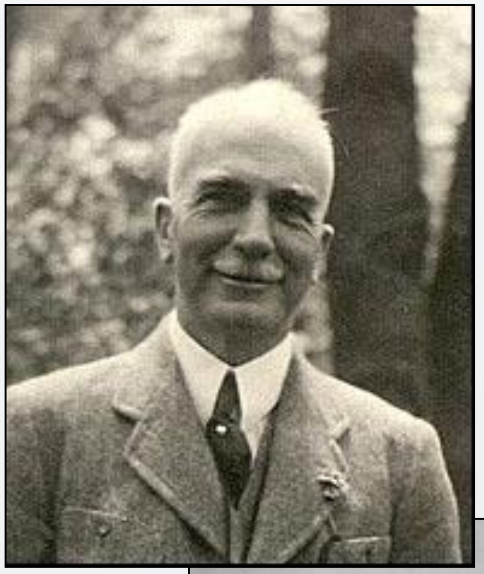
Why we are doing it?

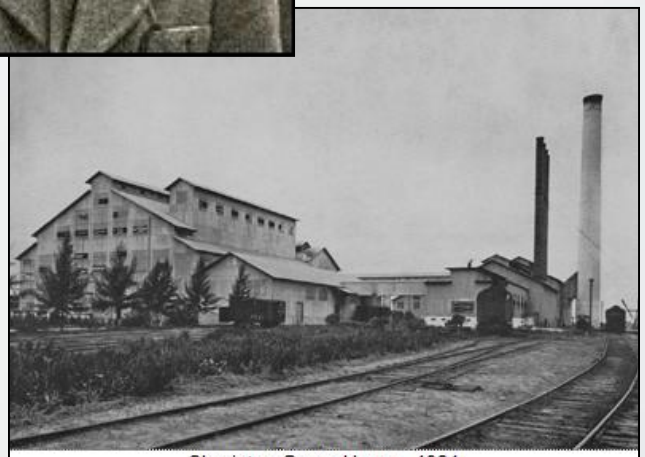
UNITED STATES SUGAR CORPORATION

SOUTHERN GARDENS CITRUS

U.S. Sugar Corporation / Southern Gardens Citrus

- Began as Southern Sugar Company in 1926
- Acquired by Charles Stewart Mott, principal shareholder and co-founder of General Motors, and restructured as U.S. Sugar in 1931
- Rapidly became America's largest and most productive sugarcane farming and sugar manufacturing business
- Expanded to Citrus groves in 1980's and Citrus processing in 1990's





Clewiston Sugar House, 1931

UNITED STATES SUGAR CORPORATION

SOUTHERN GARDENS CITRUS

Farming and Processing Basics

- 188,600 gross acres (owned)
  - 173,000 acres sugar
  - 15,000 acres citrus
  - 600 acres other misc.
- North America's most efficient sugar milling operation
  - Design capacity of 42,000 tons per day mill that was completed October, 2007
  - Built in 3 phases over 3-year period while original mill was operating
- Integrated refinery with packaging: 17 million cwt. capacity

- Railroad operations
  - 278 miles of track
  - 23 locomotives
  - 850 railcars

- Citrus processing plant with a processing capacity of 20 MM boxes
- NFC aseptic storage capacity of 56 MM gallons
- Citrus nursery – Gilchrist County

UNITED STATES SUGAR CORPORATION

SOUTHERN GARDENS CITRUS

Basics

- Citrus greening, proper name is Huanglongbing (or HLB)
- Bacterial disease caused by unculturable bacterium *Candidatus* Liberibacter asiaticus
  - Found in Brazil in 2004 and in Florida in 2005
- Spread by an insect called the Asian citrus psyllid (ACP)
  - Insect was found in Florida in 1998, now endemic
- HLB now found in virtually all groves in the state of Florida
  - Up to 100% infection in many groves

UNITED STATES SUGAR CORPORATION SOUTHERN GARDENS CITRUS

Varietal Resistance

Most Tolerant

Most Susceptible

- Poncirus trifoliata, citranges
- Grapefruit, lemons, pomelos, sour orange
- Sweet oranges, mandarins, tangelos

Varietal Resistance

Bottom line: No real resistance in *Citrus* sp.

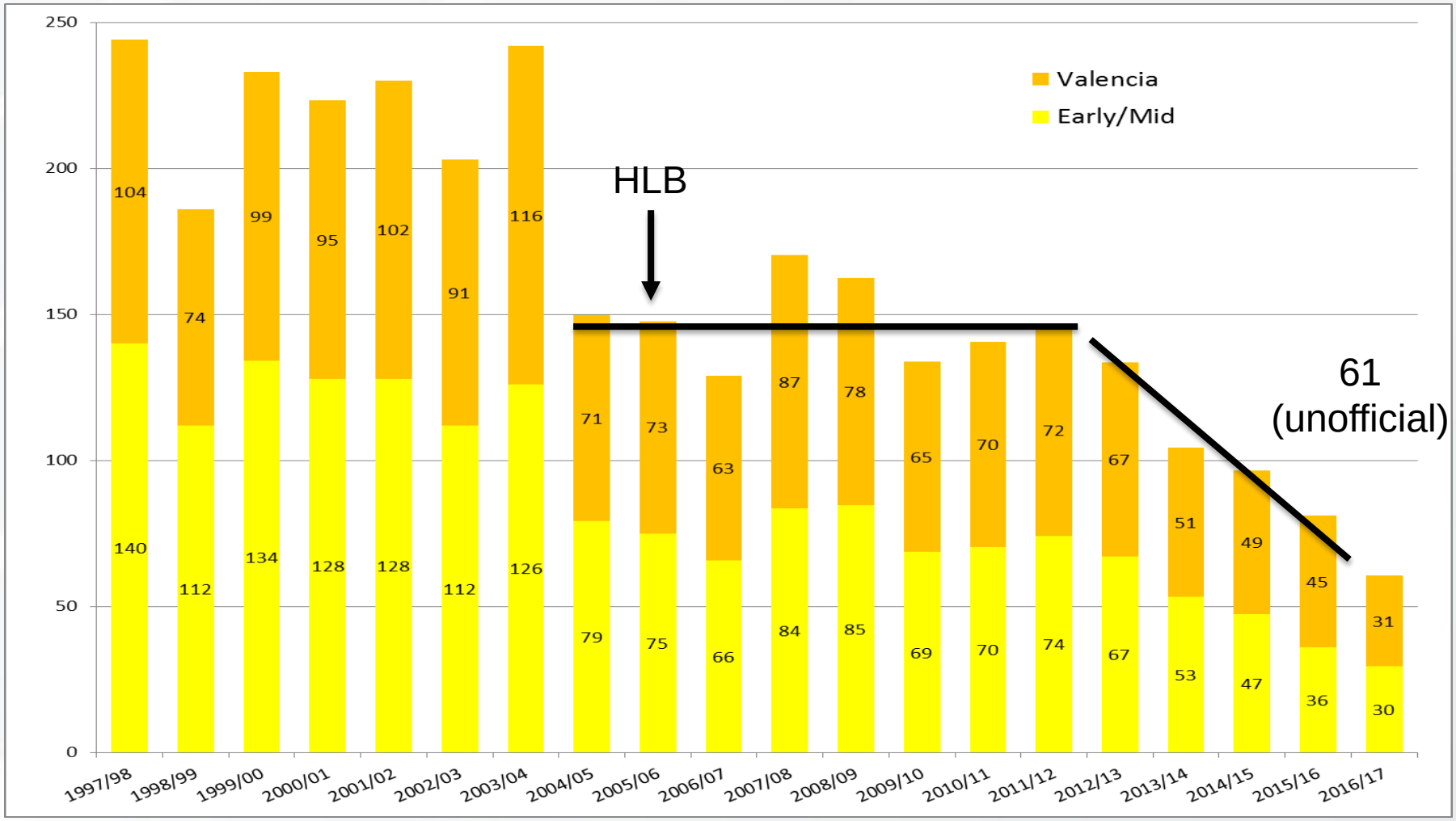
Alternate hosts: *Murraya paniculata*
Orange Jasmine
(both vector and pathogen)

What about the rest of the US?

- Texas
  - HLB is becoming more widespread in the industry
    - There are quarantine areas but the cat is out of the bag
    - ACP is endemic
- California
  - Still attempting to eradicate ACP but they are losing the battle (essentially buying time)
  - HLB in dooryard trees, no official detections in groves
- Arizona
  - No HLB, western AZ under ACP quarantine
- **Recent HLB detection in Mexicali…**

UNITED STATES SUGAR CORPORATION
SOUTHERN GARDENS CITRUS

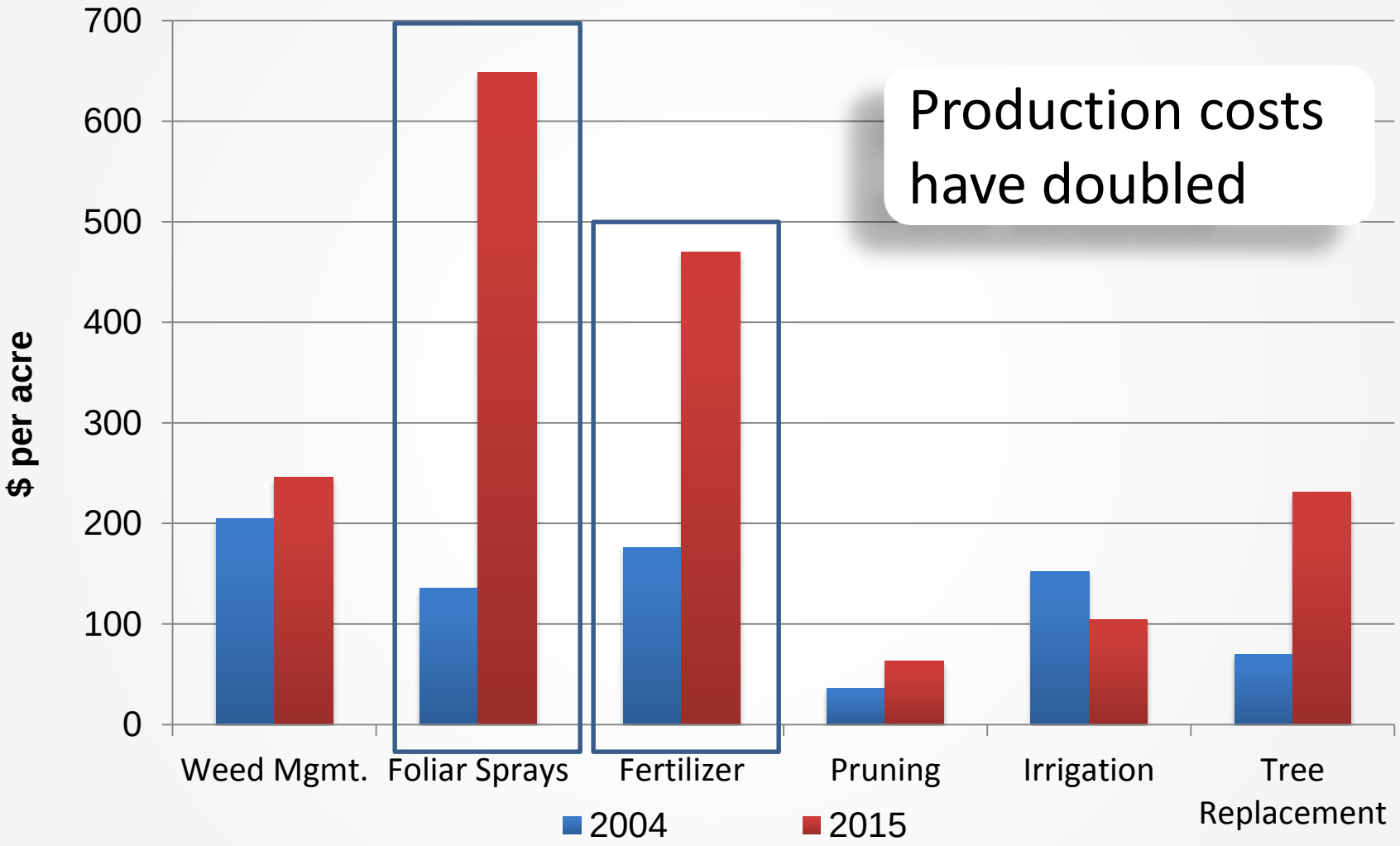
So what has happened in Florida?



UNITED STATES SUGAR CORPORATION

SOUTHERN GARDENS CITRUS

Cost of Production for Processed Oranges in Central Florida by Program, 2004 vs. 2015



Source: University of Florida, UF/IFAS, CREC

Florida Citrus Industry

2005-2006: Pre HLB

- Primarily a juice industry
- Ac in production: 578,586 (all varieties)
- Number of trees:
  - E/M: 28.96 MM
  - Lates 37.25 MM
- Production: 151,000 boxes

2016-2017

- Still a juice industry, but now severely impacted
- Ac in production: 436,867 (all varieties)
- Number of trees:
  - E/M: 21.45 MM*
  - Lates: 29.79 MM*
- Production: 61,000 boxes*

UNITED STATES SUGAR CORPORATION SOUTHERN GARDENS CITRUS

Current management programs:

- Trees are no longer being removed
- Aggressive management of ACP
  - Up to weekly applications
  - Not sustainable Aggressive fertility programs
- Stress management
  - Bicarbonates
  - Root health
- Antibiotics
- The kitchen sink

UNITED STATES SUGAR CORPORATION
SOUTHERN GARDENS CITRUS

Solutions:

Zincocide

Thermotherapy

Enhanced Nutrition

Business & Economy

UCFTODAY Arts Business Colleges Community Health Opinions Science & T

UCF Researcher May Have Found Key to Plague of Citrus Greening

Home > Citrus

Researchers Look To The Sun For Help In Citrus Greening Fight

By: Frank Giles | November 19, 2013 Email Print

Antibiotics

Nano particles

s against '*Candidatus* Liberibacter asiaticus' in

ed via the Graft-Based

Posted: Apr 02, 2015

Could a nanoparticle stem the plague of citrus greening?

d, Chuanyu Yang, Yongping Duan

halt the spread of citrus greening.

Published: November 5, 2014 • DOI: 10.1371/journal.pone.0111032

Resistant Varieties

Breeding disease-resistant citrus for Florida:

adjusting to the car ker/HLB world part 2: rootstocks

By Jude W. Grosser and

Bicarbonates and roots

What growers need to know about bicarbonates and root health

By Jim Graham, Evan Johnson and Kelly Morgan

Huanglongbing (HLB), associated with *Candidatus* Liberi-

without HLB. Symptoms exhibited by HLB-infected trees are typical of

loss, so starch concentration in r was determined. Surprisingly, ea

ıetic origin of our commercial rootstocks. There are four progenit species in citrus: namely pummelo, mandarin, citron and papeda. All o

UNITED STATES SUGAR CORPORATION

SOUTHERN GARDENS CITRUS

Solutions:

Mimicking Insect Mating Calls Could Combat Citrus Greening

NEWS

GMO targets citrus greening

By Doug Ohlemeier August 11, 2015 | 2:33 pm EDT

UF Researchers Release Possible Citrus Greening Solution

By JESSICA MESZAROS • AUG 24, 2015

Growing Produce | Vegetables | Fruits | Nuts | Citrus | Farm Marketing

Trending Now: #ClimateChange

Home > Citrus > Insect & Disease Update

Products To Help Your Trees Fight Off Citrus Greening

Posted By: Christina Herrick | August 31, 2015 Email Print Facebook Google LinkedIn Twitter

HOME > ORCHARD CROPS > SECTION 18 EXEMPTION SOUGHT IN FIGHT AGAINST HLB

Section 18 exemption sought in fight against HLB

Logan Hawkes Oct 19, 2015

NATIONAL NEWS

HOME » NEWS » NATIONAL NEWS

Scientists use wasps to protect citrus crops from disease

Prescription for Curing Citrus Greening: Apply Heat and Wait

Florida Scientist Finds Promising Treatment For Deadly Citrus Disease

The Huffington Post | By Joe Satran

Florida Researchers Find Temporary Citrus Greening Cure

UF/IFAS Research- Fighting Citrus Greening with Steam

University of Florida IFAS Video

UF researchers say citrus greening can be managed with new biological and chemical controls

UF team finds possible treatment for citrus greening

UCF prof tests treatment for citrus greening

Gout treatment halts spread of citrus greening disease, Florida scientists say

'Good' and 'bad' bacteria in the fight against citrus greening disease

November 19, 2015

New genetic technology in Lake Alfred could lead to citrus greening solution

So are the solutions real?

- So are they real?
  - Yes/Possibly/Maybe not/Probably not
- Are they practical?
  - Yes/Possibly/Maybe not/Probably not

- **Virtually all scientists and producers will say that plant resistance is the only real solution**
  - Many also think that near-term durable resistance will only come through biotech approaches

UNITED STATES SUGAR CORPORATION
SOUTHERN GARDENS CITRUS

Current management programs

- Current management programs are:
  - Not sustainable (economically, biologically)
  - Not working
- “Getting used to the dark”

So you get the picture…..

- The Florida citrus industry is in a fight for the survival of the industry

- As far as the trait:

We are in a “need to have” vs. a “nice to have” situation

and the clock is ticking…

UNITED STATES SUGAR CORPORATION SOUTHERN GARDENS CITRUS

Working with HLB

- Nothing is easy with HLB
  - Can’t be cultured
  - No standardized method to screen
  - Long latent period
    - Months to years
  - No validated early detection procedure other than PCR
  - No good source of resistance in anything near a commercial species/variety
  - Tolerance vs. immunity
    - Tolerance still provides inoculum sources which makes everything harder (circular reference)

UNITED STATES SUGAR CORPORATION
SOUTHERN GARDENS CITRUS

Other difficulties

- Long lead time to produce trees for trials
- Trials are expensive and multi-year
- Juvenility is an issue
- Not many GMO trials had been done with citrus
  - No guidance documents
- Material was coming from Texas (Florida quarantine issues)
- Sense of urgency dictates the pace of the work…..

UNITED STATES SUGAR CORPORATION
SOUTHERN GARDENS CITRUS

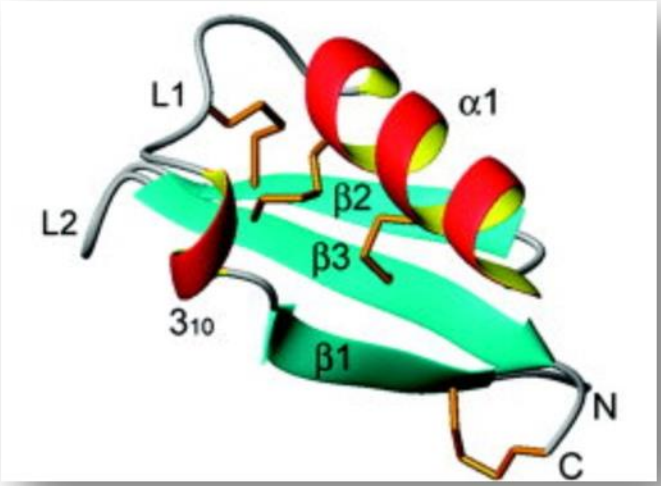
Basics of the Project

- Built on work done by Dr. Erik Mirkov, Texas A & M, Weslaco
  - Originally targeting another bacterial disease (citrus canker)
  - Spinach defensins (SoD) based on work by Segura (1998)
  - Initial screen against HLB late 2005/early 2006
- Standard GMO technology
  - Agrobacterium mediated transformation
  - Selection on kanamycin
  - All of the components (except SoD) have been through the system

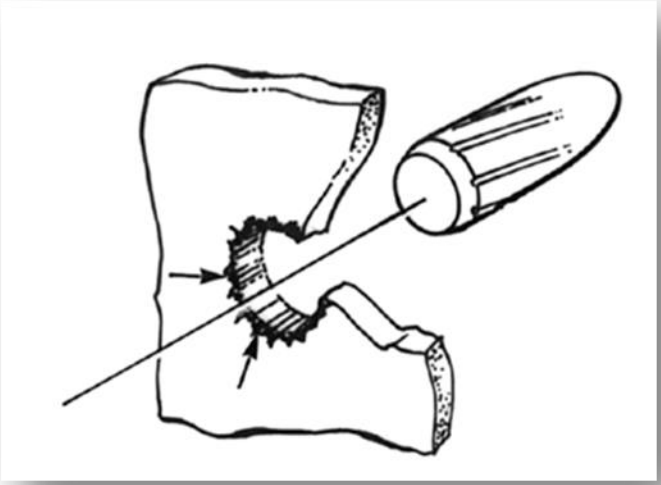
UNITED STATES SUGAR CORPORATION SOUTHERN GARDENS CITRUS

Defensins

- Defensins are small (c.a. 5 kDa), highly stable, cysteine rich peptides; first line of defense for multi-cellular organisms
  - Active against bacteria, fungi, and many viruses



- Highly conserved structure – most function by binding to the microbial cell membrane forming pore-like membrane defects that allow efflux of essential ions and nutrients



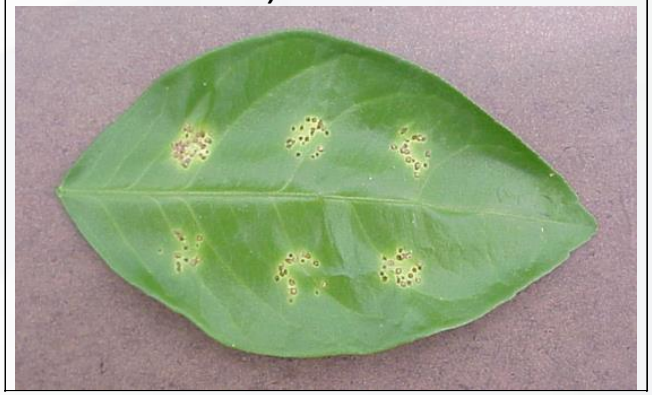
Defensin-like Proteins in Spinach

- Initial work was done with SoD2 and SoD7
  - SoD7 is a truncated version of SoD2 and was probably a lab artifact based on recent data mining of *S. oleracea* sequences in GenBank
- Recently additional defensins in *S. oleracea have* been identified and work will begin with at least one of the new sequences

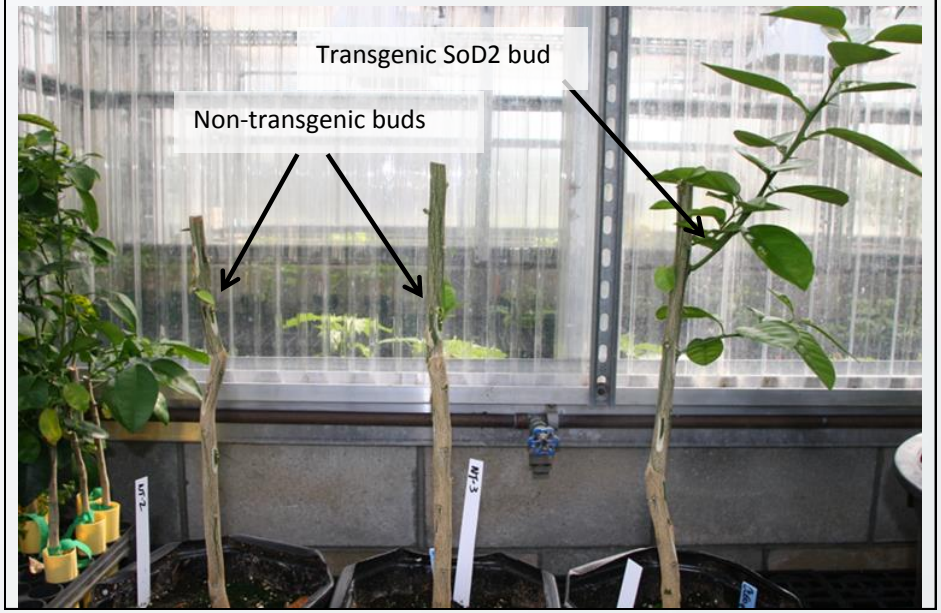
The beginning

Detached non-transgenic grapefruit leaf inoculated with citrus canker *(note leaf chlorosis, necrosis around lesions, and lesion size)*

Detached SoD2 transgenic grapefruit leaf inoculated with the same concentration of citrus canker as in 1a *(note lack of chlorosis and necrosis, and reduced lesion number and lesion size)*



Rootstocks budded with non-transgenic grapefruit (left two plants) and SoD2 transgenic buds (right) inoculated with HLB.



UNITED STATES SUGAR CORPORATION

SOUTHERN GARDENS CITRUS

Defensin Project – complicated from the start

- Initial priority was to get the existing material and new lines from Texas to Florida
  - State Regs: not much different from international quarantine protocol
  - Caused a delay in getting the material to the field
  - Had to develop a system to move the material that satisfied all
    - APHIS movement notifications
    - State quarantine efforts
  - Technology improvements were sometimes quick and unanticipated so lead time for the process(es) was important

Permitting with little history…..

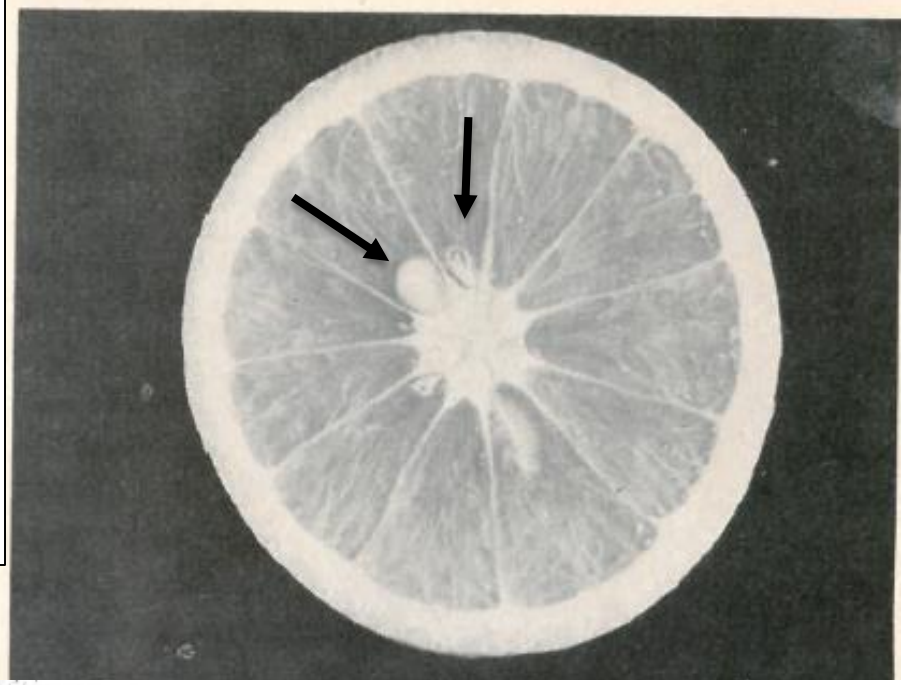
- Initial APHIS permit for the technology: 3/1/2009
- At that time:
  - 15 Citrus permits issued
  - 3 Institutions
  - 4 Phenotypes* (IR, VR, FQ, MG, BR*)
  - All very small, most 0.1ac and were renewals of original plantings, so essentially <5 individual plantings
  - All grapefruit

Permitting with little history……

- Standard issues that needed to be addressed:
  - Confinement/Pollen flow
    - Isolation distance
    - Border rows
    - Seed
- Citrus is different
  - Many species of Citrus are apomictic
    - Nuclear embryony: embryo is from maternal tissue
    - Not 100%
    - Definition of seedy…
  - Juice industry, so seeds are not an issue
    - Unless they are…..

UNITED STATES SUGAR CORPORATION SOUTHERN GARDENS CITRUS

- 0-6 seeds = Seedless (USDA??)
- Valencia: Few to none



Valencia (Valencia Late, Hart Late, Hart's Tardiff) (fig. 4–10)

Fruit medium-large, oblong to spherical; areole ring faint or lacking; seeds few or none. Well-colored at maturity, but regreens thereafter under certain conditions. Rind medium-thick, tough, and leathery; surface smooth to faintly pebbled. Juice abundant and flavor good but commonly somewhat acid. Fruit holds exceptionally well on tree with little deterioration in quality and ships and stores well. Excellent for processing. Latest maturing of all commercial varieties.

Tree vigorous, somewhat upright, large and prolific, but with alternate-bearing tendency. Very wide range of adaptation.

This variety should not be confused with the Spanish *Valencia Temprana* and various seedling clones grown in the region of Valencia, Spain.

The total heat requirement for maturity of

TABLE 3. Continued

Seed parent	No. of pollen-parent cultivars[a]	No. of seeds	Seedlings per seed		Nucellar seedlings as percentages of all seedlings
			Total	Zygotic	
Szinkom	7	1683	1.50	0.25	83
King	7	387	1.01	0.80	21
Satsuma	11	323	1.44	0.14	90
GRAPEFRUIT GROUP					
Marsh	6	207	1.08	0.05	96
Imperial	8	626	1.26	0.42	66
Imperial	Selfing	99	1.22	0.10	92
Panuban	1	70	1.11	0.83	26
Sukega	2	116	1.00	1.00	0
PUMMELO					
Four cultivars	14	1954	1.001	1.001	0
Seven cultivars	8	610	1.00	1.00	0
SWEET ORANGE					
Maltese Oval	8	167	1.09	0.66	39
Paperrind	5	82	1.56	0.26	84
Valencia	8	106	1.35	0.21	85
Washington navel	6	24	1.33	0.04	97
Washington navel	1	32	2.00	0.62	69

Initial trial

- Conditions proposed by the applicant
- Initial trials
  - 1 mile isolation distance to nearest citrus
    - Now 200-900’
  - No flowering was allowed (not really an issue other than inspection and documentation)
    - Now allowed
  - Border trees: hard to keep in good shape (HLB)

Typical permit conditions or requirements that work for other crops may not work for citrus

UNITED STATES SUGAR CORPORATION SOUTHERN GARDENS CITRUS

Volunteer monitoring

Lead times, science, biology and the process

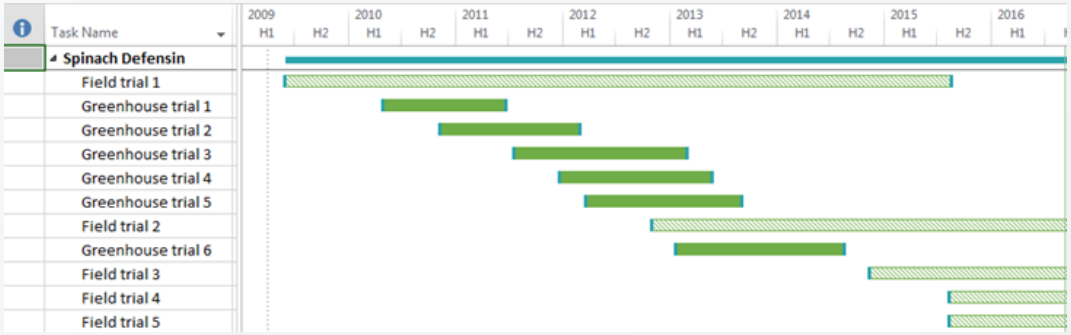
- Regulatory lead times are real….
  - Everybody has a job to do (to keep agriculture and people safe) and there is a process that has be followed
- Science
  - Some advances are methodical and planned
  - Others are unanticipated, fortuitous, and very much welcomed…..
- Biology
  - Hard to make it go too much faster
- The process
  - For the most part is sequential

UNITED STATES SUGAR CORPORATION
SOUTHERN GARDENS CITRUS

Lead times, science, biology and the process

- Variables
  - Which gene (s)
  - Codon optimization
    - Yes or no
    - Which one
  - Signal peptide
  - Varieties
  - Enough events
- Was a process

  Driven by lab and biology, not the plan



- Movement notification: 10 days
- TX to FL movement: 30 - 60 days
- Propagation:
  - Greenhouse: 30 days
  - Field: 6-12 months
- APHIS Permit: 120 days
- Trial: 1-3 years

Before you finish with the process for 1, you are starting over due technology improvements

UNITED STATES SUGAR CORPORATION

SOUTHERN GARDENS CITRUS

Lead times, science, biology and the process

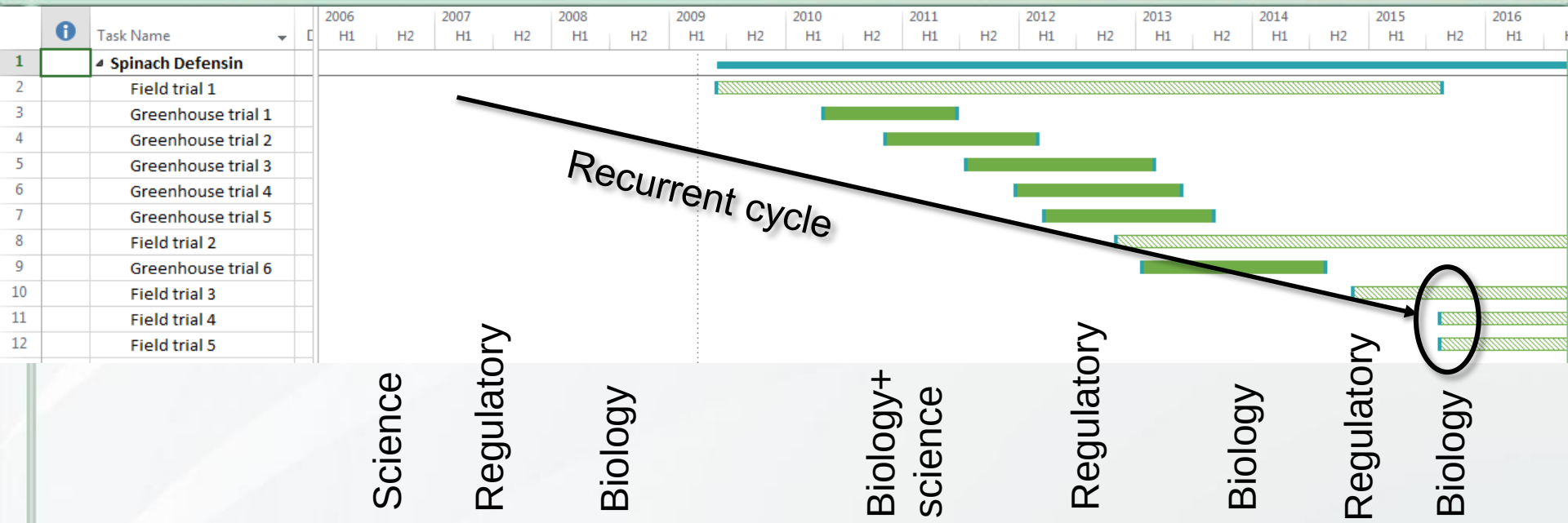
Initial Field Trial 5/2009

- New lines of all of the below 10/2015
- 2 of same varieties + 2 new varieties, 2 genes stacked, 2 codon optimizations, with and without signal peptides 11/2014
- Advanced lines from early trial 10/2015
- 4 varieties, 2 genes X 3 codon optimizations, with signal peptides 11/2012

UNITED STATES SUGAR CORPORATION

SOUTHERN GARDENS CITRUS

Lead times, science, biology and the process



- 4 Permits plus 1 amendment
  - Added sites
  - Added constructs
  - Extended time
- Only one complete recurrent cycle

Lead times, science, biology and the process

- Cycles are getting shorter
  - Much of it due to getting the regulatory process:
    - Figured out
    - Adapted to citrus
- Key is regular interactions with APHIS
  - The Agency/Staff is aware of the urgency and they have been willing to work with and help us
  - Processes have changed on both sides
    - Better planning and anticipation on our end
    - Process changes on the Agency end
      - Better understanding of the crop (both sides)
      - Dedicated Citrus reviewer
      - Standardized conditions

UNITED STATES SUGAR CORPORATION
SOUTHERN GARDENS CITRUS

As we increase our acreage….

- Testing over 10 ac (per construct)
  - EUP required (required because intended use of the product is as a pesticide)
  - Temporary Tolerance Exemption
  - Now dealing with different Agency: EPA
- EPA
  - Different requirements
    - More stringent in some respects (data requirements)
  - Different lead times
    - 10 months for EUP
  - Now involves fees

Initial EUP – Specific to the event(s)

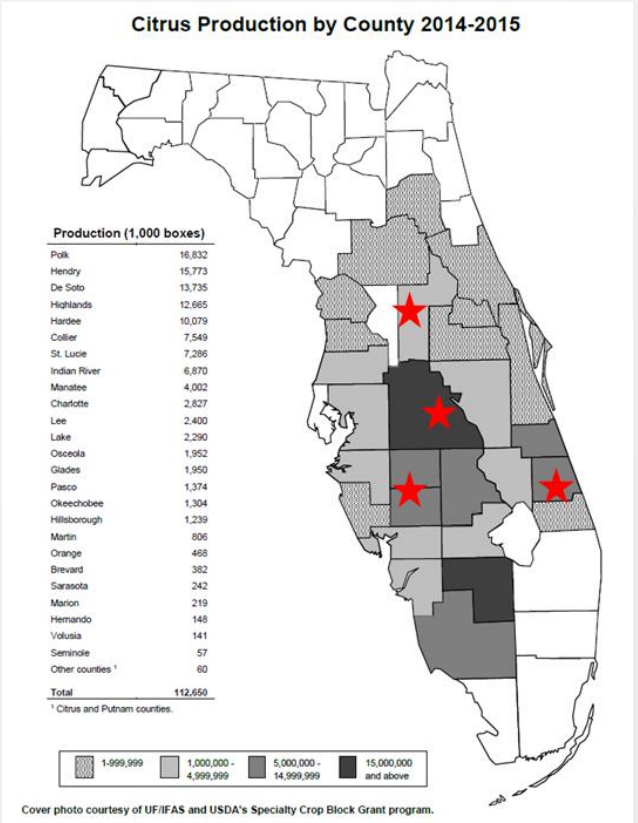
- EUP/Temporary Tolerance Exemption
  - 88232-EUP1 issued 4/30/2015
    - 600 total ac in Florida (400ac 1 county) and Texas (200ac 1 county)
    - 4 plant lines: 2 sweet orange, 1 lemon, 1 grapefruit
    - 3 constructs: SoD2, SoD7, SoD2+SoD7
  - Final Rule for temporary exemption from the requirement of a tolerance issued on 5/6/2015
    - SoD2 and SoD7
    - Expires 4/18/2018

By the time the EUP issued.....we realized that we would have to amend it

- Applied for an Amendment the EUP to give us more flexibility given the pace of the work and the long lead times for the science and the Regulatory process
- 88232-EUP Amendment 1 final version issued December 23, 2015
  - Same acreage, same locations
  - Any plant lines using the original 3 constructs (with and without signal peptide)
  - All future plantings will go under this EUP

Since then……

- New defensins have been found and plants are in the field (will skip greenhouse and go direct)
- We are expanding the scope of the field testing
  - Testing in all five major citrus production regions
- Will require another amendment to the EUP to add both:
  - Locations
  - SoDs
- Will have to amend or obtain another Tolerance Exemption



Lead times, science, biology and the process

- Cycles are getting shorter
  - Much of it due to getting the regulatory process:
    - Figured out
    - Adapted to citrus
- Key is regular interactions with EPA
  - The Agency/Staff is aware of the urgency and they have been willing to work with and help us
  - Processes have changed on both sides
    - Better planning and anticipation on our end
    - Process changes on the Agency end
      - Better understanding of the crop (both sides)
      - Willing to adapt the process to work with Citrus
      - Closer partnership with APHIS

UNITED STATES SUGAR CORPORATION SOUTHERN GARDENS CITRUS

Concurrent processes

- As much as the process will allow, much of the work is being done concurrently:
  - Science
  - Testing
  - Regulatory
- This is a business decision that is being made due to the urgency of the situation
  - None of the Agency missions are circumvented
  - SGC assumes the risk because the industry cannot afford the normal “Staged Gate” approach

FDA

- Haven’t said much about the FDA because we are not there yet, but we have been meeting with them as well
  - Not as frequent
  - More informational
    - Both sides

That takes us to where we are now

- Many more steps to go:
  - Regulatory package
    - Product characterization
    - Biology and efficacy
    - Effects on other agricultural products
    - Plant pest risk
  - NEPA (National Environmental Policy Act)
    - Environmental impact compared to reasonable alternatives
      - Agriculture
      - Environment
      - Animal health
      - Human health

A word about the Agencies and their Staff

- All of the Agencies have been great to work with
  - Extremely knowledgeable
  - Extremely professional
  - Extremely cooperative
- They have a job to do to protect agriculture, the environment, and our food supply and they take it seriously and they do it well
- And lastly, they have done what they said they would do…..which is not to put up any road blocks in process as long as we met the criteria of the processes

UNITED STATES SUGAR CORPORATION SOUTHERN GARDENS CITRUS

Thank you for your attention!