Progress on HLB Diagnosis and Liberibacter Detection

A Short Summary of Some Approaches

Mark E. Hilf
USDA ARS USHRL
Fort Pierce, FL

Current Widely Used Methods

- HLB Diagnosis
  - Whole plant assessment
  - Characteristic foliar symptoms
    - Infection well established
- Liberibacter Detection
  - Subsample analysis
  - Polymerase chain reaction (PCR)
    - Conventional PCR
      - lesser sensitivity (symptomatic tissue)
      - lesser cost
    - Real time PCR (qPCR)
      - Increased sensitivity
      - greater cost

Early Responses

- Specific changes in host physiology
  - Indicative of infection
  - Independent confirmation of infection without detection of the HLB-associated pathogen

Christina E. Davis & Co.

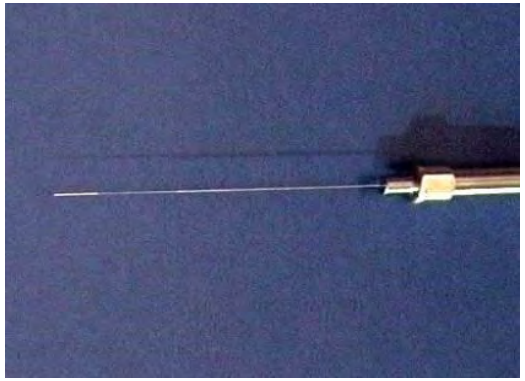
Mechanical and Aerospace Engineering
Clinical and Translational Science Center UC Davis

- Whole Tree Sampling
- Analysis of Volatile Organic Compounds (VOC)
- Differentiation of infected/diseased trees from not
  - Huanglongbing (HLB)
  - Citrus tristeza virus (CTV)
  - Citrus Variegated Chlorosis (CVC)

VOC sampling and collection

- Multiplexed sampling approach:
  - Twister- and SPME- GC/MS lab based analysis
    - Varietal fingerprinting biomarker discovery and profiling
  - GC/DMS for field studies
    - Near real time analysis.
  - Combinations of in field and lab based instrumentations
    - VOCs fingerprinting and profiling

Twisters

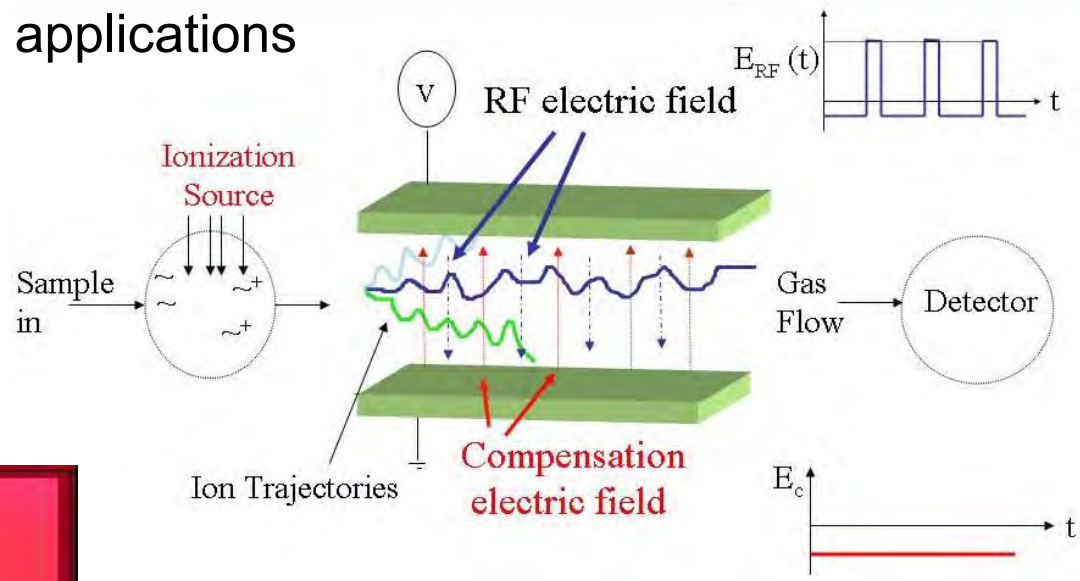


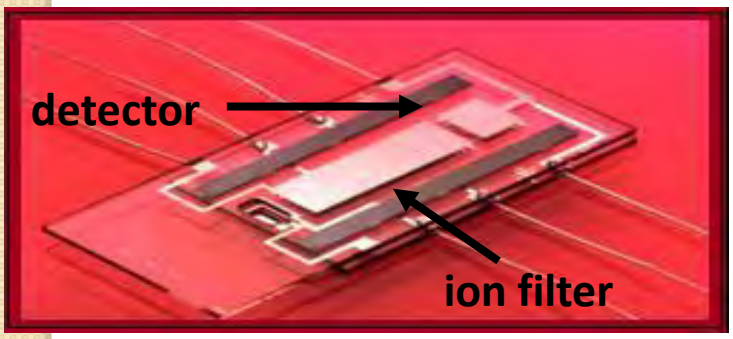
Solid phase micro extraction fibre (SPME)

GC/DMS
(Lindcove Research & Education Center)

Differential mobility spectrometry (DMS)
1st generation chip-based sensor

- "Sniffer" sensitive to below ppb levels
- Robust and reagentless
- Software upgrades for new applications
- Compact and portable
- Low cost-of-ownership
- Minimal maintenance

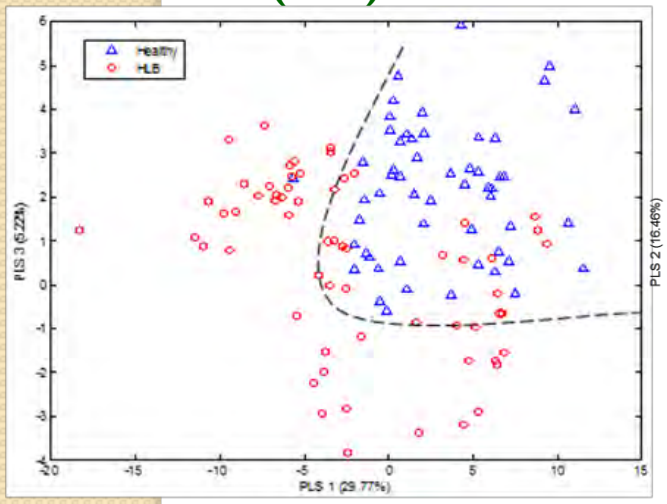




Approximate development timeline (2001-2013)

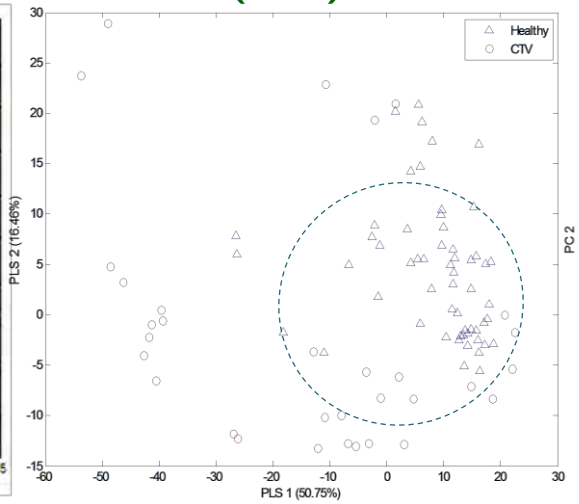
GC/DMS results for various citrus diseases (HLB, CTV and CVC)

GC/DMS data (HLB)



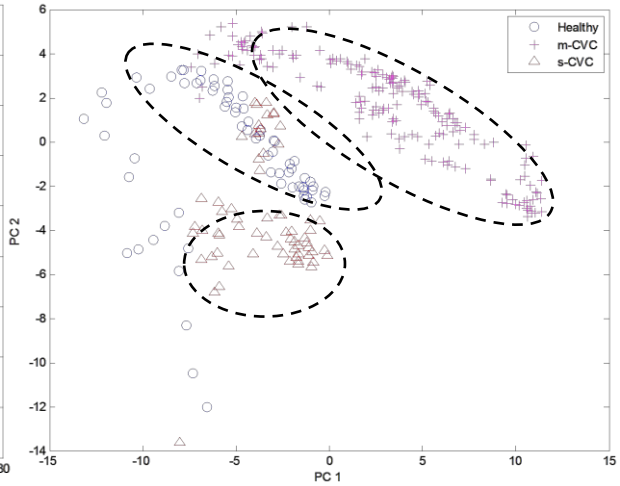
Accuracy: >80%

GC/DMS data (CTV)



Accuracy: >95%

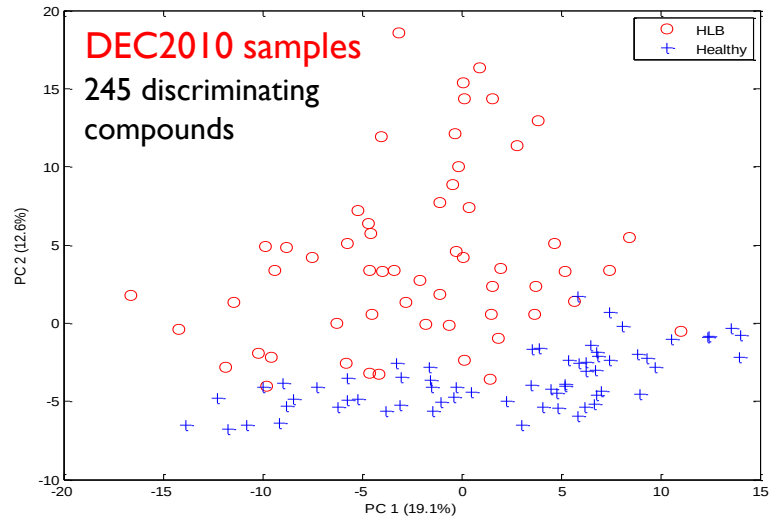
GC/DMS data (CVC/greenhouse)

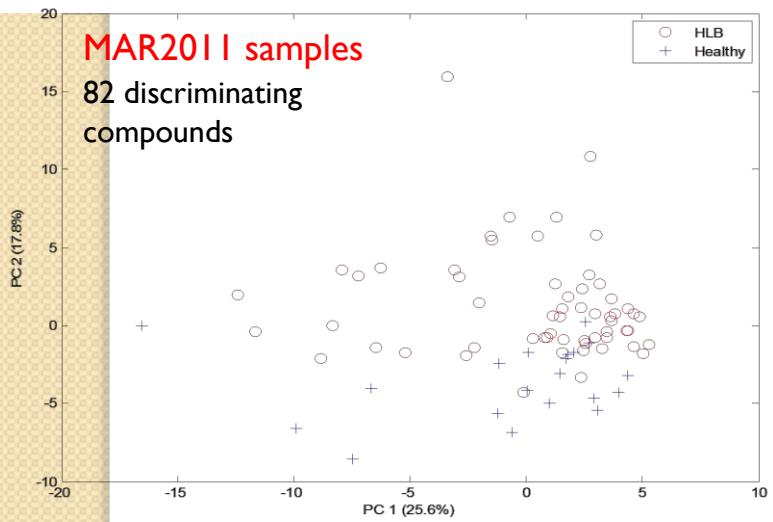


Accuracy: ~80%

Data generated by Dr. Alexander Aksenov, Dr. William Cheung; analyzed by Dr. Weixiang Zhao.

HLB Twister biomarker study: Classification accuracy using 16 common compounds:





- 93.4% (54/57 for HLB and 59/64 for healthy) for the DEC2010 samples
- 83.5% (10/20 for healthy and 56/59 for HLB) for the MAR2011 samples

Dr. Hailing Jin[1] & Dr. Kim Bowman[2]

[1]Dept. of Plant Pathology and Microbiology & Institute for Integrative Biology, UC Riverside
[2]USDA, ARS US Horticultural Research Laboratory, Fort Pierce, FL

- Subsample analysis
- Detection of host nucleic acid
  - Small RNAs (sRNAs) induced by infection by Liberibacter
- Profiled sRNAs induced by graft-infection of sweet orange with Liberibacter
  - Identified sRNAs highly induced by infection

Dr. Hailing Jin[1] & Dr. Kim Bowman[2]

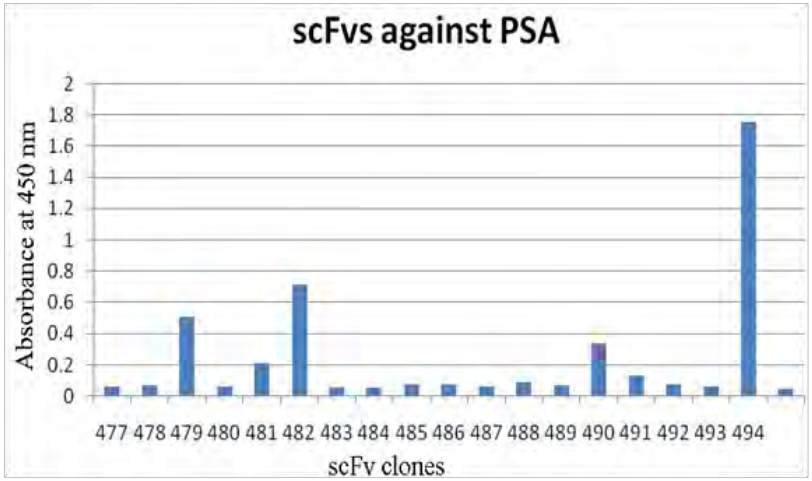
- A class of sRNAs are known as micro-RNAs (miRNA)
  - 18-40 nt
  - Bind to messenger RNAs (mRNAs) and suppress translation (protein synthesis)
- Identified miR399 in Liberibacter but not Spiroplasma infected sweet orange trees
  - miR399 is induced by phosphorous starvation in other plants

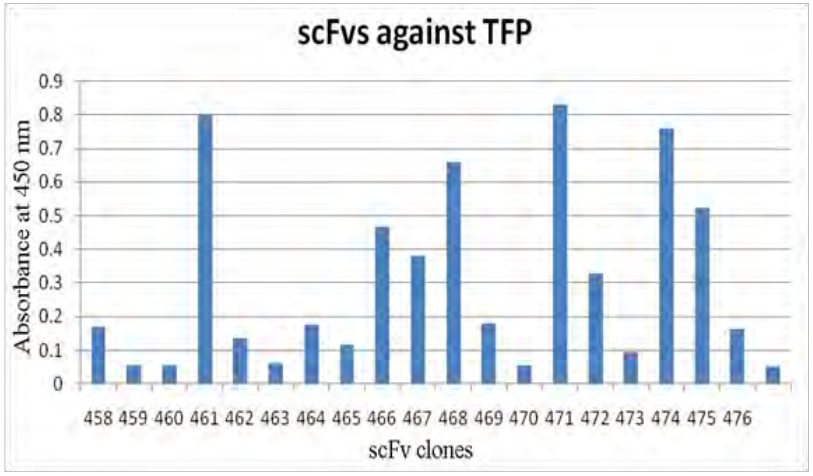
Dr. John Hartung

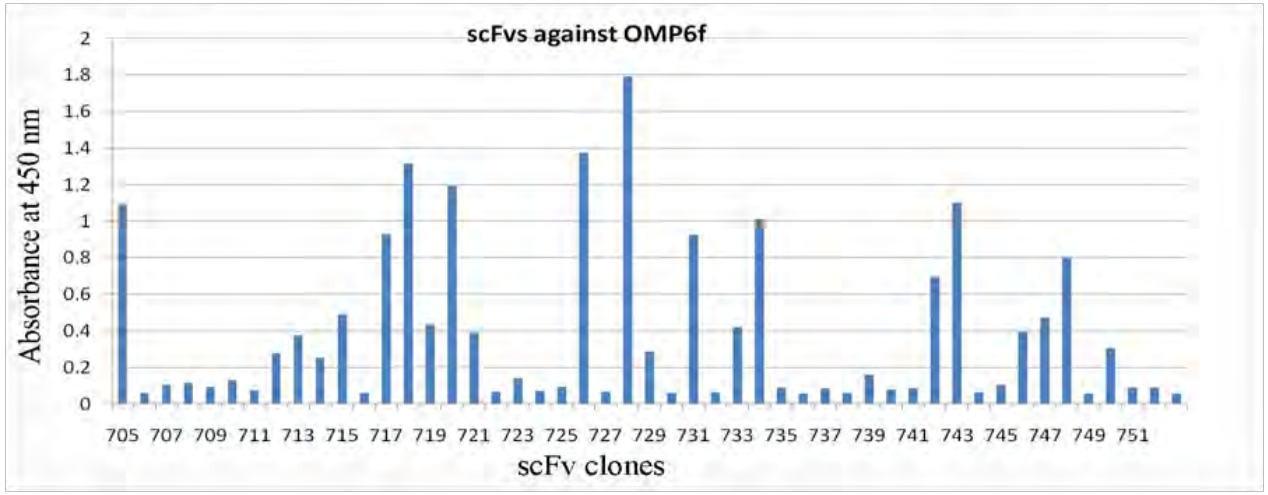
USDA ARS MPPL

Beltsville, MD

- Subsample analysis
- Serological analysis
  - Detection of Liberibacter proteins
- Cloning of genes which produce antibodies to Liberibacter proteins
  - Genes for short chain variable fragments (scFvs)
  - Screen scFv genes for antibodies to Liberibacter proteins
  - Application of expressed antibodies for detection of Liberibacter proteins


scFvs against PSA
Absorbance at 450 nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494
scFv clones


scFvs against TFP
Absorbance at 450 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476
scFv clones

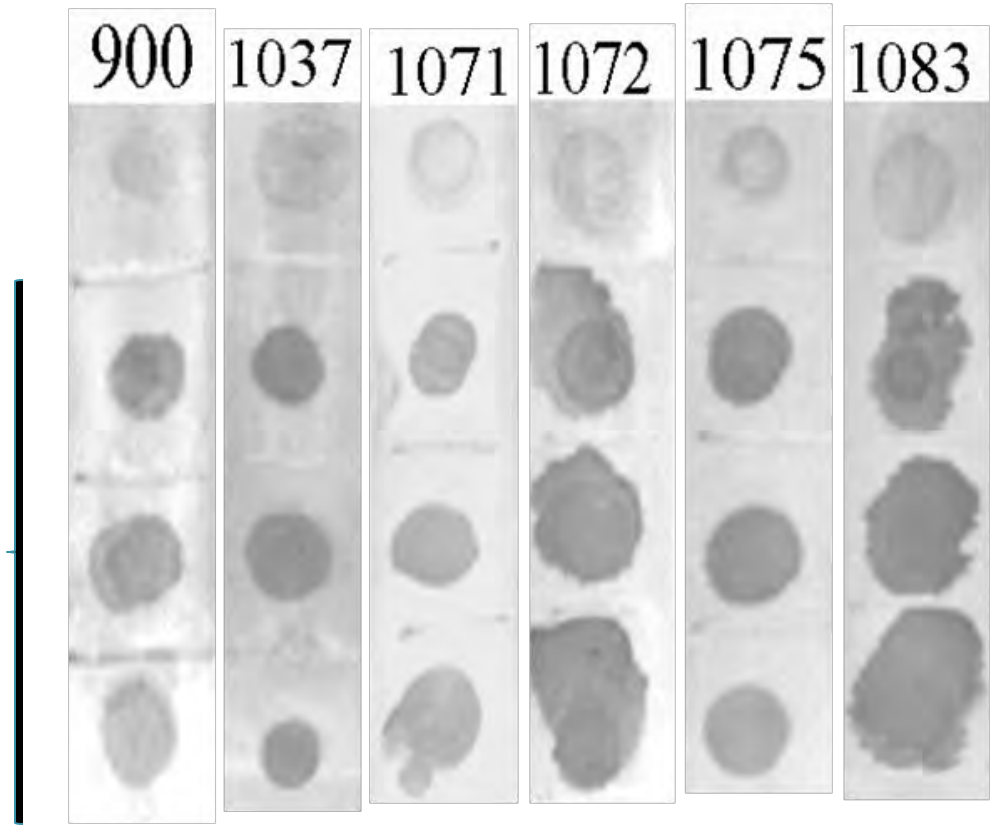

scFvs against OMP6f
Absorbance at 450 nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
705 707 709 711 713 715 717 719 721 723 725 727 729 731 733 735 737 739 741 743 745 747 749 751
scFv clones

J. Hartung

Antibody (scFv) Gene Clones

Healthy

Infected



J. Hartung

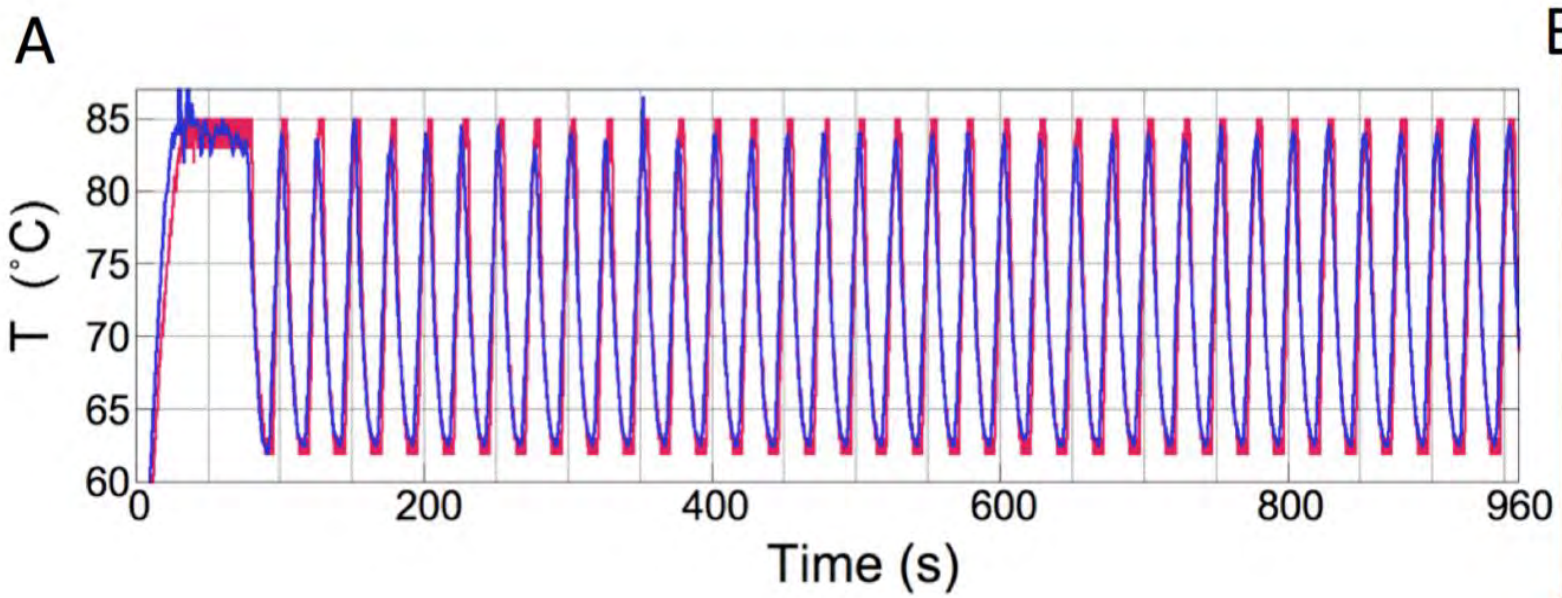
Dr. R. Bruce Cary

CSO, Mesa Tech International Inc.
Santa Fe, NM

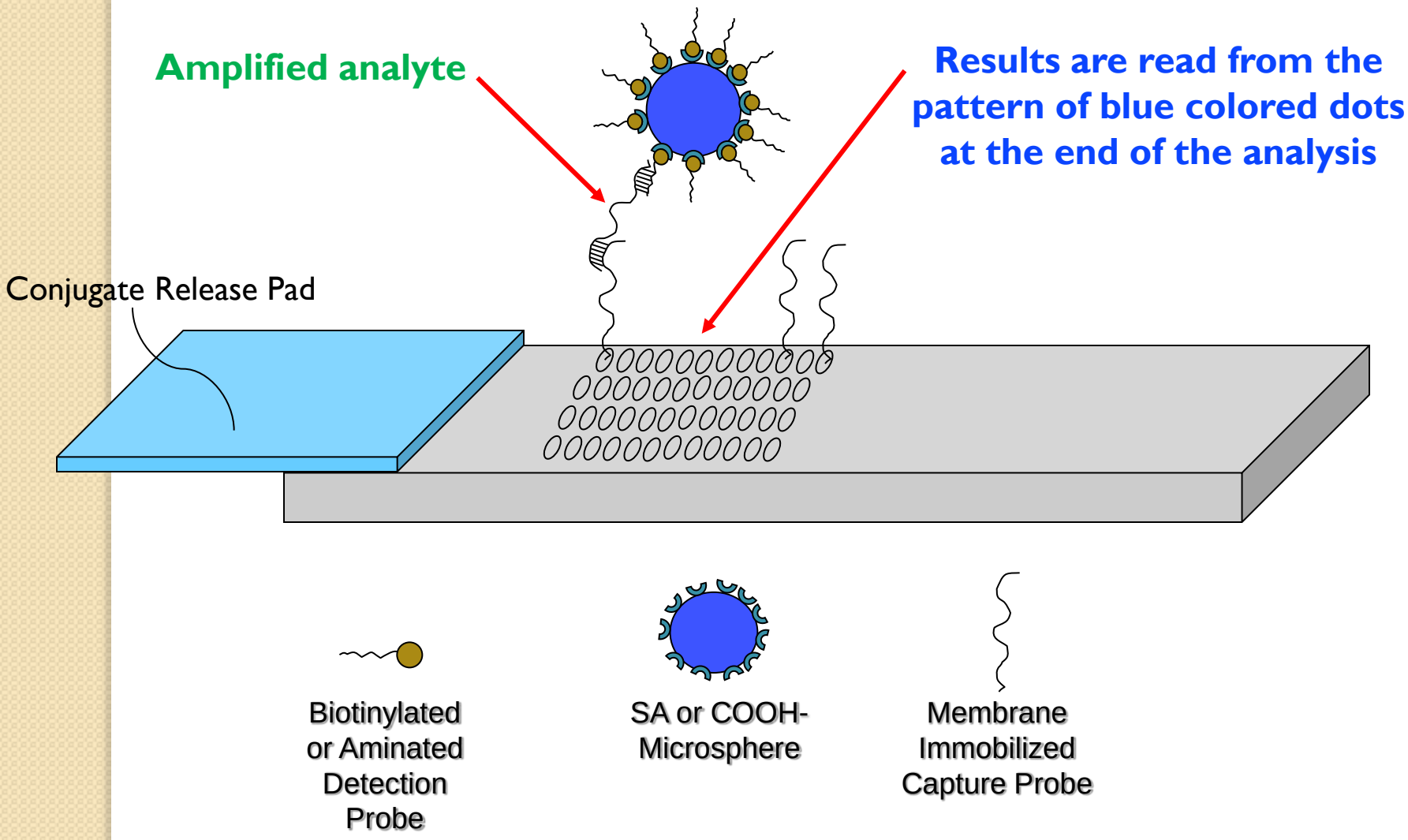
- Subsample analysis
- Oscillating Amplification Reaction (OscAR)
  - Detection of pathogen DNA
  - Integrated device for use in the grove

Oscillating Amplification Reaction (OscAR): A Novel NA Amplification Chemistry

- **Rapid Two-temperature, Low ΔT Amplification Method**
  - FAST! Exponential amplification from Passive Buffer Exchange processed samples using low cost disposable microheaters
  - HLB marker detection from real-world infected citrus leaf tissue has been demonstrated
  - Optimized for use in Mesa Tech integrated molecular diagnostic platform



Lateral Flow Sandwich Hybridization Assay Schematic



Candidatus Liberibacter DNA Test Kit

- **Integrated system**
  - Provides DNA-based test results with PCR-like sensitivity and specificity in as little as 23 minutes
  - Yes/No DNA test read by eye
  - Low cost & completely self-contained kit
  - No laboratory equipment or specialized instruments
  - 9V battery or A/C wall adapter powered base-station
  - Sample preparation, DNA amplification and sequence-specific detection in an easy to use disposable cartridge
- **DNA Tests Anyone Can Perform**
  - Place tissue to be tested in tube containing sample extraction buffer, mix by inverting tube
  - Use included volumetric dropper to add sample to test cartridge
  - Press the start button
  - When the light turns green: Read test results (approximately 25 minutes sample-to-result)

Molecular Testing Platform Provides Rapid DNA Tests for the Point-of-Use

- MesaTech's Test Shown to Detect HLB Bacterial DNA in Diverse Specimens
  - Green house citrus leaf samples
  - Field collected citrus leaf samples
  - Asian Citrus Psyllid, whole insect
  - Dodder
  - Sample to DNA test result in 23 minutes
- Prototype designs have guided the development of a pilot product now entering production
  - Sample to DNA test result in 23 minutes
  - Targets genetic marker for *Ca*. L. asiaticus and *Ca*. L. americanus

Bruce Cary/Mesa Tech International

Dr. P. Scott White

Los Alamos National Laboratory
Los Alamos, New Mexico

- Subsample analysis
- Development, validation, and deployment of rapid, inexpensive, multiplex assays for simultaneous detection and strain characterization of multiple citrus pathogens **(CRB Project No. *5300-10K*)**
- Multiplex Oligonucleotide Ligation PCR (MOL-PCR)
  - Detection of pathogen DNA
  - Can use up to 500 specific probes in a single reaction
    - Detection and discrimination of multiple pathogens/isolates in a single reaction
  - Designed for high sample volume

Laboratory analysis work flow

Collect field samples, transport to lab (via mail or hand-carry)

Process samples in high throughput laboratory (up to thousands/day)

(or research)
Management decisions based on rich data

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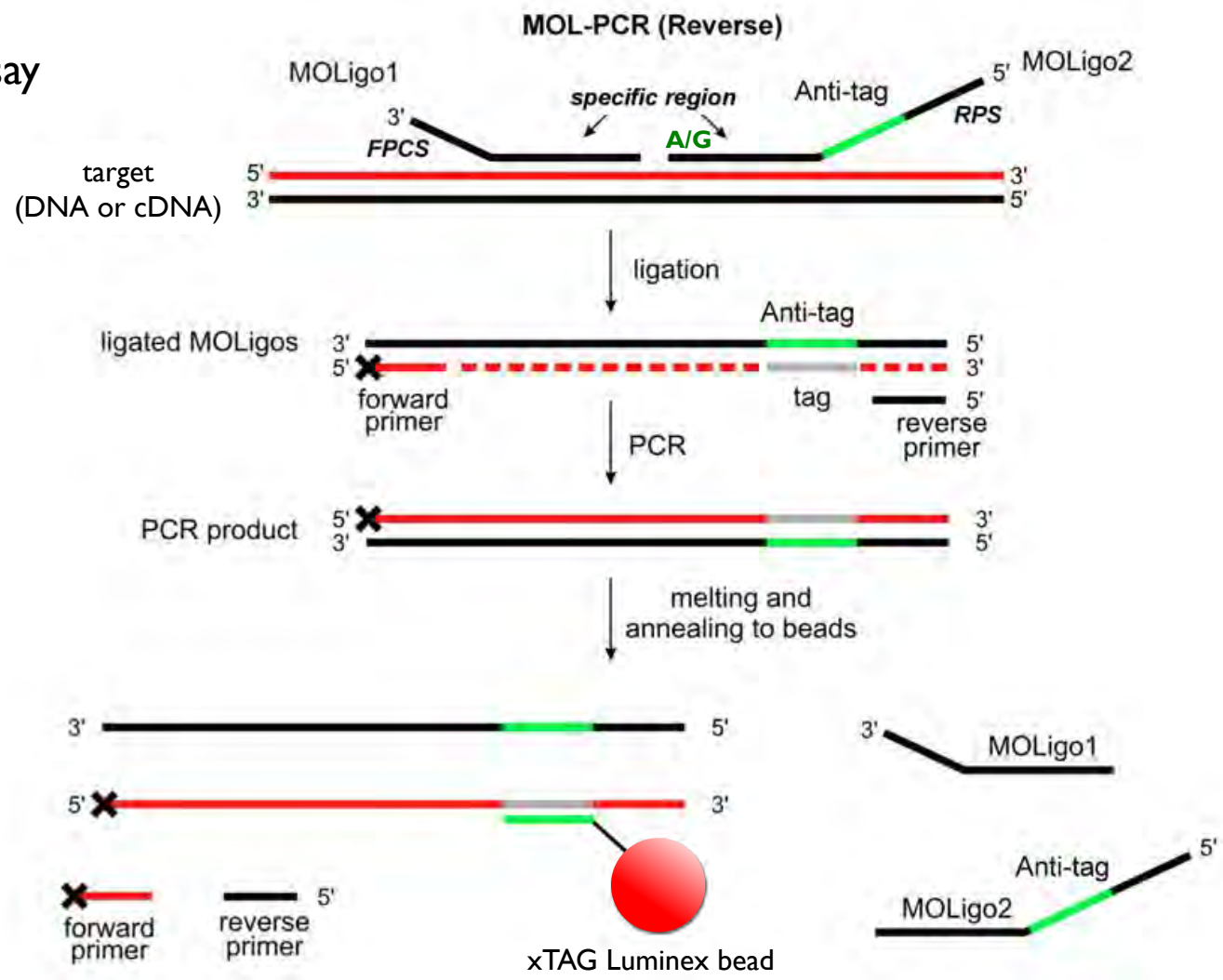
Multiplex Oligonucleotide Ligation-PCR (MOL-PCR): optimized for simultaneous detection *and* SNP scoring

DNA/cDNA based assay

Note: this represents one target on one bead within a multiplex of up to 500 probes (limited by existing readout instruments) in a single reaction. Some probes can be used for detection, while others can discriminate among SNPs for fine resolution among pathovars where needed.



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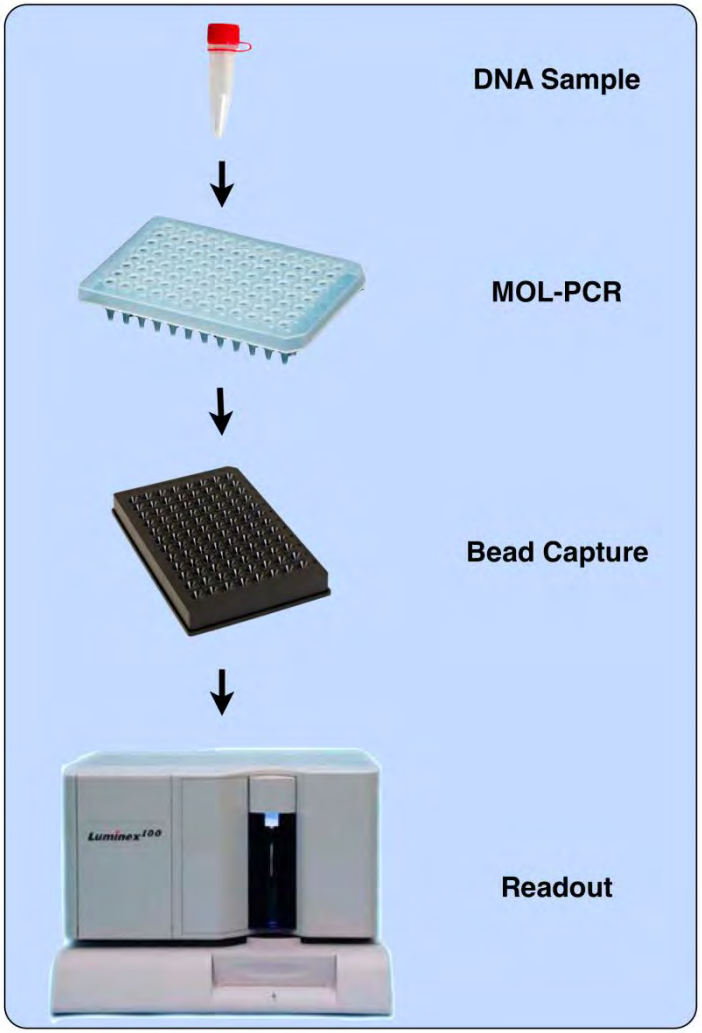
MOL-PCR Process

- Nucleic acid detection and analysis for high specificity
- DNA or cDNA from samples
- Up to 500 markers per sample in one tube (each well)
- Compatible with 96 or 385-well plates
- Suitable for host AND pathogen markers in same assay
- Streamlined process
- "Universal" PCR
- Direct labeling
- Minimal sample handling
- No wash steps
- No custom beads
- Readout on commercial flow cytometer (e.g., Luminex, Attune, etc)



Laboratory-based high throughput/low cost multiplex assays

Parallel processing further improves throughput.

Example:
4 dual-block PCR machines, 1 Luminex instrument can process approx. 720 samples/8 hr. shift

New Luminex instrument: >2500 samples/8 hr. shift

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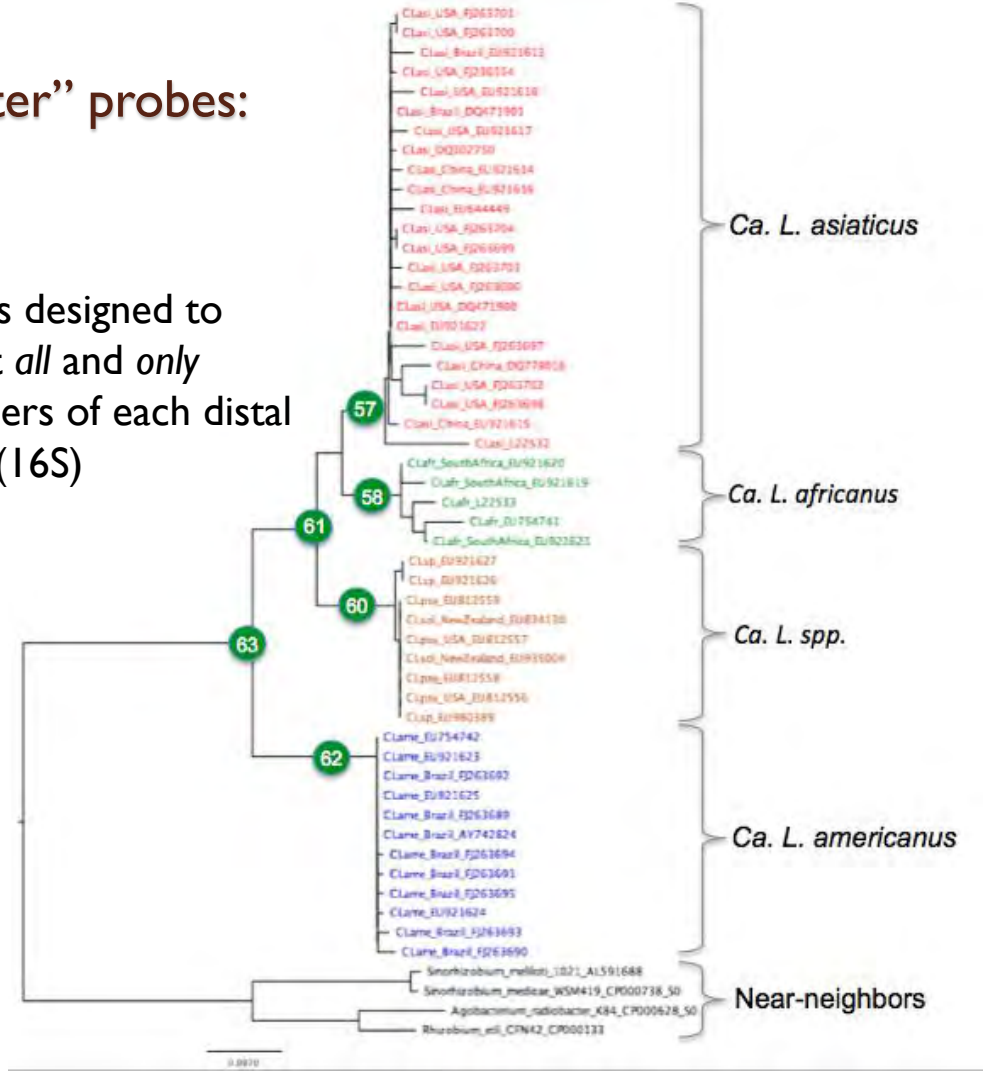
HLB species-specific and "Pan Liberibacter" probes: separate HLB assay

Species	Node	# of seqs
All HLB	N63	47
Ca. L. americanus	N62	11
N57+N58+N60	N61	36
Ca. L. spp.	N60	9
Ca. L. asiaticus	N57	23
Ca. L. africanus	N58	4

- Probes designed to detect *all* and *only* members of each distal clade (16S)



All problem probes have been redesigned and passed specificity testing.

Ca.Liberibacter 16S SNPs detected by probes, and their location

			Position																														
Species	Node	# of seqs	55	56	71	128	170	175	267	313	329	358	413	469	635	768	810	888	891	901	902	903	904	905	906	937	1014	1018	1052	1057	1102	1149	1196	1222
All HLB	N63	47				G[3]	G[4]	C[4]		G[1]	A[1.1]																					T[2]		
Ca. L. americanus	N62	11			T[4]								T[5]	T[2]	G[1]				T[3]															
N57+N58+N60	N61	36																C[3]		A[1.1]	G[1.2]	T[1.3]	T[1]	T[1.4]	A[1.5]		T[2]	A[2.1]						
Ca. L. spp.	N60	9							T[2]			A[1]														T[3]			A[4]				T[5]	
Ca. L. asiaticus	N57	23	G[2.1]	A[2]													C[3]														C[1]			
Ca. L. africanus	N58	4														T[3]														T[1]				G[2]

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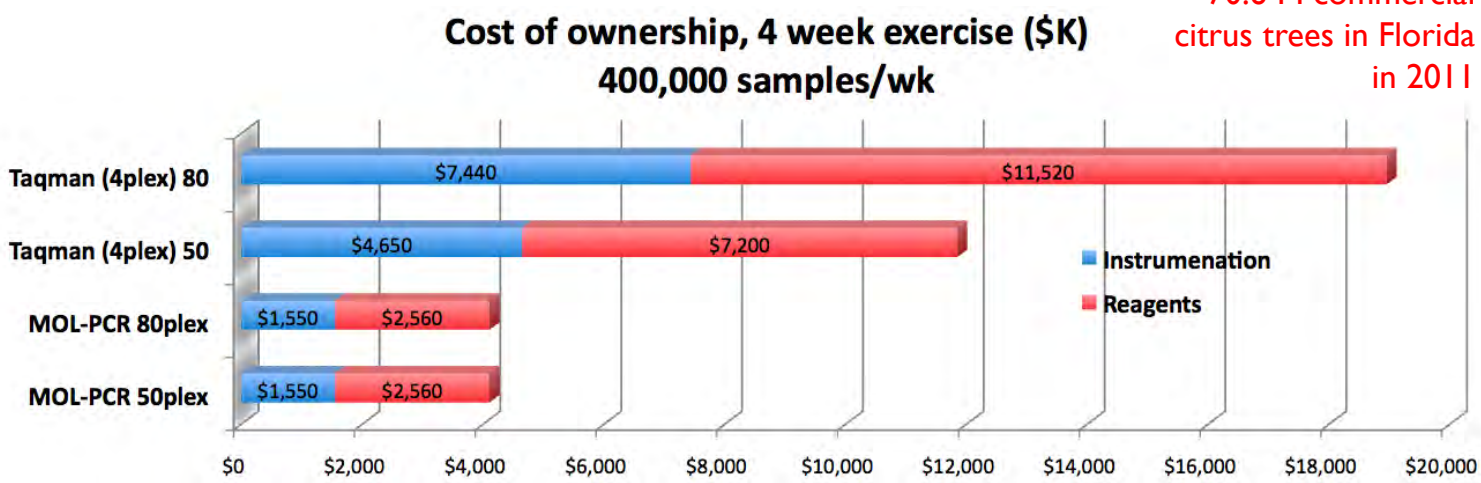
Platform and assay comparison

Assay/platform	Specificity	Sensitivity	Instrument Cost	Operational cost	Multiplex	Throughput
Taqman PCR	High	High	Moderate to High*	Moderate*	Limited to 4	Moderate
qPCR (RT-PCR)	High	High	Moderate to High*	Moderate*	No	Moderate
ELISA	Low to Medium	Low to Medium	Low	Low	Single to 2-plex	Moderate
Sequencing	High	Low to Medium**	Extreme	Extreme	Possible with some platforms	Extremely Low
MOL-PCR	High***	Medium to High	Moderate	Low to Moderate	500+ possible	Med to High

*Depends on required "bandwidth". Costs increase linearly with additional markers/samples.
**Large amounts of enriched or purified DNA are generally required, but whole genome amplification (WGA) is sometimes used to increase sensitivity.
***Includes SNP discrimination for higher resolution among pathovars, where needed.

70.6 M commercial citrus trees in Florida in 2011

Luminex will market kits for citrus pathogen detection, and National Agricultural Genotyping Center will be one of several testing service providers.



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Parameters to consider in support of Citrus industry

- Platform (flow cytometer; current favorite is Luminex 100/200, but there are many)
  - *Fully mature, commercial technology with thousands of installed instruments worldwide*
  - *Several application options for instrumentation, including small benchtop to high throughput*
  - *Multiple vendors produce and sell flow cytometers, so there are many choices*
- Assay (MOL-PCR)
  - Mature assay developed with over $3M in investments from US Gov and Industry
  - Developed for human health and biodefense, applied to Citrus pathogen characterization and detection
  - Patented LANL technology; US Patent # 7,153,656
  - Licensed to American Qualex (San Clemente, CA) and sublicensed to Luminex Corp. (Austin, TX)
  - Partnership with Luminex for kit manufacturing
  - Tech transfer in progress to USDA ARS Riverside from LANL
- Validation panels
  - Required for validating any platform/assay
  - Should include all available isolates of all pathogens included in the assay
  - Samples scattered across the globe
  - Working with UC Riverside and USDA-ARS Parlier to collect and test samples.

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Brief Update on Seed Transmission

- Published studies focused primarily on scion varieties
- Focus of current study was on rootstock varieties
  - 2010 Dept. Plant Industry (Florida) list of most popular rootstocks
- Purpose was to find trends among many varieties; not focus on large numbers of a limited number of varieties

Brief Update on Seed Transmission

- Seeds collected in February, 2012 from trees verified to be infected with '*Ca.* Liberibacter asiaticus' (Las)
- All trees are part of germplasm collection at USHRL farm, Fort Pierce, FL
- Real time PCR (qPCR) used to assess Las
  - 16S rRNA gene assay (Wenbin Li, APHIS)
  - Log CN=Ct-36.28/-3.16
    - 1 copy of target gives Ct ~36.3

Analysis

- Dissection of Seeds
  - Seed coat removed and analyzed
  - Seed dissected into
    - Embryo(s)
    - Cotyledons
- Planting of Seeds
  - Seedling recovered soon (~7-10 days) after germination
    - Hypocotyl
    - Root

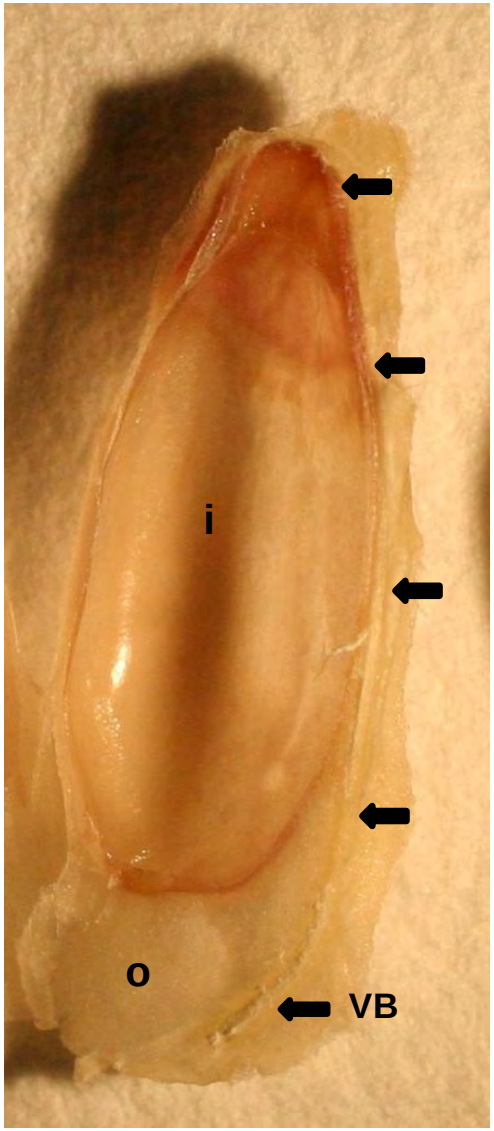
Rootstock Varieties

- Swingle
- Carrizo
- Sour Orange
- Cleopatra
- Volkamer
- US 812
- Sun Chu Sha
- US 897
- US 802
- US 942
- Rough Lemon
- Smooth Flat Seville
- P. trifoliata (Flying Dragon)
- C-35 Citrange
- P. trifoliata (English Dwarf)
- Ridge Pineapple
- Bitter Sweet
- Rangpur

Rootstock Varieties:

Seed Coat QPCR: Incidence and Mean Ct Values

Swingle	9/12	33.0	Rough Lemon	7/12	33.9
Carrizo	4/12	33.8	SmFlatSeville	12/12	24.4
Sour	6/12	26.0	Flying Dragon	0/12	
Cleopatra	6/12	31.6	C-35 Citrange	9/12	33.2
Volkamer	8/12	31.9	English Dwarf	2/12	33.2
US 812	0/12		RidgePineapple	7/12	32.0
SunChuSha	6/12	31.4	Bitter Sweet	5/12	33.6
US 897	0/12		Rangpur	12/12	29.5
US 802	0/12				
US 942	0/12				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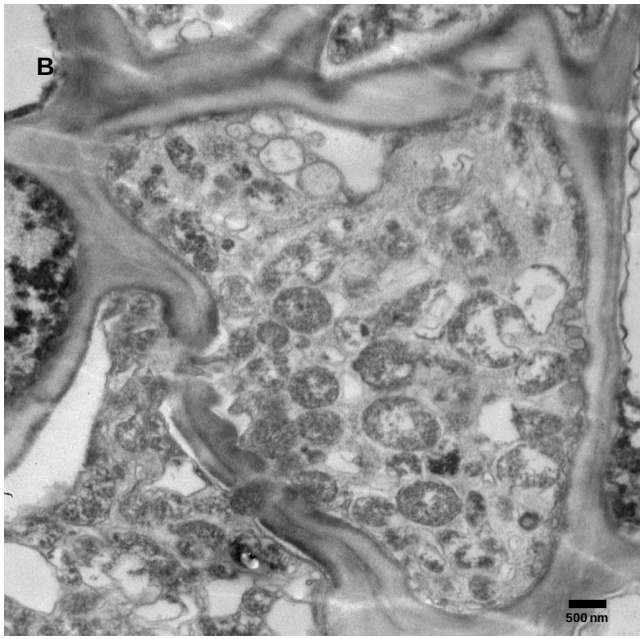
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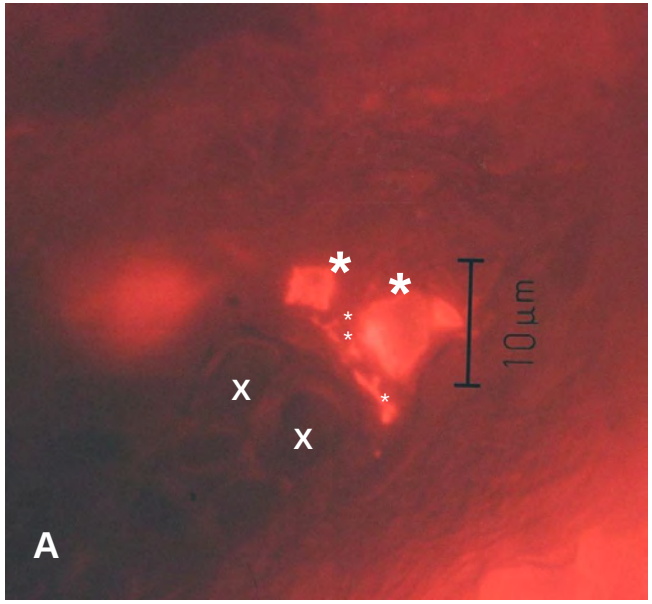
500 nm


*
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*
X
X
10 µm
A

Liberibacter in Seeds

- Embryos and cotyledons from seeds which were the source of seed coats
  - Cotyledons: 0/371
  - Embryos: 0/373
- Germinated seedlings
  - Shoots: 0/425
  - Roots: 6/425*

*Smooth Flat Seville; high Ct values likely remnant seed coat on attached cotyledon.

Seed Transmission

The bacteria are on the outside (seed coat) and not on the inside (cotyledons/embryo). If bacteria are not on the inside, seed transmission will not occur.