

Ralstonia solanacearum Race 3 biovar 2 (*Phylotype II, sequevar 1*)

From the field to the lab:
Towards accurate identification of a select agent pathogen

Patrice Champoiseau, University of Florida

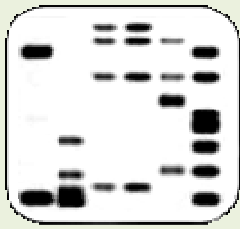
Second National Meeting of the National Plant Diagnostic Network

December 6-10, 2009 ♦ Miami, Florida

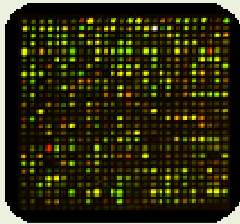


Introduction

USDA-NRI Project: *Ralstonia solanacearum* race 3 biovar 2: Detection, exclusion, and analysis of a select agent pathogen.



Aim 1. Develop rapid, robust, and reliable diagnostic assays for R3b2, using both immunological and DNA-based approaches.




Aim 2. Identify R3b2 genes involved in cold adaptation and growth in plant hosts, using a microarray-based post-genomic approach.



Aim 3. Develop and deliver a package of optimized education and management training modules.
Use **evaluation tools** to assess program effectiveness.

Outlines

- Introduction
- Current situation
- Detection, identification
- Coming diagnostic tools
- Educational resources



Friday,
December 13, 2002

Part V

**Department of
Agriculture**

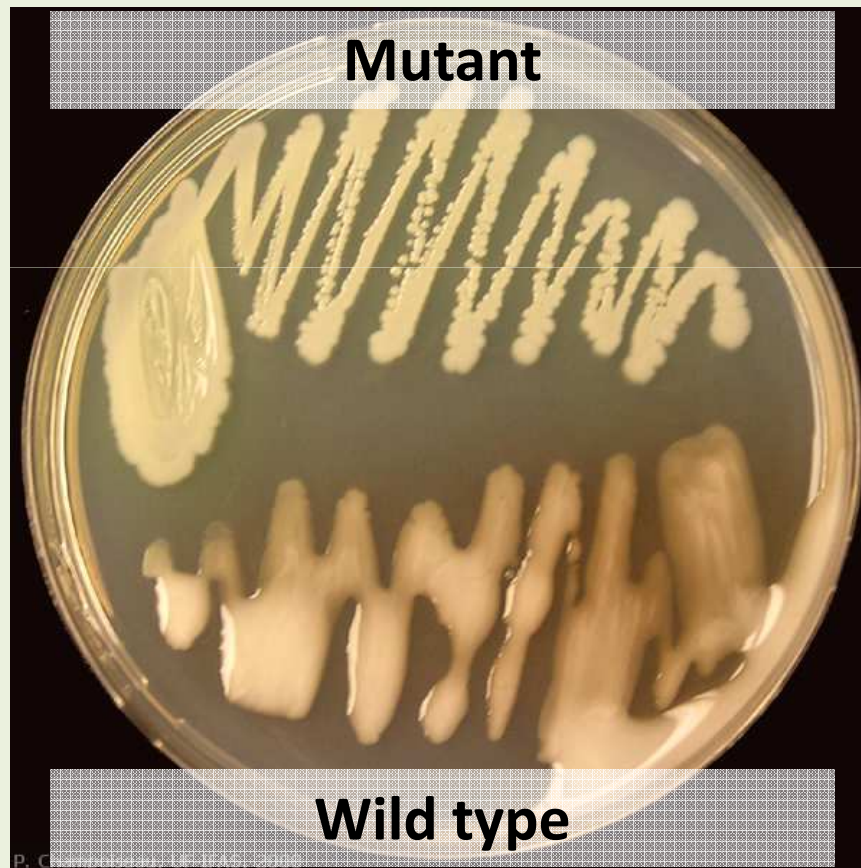
Animal and Plant Health Inspection
Service

7 CFR Part 331
9 CFR Part 121
Agricultural Bioterrorism Protection Act of
2002; Possession, Use and Transfer of
Biological Agents and Toxins; Interim
Final Rule

Federal Register

Introduction

- ◆ *Ralstonia solanacearum* is a plant pathogenic bacterium.
- ◆ Formerly known as *Pseudomonas solanacearum*.



Cultures of virulent (bottom) and non-virulent (top) colonies of *R. solanacearum*.



Culture of *R. solanacearum* on TZC agar medium

Introduction

- ◆ Bacterial wilts: >200 plant species, > 50 families (trees, cultivated crops, ornamentals, and weeds).



- ◆ Worldwide distribution from tropical to cold-temperate areas (Europe, North Asia).
- ◆ Soil-borne and water-borne pathogen.

Introduction

- ◆ *R. solanacearum*: a “species complex”

Species	<i>Ralstonia solanacearum</i>															
Strains	[Grid of 16 columns and 2 rows of empty cells]															
Races	1		4	5	1		2	3	Not determined			1	3	R. sy	BDB	
Biovars	3		4		5	2T		1		2	2T		1	2	2T 1 2	
MLGs*	8	9	11		15	21	19		21	30	1	2	24	26		
	10	12	11		17	23	20		23	32	3	4	25	27		
	13	24	11		16	22	20		22		5	6	28	27		
RFLP Groups	Division 1 “Asiaticum”					Division 2 “Americanum”					Not determined					
Sequevars	Analysis of partial gene sequences (endoglucanase, <i>hrpB</i> , and ITS region)															
Phylotypes	Phylotype I “Asia”					Phylotype II (A-B) “America”					Phylotype III “Africa”			Phylotype IV “Indonesia”		

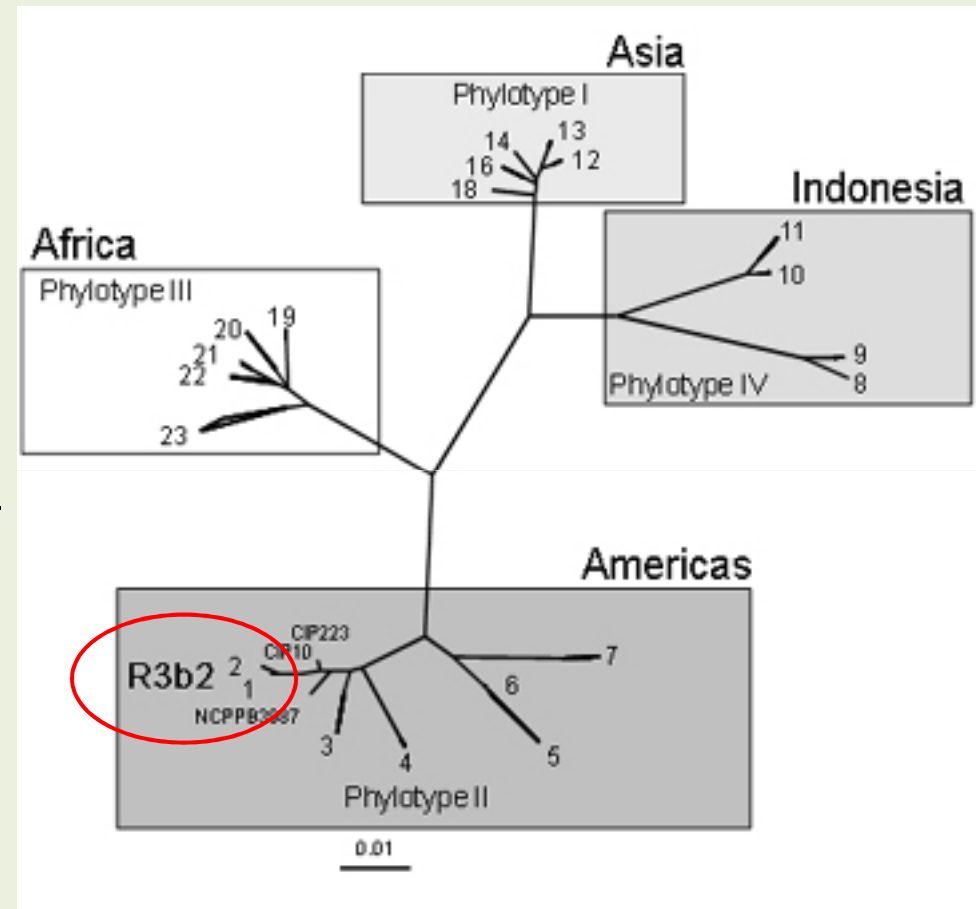
*MLGs= Multilocus Genotypes

(Fegan and Prior, 2005)

Introduction

- ♦ Race 3 biovar 2 (R3b2): *Phylotype IIB, sequevar 1 (2)*

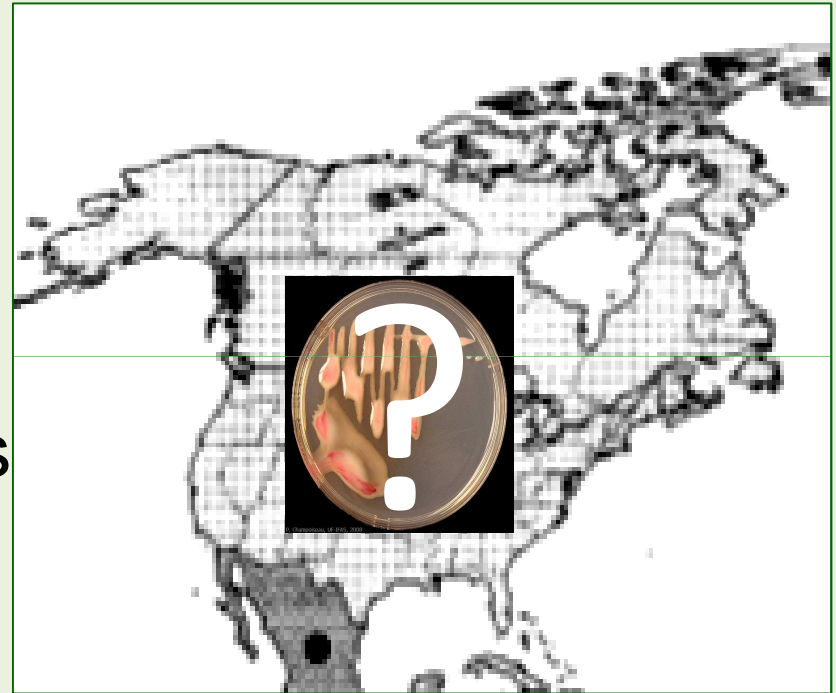
- Select Agent pathogen in the US:
Dec. 13, 2002; Nov. 14, 2008
- Origin: tropical highlands
("Cold tolerant" ?)
- Worldwide except US and Canada
- Primary hosts: potato and tomato
- Other hosts: pepper, tobacco,
geranium, weeds
- Potato: 1\$ billion losses yearly
- Threat: US potato industry



From Fegan and Prior, 2005

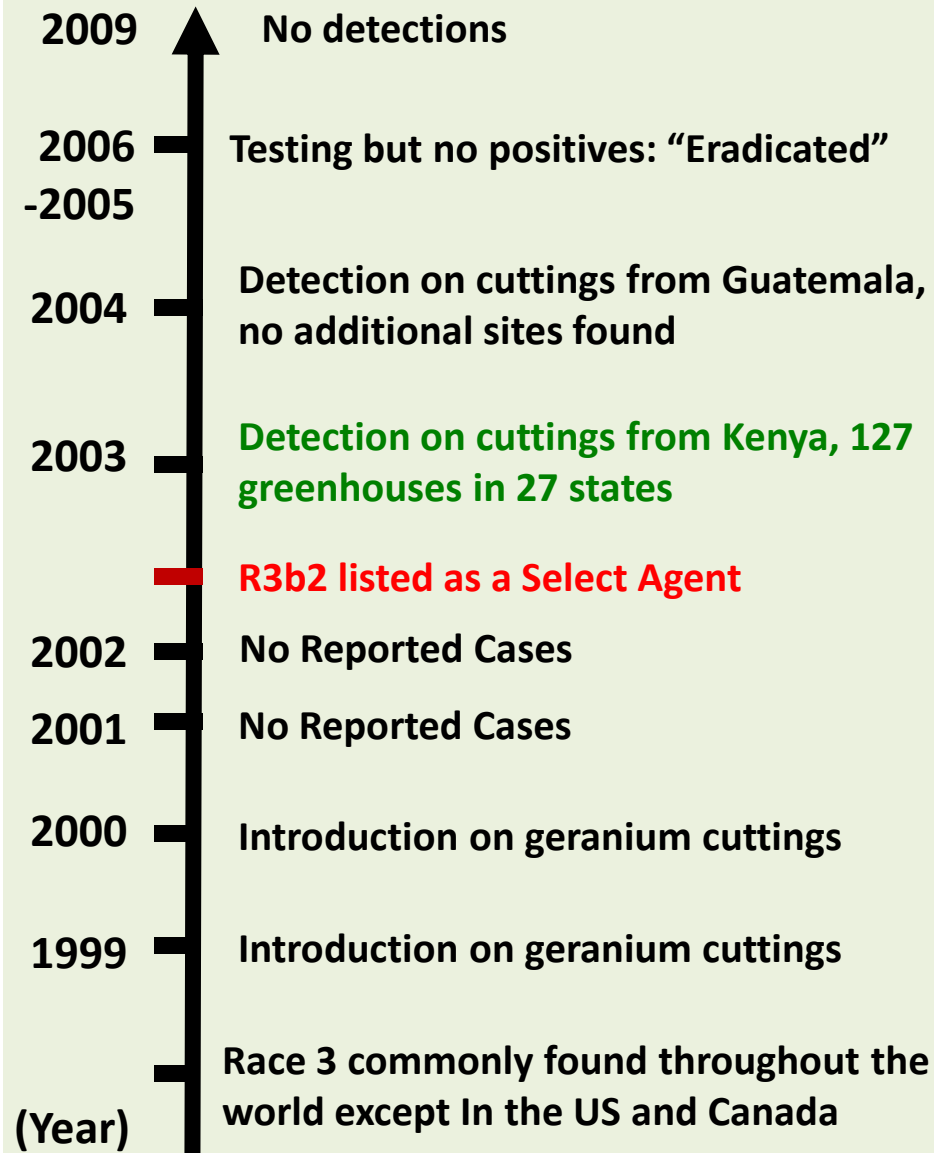
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Current situation

◆ History of introduction of R3b2 in the US



Symptom caused by *R. solanacearum* R3b2 on geranium. Photo: C. Allen, U. Wisconsin.

Current situation

- ◆ Risk for re-introduction in the US

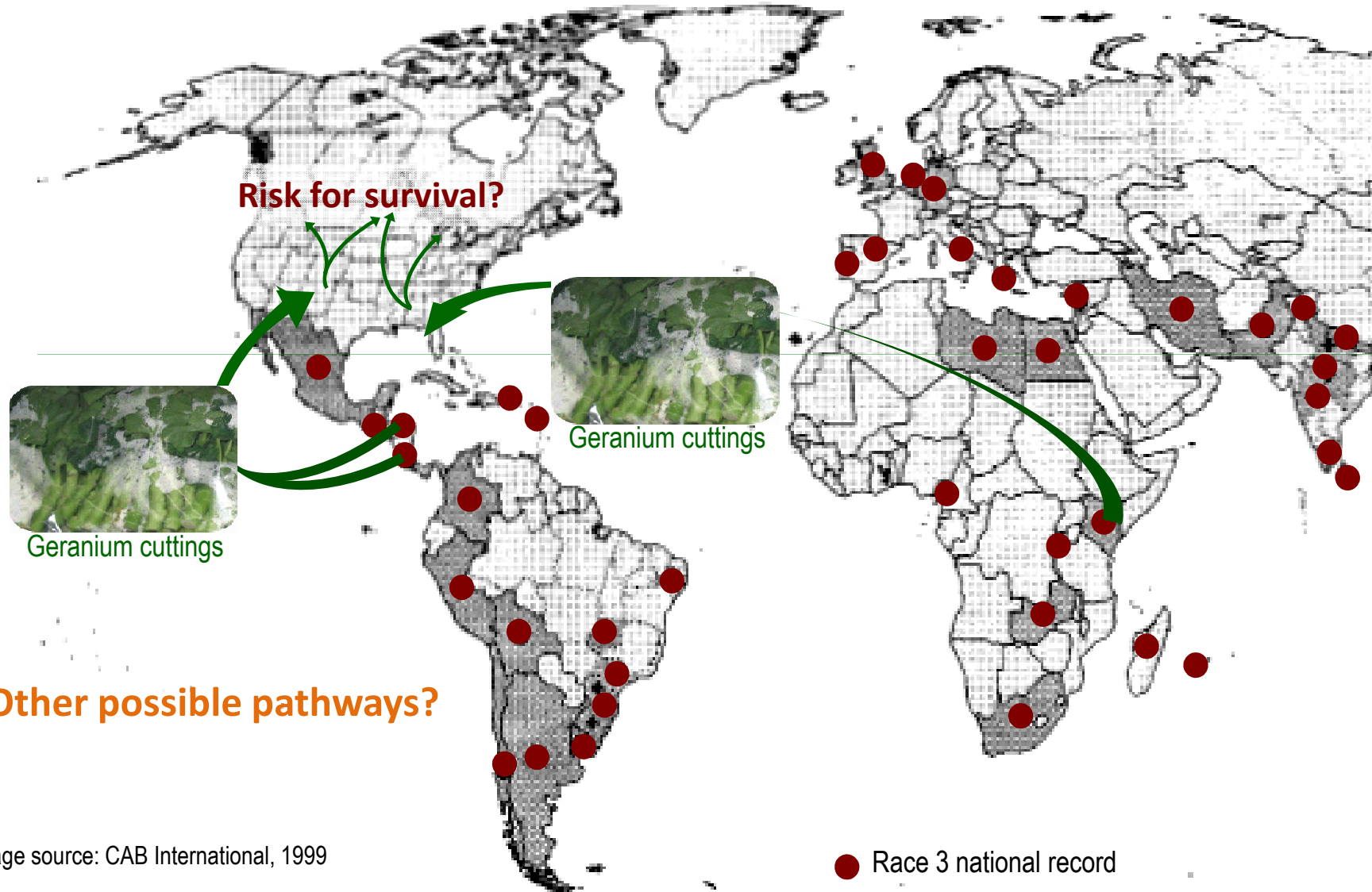


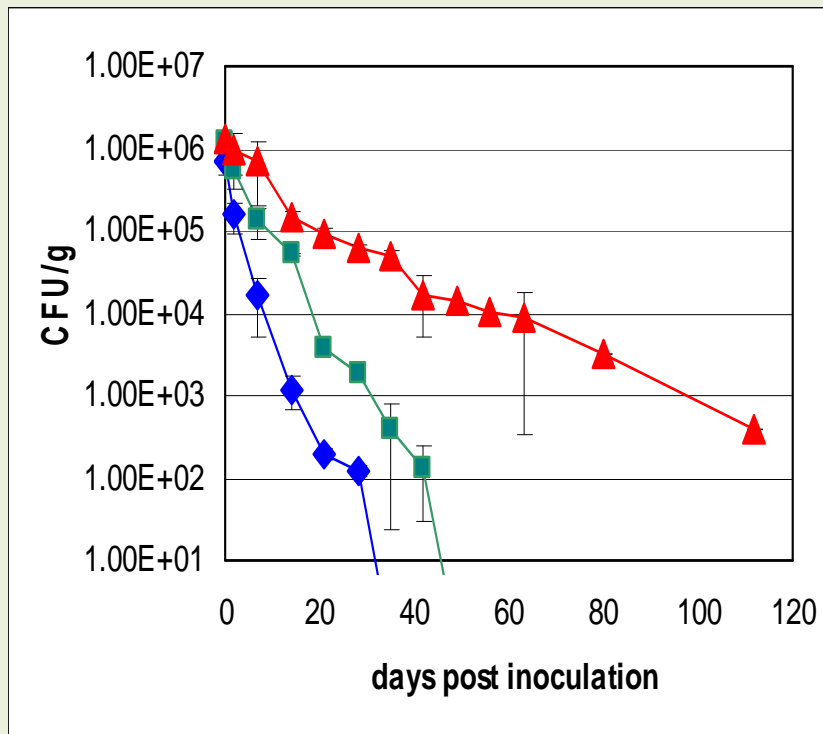
Image source: CAB International, 1999

Current situation

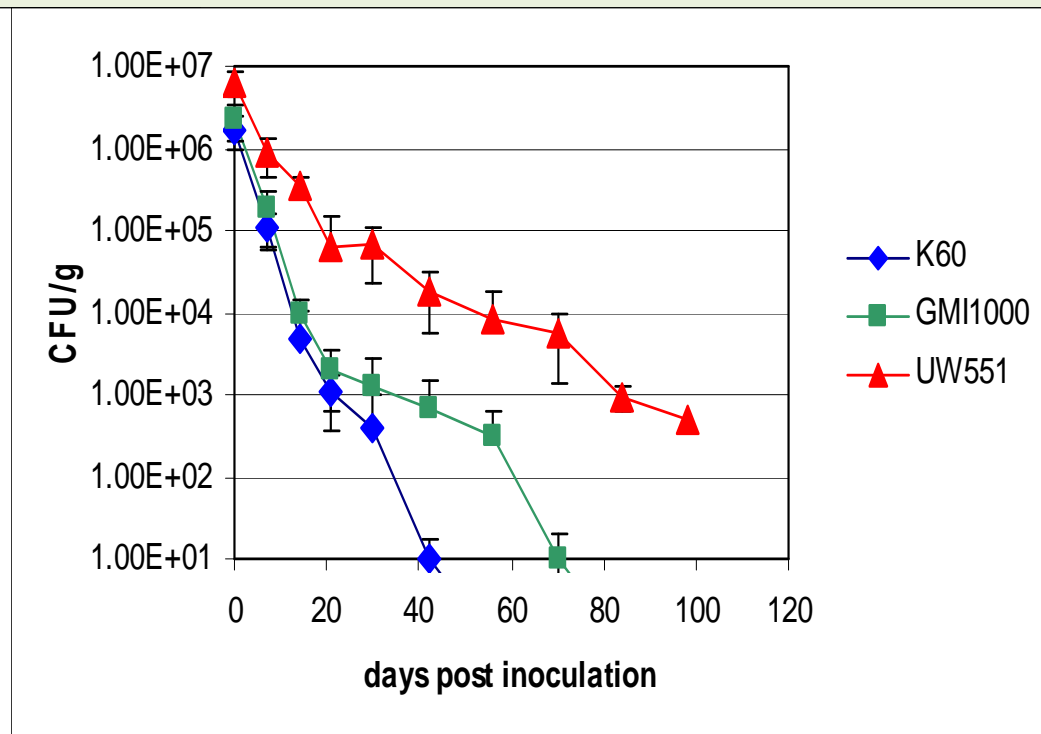
◆ Survival of R3b2 on potato at 4°C

Milling et al., 2009. *Phytopathology* 99:1127-1134

A: Russett Norkotah



B: Shepody



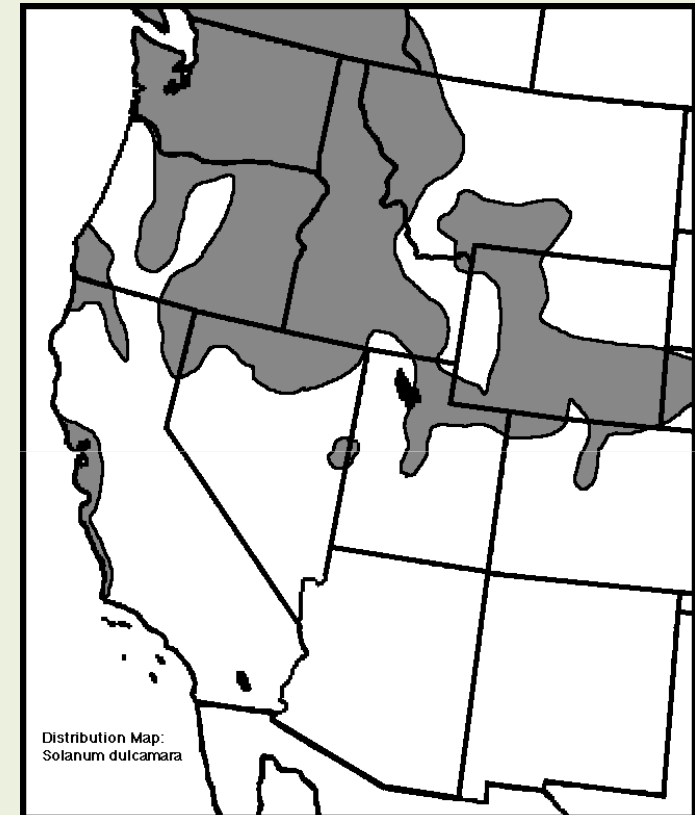
Immediate incubation at 4°C

Current situation

- ◆ Survival of R3b2 on semi-aquatic weeds
 - *Solanum dulcamara*
(bittersweet or climbing nightshade)
 - In Europe: responsible for outbreaks of brown rot of potato (20 years)
 - Overwinter



Photo: J. Elphinstone, CSL, UK



S. dulcamara: distribution Map. D. Chesner, SWSBM, US.

S. dulcamara: key features for identification.
J. Elphinstone, CSL, UK

Current situation

- ◆ Other genotypes of *R. solanacearum* in the US

Race 1 biovar 1 (phyloptype II, seq 7)

- Type strain : endemic/limited to the Southern US
- Causes bacterial wilt on potato, tobacco, and tomato
- Also pathogenic on geraniums



Photo: T.M. Momol, UF-IFAS

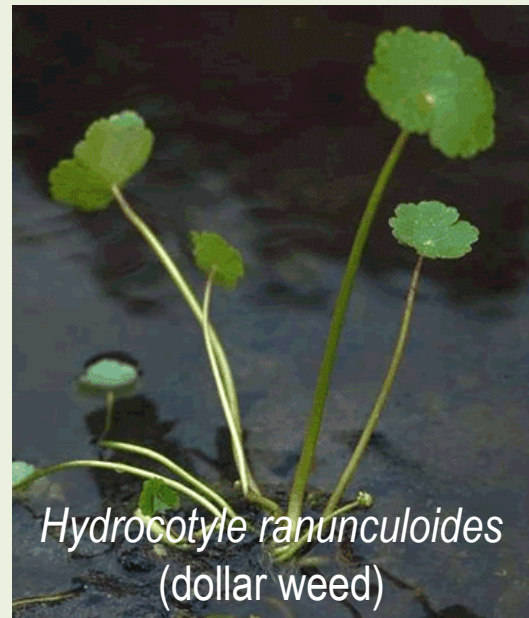
Current situation

- ◆ Other genotypes of *R. solanacearum* in the US

Race 1 biovar 3 (phyloptype I, seq 13) - Asia

Race 1 biovar 1 (phyloptype II, seq 38) - Caribbean

- Broad host range: eggplant, tomato, tobacco, and potato but also anthurium, cucurbits, and pothos
- Found in common aquatic weeds in Florida:



Hydrocotyle ranunculoides
(dollar weed)



Polygonum pennsylvanicum
(Pennsylvania smart weed)

Ji et al., 2007. Plant Disease 91:195-203; Hong et al., 2008. Plant Disease 92:1674-1682

Current situation

- ◆ Status as a Select agent:

Positive identification of R3b2 in the US



**Destruction of all infected or potentially infected plants
Quarantine of production facilities and equipment
Surveys**

**Could result in \$ millions losses due to application of effective
regulatory eradication protocols**

**Critical to prevent re-introduction and spread of R3b2 in the US
Ensure early and accurate identification to prevent misdiagnosis**



**Effective procedure and use of rapid, sensitive, and reliable diagnostic
assays for identification of R3b2**

Outlines

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Bacterial streaming test

Detection, identification

- ◆ **Step 1:** Symptom identification (field/greenhouse)



Detection, identification

◆ Step 1: Symptom identification - Early stages

Photo: D.P. Weingartner, U. Florida



Potato/Tomato
Wilting of the
youngest leaves

Photo: U. Georgia



Photo: D. Norman U. Florida



Geranium

Upward curling

Wilting of lower leaves

Detection, identification

◆ Step 1: Symptom identification - Late stages

Potato/Tomato

Wilting of foliage
Drying of leaves
Stunting of plants

Plant death



Brown discoloration
of vascular tissue

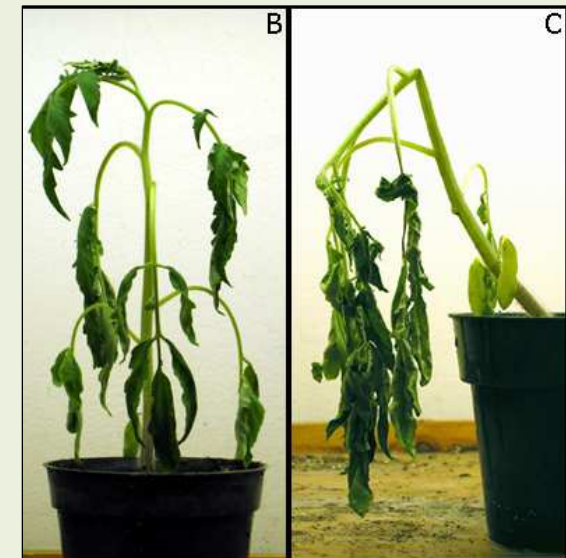


Photo: D. Thurston, Cornell U.



Photo: T.M. Momol, UF-IFAS

Stem collapse



Detection, identification

◆ Step 1: Symptom identification - Late stages

Geranium

Wilting of the upper leaves

Yellowing, drying of leaves



Photo: C. Allen, UWisconsin



Photo: T.M. Momol, UF-IFAS

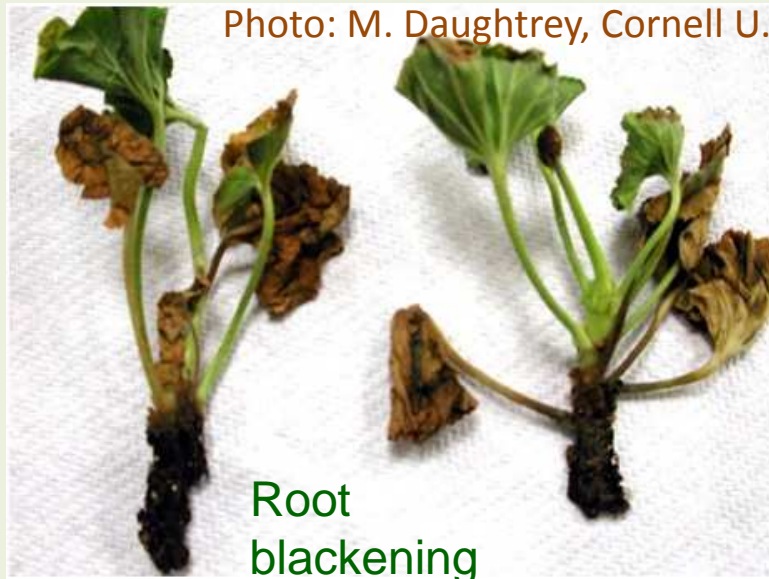


Photo: M. Daughtrey, Cornell U.

Root
blackening



Plant death

Detection, identification

- ◆ **Step 1:** Symptoms look-alike

Root damage, drought, nutrient deficiency or other plant pathogens, such as the *Verticillium* and *Fusarium* fungi (potato, tomato), or *Xanthomonas campestris* on geranium.



Photo: RG. Gardner, NC State U.

Fusarium wilt of tomato



Source: UNECE

Verticillium wilt of potato



Photo: SGP Nameth, Ohio State U.

Bacterial blight of geranium

Detection, identification

◆ **Step 1:** IMPORTANT notes on symptoms

Symptoms induced by R3b2 (PII, seq1) **CANNOT BE** distinguished from those induced by other strains (PII, seq 7 or 38)

LATENT INFECTIONS

- Infected but symptomless plant
- Tolerant cultivars, secondary hosts
- Vegetative propagating material
- Major epidemiological trait

Geranium cuttings ready for shipment to the US



Detection, identification

- ◆ **Step 1:** Signs of the pathogen - later stages



Photo: M. Daughtrey, Cornell U.

Bacterial ooze



P. Champoiseau, UF-IFAS, 2008



Photo: D. B. Langston, UG,
Bugwood.org

Bacterial streaming test

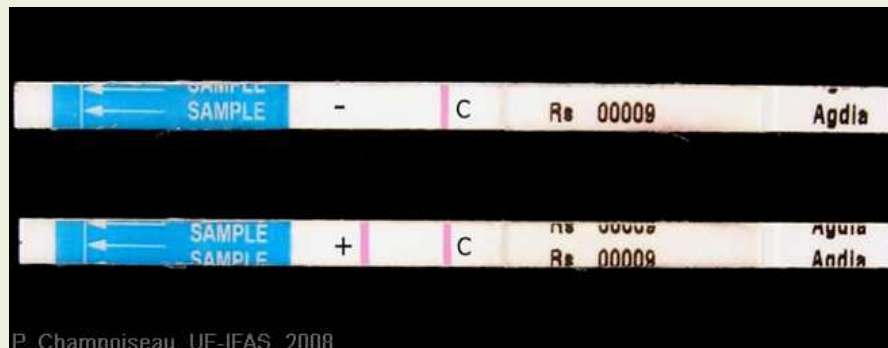
Detection, identification

◆ Step 2: Early screening tests

Species level
Fast and easy
Field or lab



- Late stages
- Low sensitivity



Use of immunostrips

Detection, identification

- ◆ **Step 2:** Early screening tests

Species level
Fast and easy
Field or lab

USDA-APHIS-PPQ Commercially Available Recommended Immunodiagnostic Tests:

Address **Rs ImmunoStrip® Test**
Agdia, Inc.
30380 County Road 6
Elkhart, IN 46514
Web site: <http://www.agdia.com>
T: 800-622-4342
F: 219-264-2153

Address **Potato Brown Rot Pocket™ Diagnostic**
Central Science Laboratory (CSL)
Sand Hutton, York, YO41 1LZ
Web site: <http://www.csl.gov.uk>
T: 44 1904 462600
F: 44 1904 46211

Address ***Ralstonia solanacearum* SPOTCHECK LF™**
Adgen, Ltd.
Nellie's Gate, AYR
Scotland, KA6 5AW
Web site: <http://www.adgen.co.uk>
T: 44 1292 525275
F: 44 1292 5255477

Detection, identification

- ◆ **Step 3:** Confirmation of the genus/species

Species level
Approved diagnostic
screening lab
(PPQ permit)

Immunodiagnostic assays (species-specific Abs)

Enzyme-linked immunosorbent assay (ELISA), immunofluorescence-antibody staining (IFAS), or immunofluorescence-colony staining (IFCS).

DNA-based assays

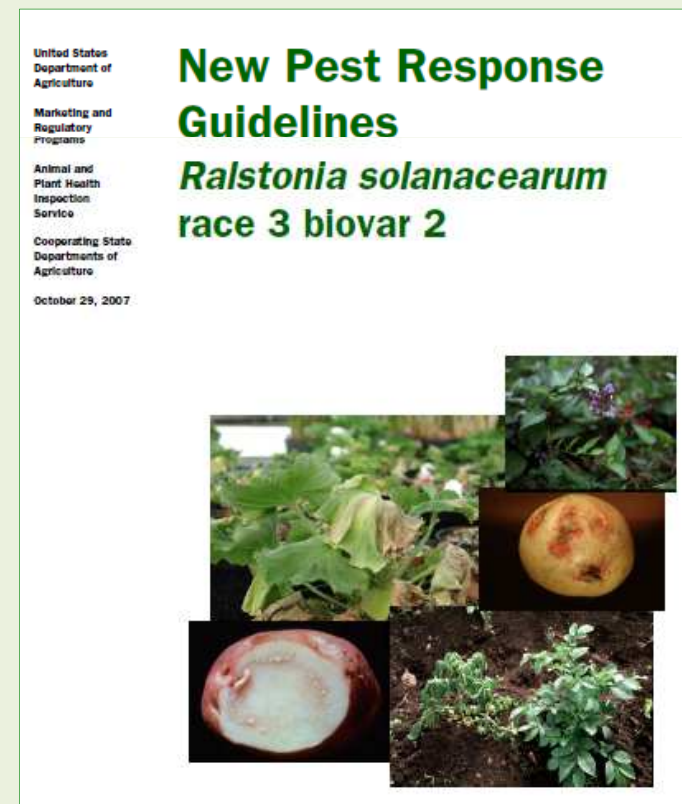
Polymerase chain reaction (PCR)

Universal species-specific primers

Primer set 759/760: 280bp fragment
(Opina et al., 1997)

Sampling protocols

Plant tissues, water, soil

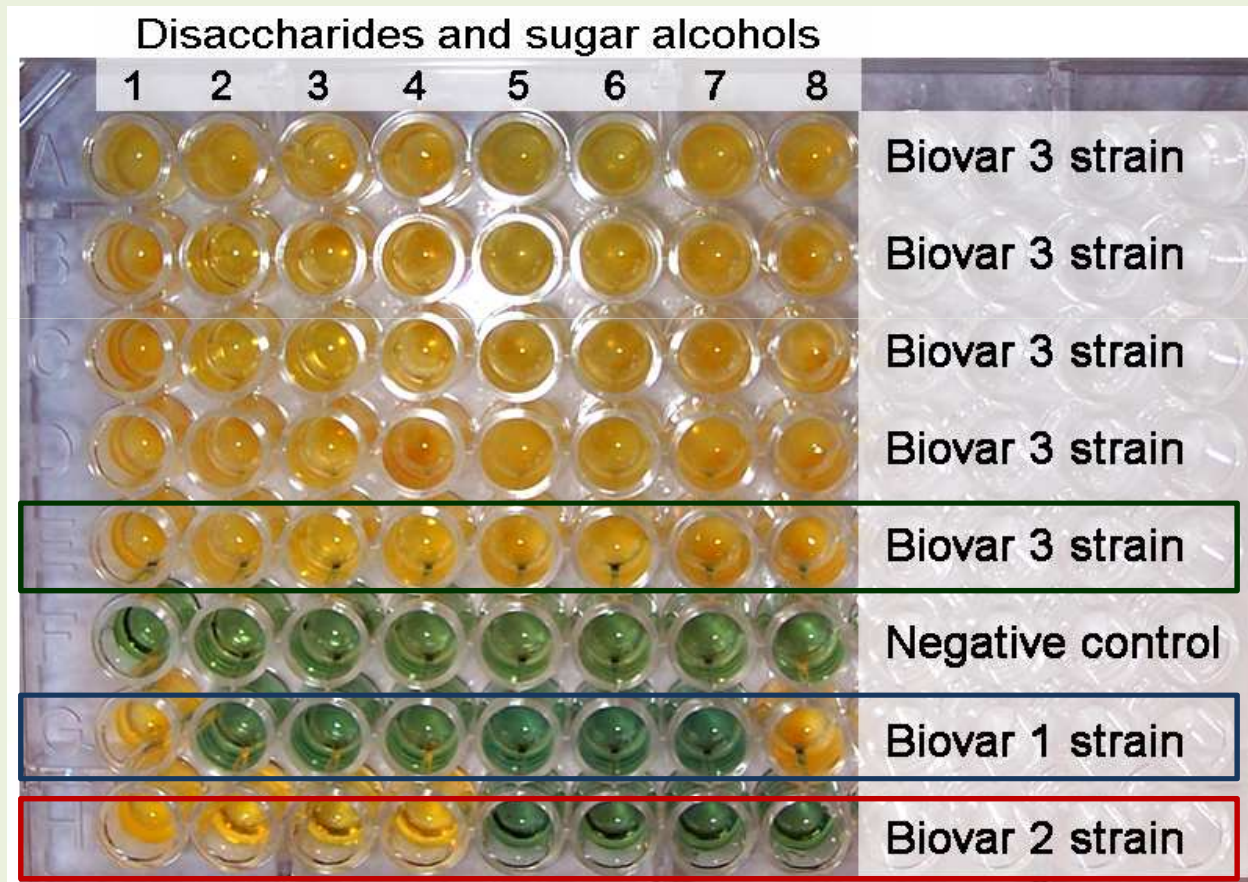


Detection, identification

- ◆ **Step 4:** Identification of the biovar

Biovar test

Sub-species
level
USDA-APHIS-PPQ-
NPGB Laboratory
Beltsville, MD



1 = Glucose ; 2 = Maltose ; 3 = Lactose ; 4 = D-(+)-Cellobiose
5 = Mannitol ; 6 = Sorbitol ; 7 = Dulcitol ; 8 = Trehalose

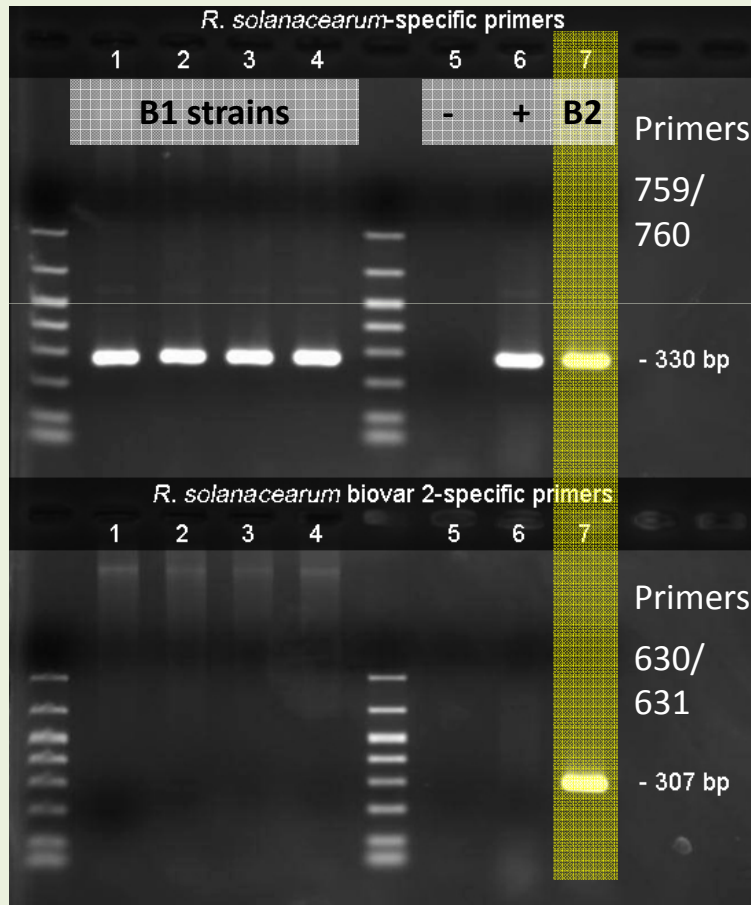
E.N. Twieg, USDA-APHIS-PPQ-CPHST-National Plant Germplasm and Biotechnology Lab., Beltsville, MD

Detection, identification

◆ Step 4: Identification of the biovar

PCR using biovar 2-specific primers

Sub-species level
USDA-APHIS-PPQ-
NPGB Laboratory
Beltsville, MD

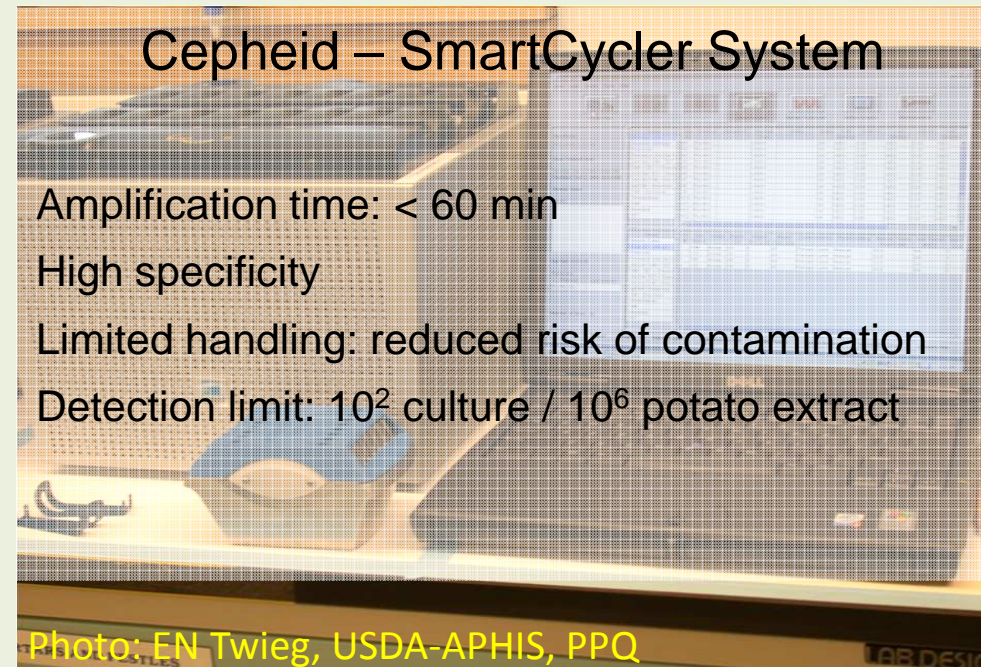


1 to 4 = Biovar 1 strains ; 5 = Negative control
6 = Biovar 1 positive control ; 7 = Biovar 2 positive control

Biovar 2-specific primers (630/631)

307bp fragment
(Fegan et al., 1998)

Real-time PCR (Weller et al., 2000)



Detection, identification

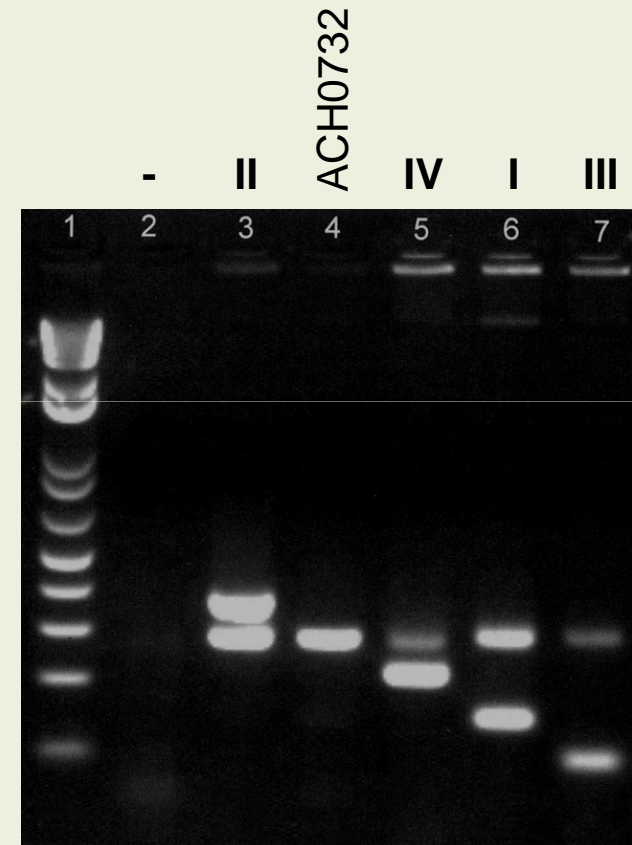
- ◆ **Step 4:** Identification of phylotype/sequence

Sub-species level

Multiplex PCR (Fegan and Prior, 2005)

PCR-Phylotype

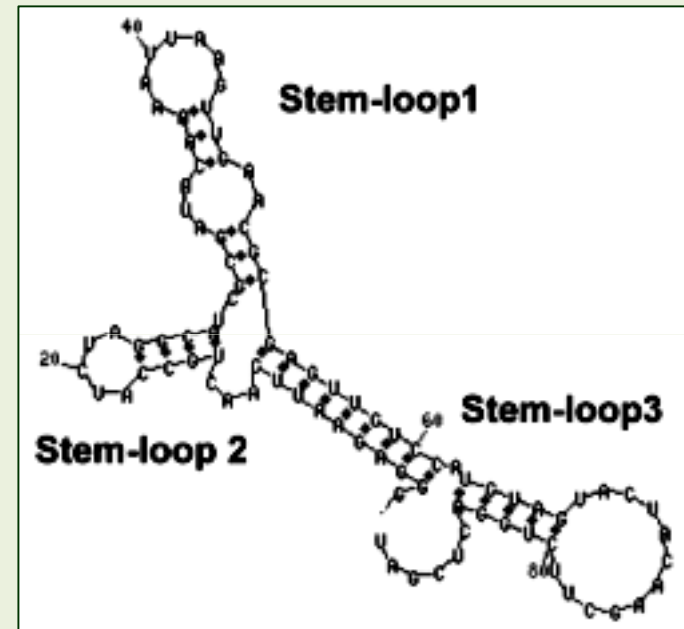
Nmult:21:1F	CGTTGATGAGGCGCGCAATT	Phylotype I (Asiaticum)
Nmult:21:2F	AAGTTATGGACGGTGGGAAGTC	Phylotype II (Americanum)
Nmult:22:InF	ATTGCCAAGACGAGAGAAGTA	Phylotype IV (tropical)
Nmult:23:AF	ATTACGAGAGCAATCGAAAGATT	Phylotype III (African)
Nmult:22:RR	TCGCTTGACCCTATAACGAGTA	Amorce reverse unique
759R	GTCGCCGTCAACTCACTTTC	Marqueur interne
760F	GTCGCCGTCAGCAATGCGGAATCG	<i>R. solanacearum</i>



Sequevar: sequencing and analysis of partial endoglucanase (*egl*) gene

Outlines

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Structure of a Molecular Aptamer

Coming diagnostic tools

- ◆ Aim 1. Develop rapid, robust, and reliable diagnostic assays for R3b2

Challenge: Achieve high sensitivity (low populations) with high specificity from plant extracts, soil, and water samples.

Approaches:

- Monoclonal antibody (3.H7)
- R3b2-specific primers
- Immunocapture Separation (IMS) - PCR
- Magnetic Capture Hybridization (MCH) - PCR
- Novel amplification method LAMP

Coming diagnostic tools

- ◆ Production of a monoclonal (IgG3) Ab 3.H7:

Bound all *R. solanacearum* strains tested (109)

Not R3b2-specific

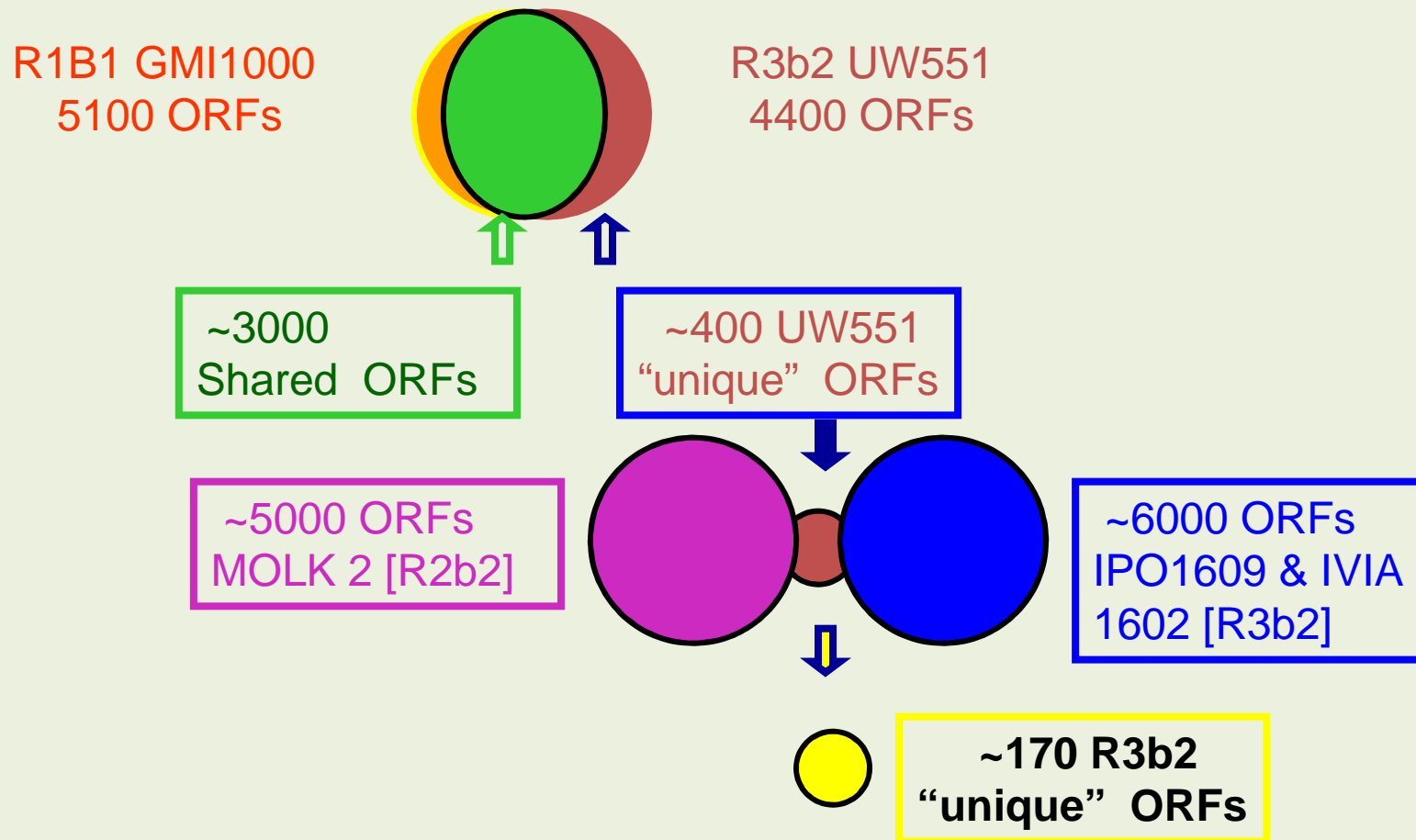
Not EPS- cells

O.D.		Name	Origin	Hosts
0.7825	R3B2	PS-13	Guatemala	Geranium
0.9925	R3B2	PS-14	Guatemala	Geranium
1.437	R3B2	PS-16	Guatemala	Geranium
0.729	R3B2	PS-17	Guatemala	Geranium
1.593	R3B2	PS-20	Guatemala	Geranium
0.903	R3B2	PS-22	Guatemala	Geranium
0.556	R3B2	UW552	Guatamala	Geranium
0.79	R3B2	UW437	Australia	Tomato
0.565	R3B2	UW491	Colombia	Potato
0.7085	R3B2	UW596	Guatemala	Potato
0.255	R3B2	UW551 EPS-	Kenya	Geranium
1.116	R3B2	UW551 LPS-	Kenya	Geranium

Coming diagnostic tools

- ◆ Identification of R3b2-specific ORFs:

In Silico Genomic Subtraction (M. Schell, U. Georgia)

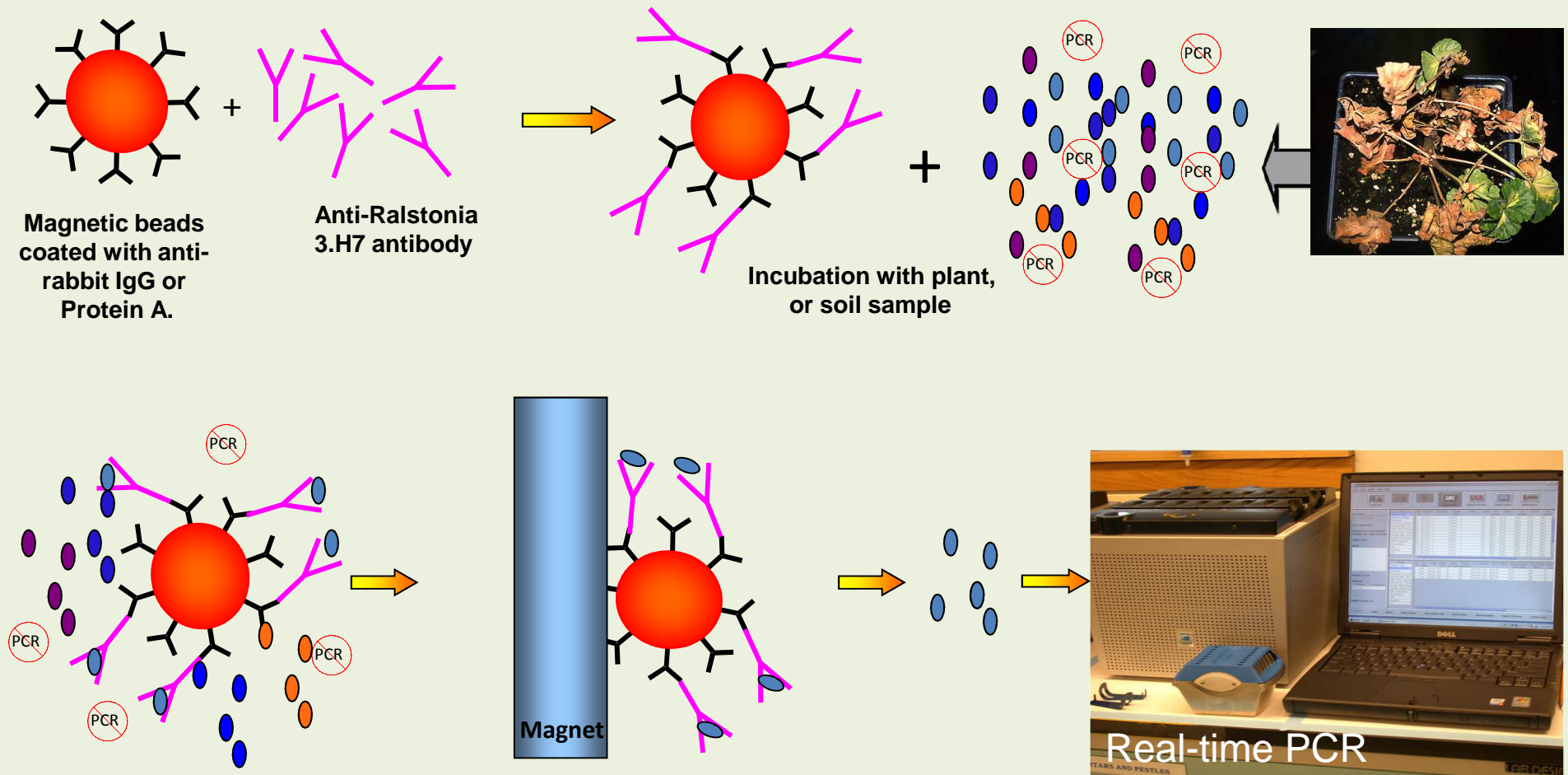


5 primer sets specific to R3b2 (52/100 strains)

Coming diagnostic tools

- ◆ ImmunoMagnetic Separation (IMS):

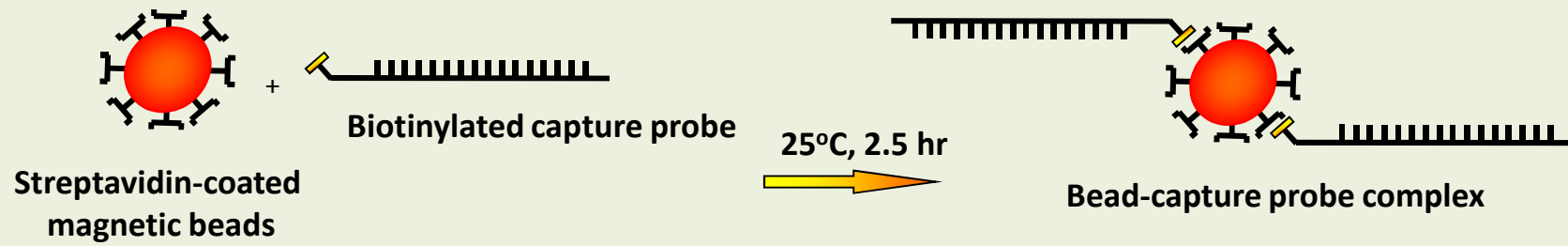
Use magnetic beads coated with Ab 3.H7 to simply and easily purify plant or soil samples from other bacteria and PCR inhibitors.



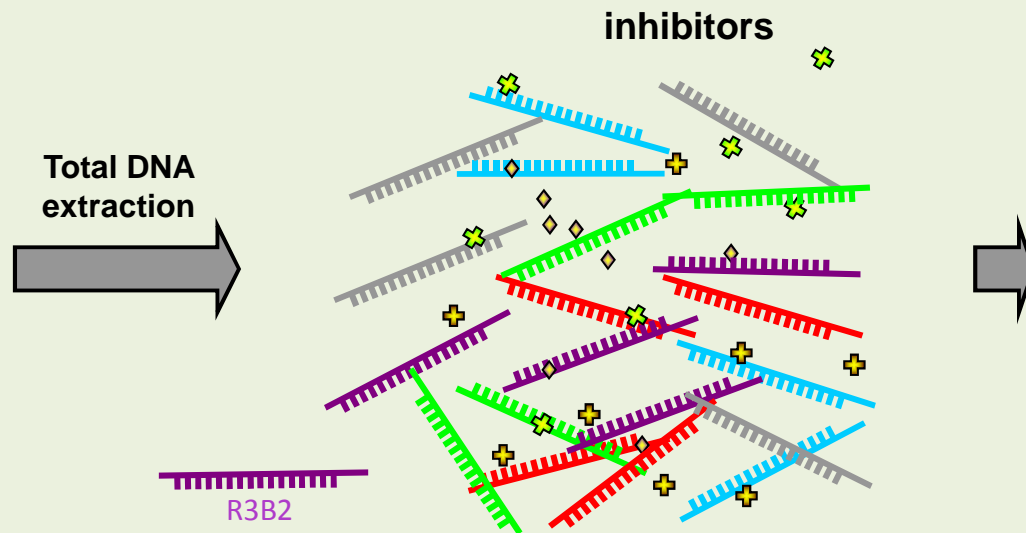
Coming diagnostic tools

- ◆ **Magnetic Capture Hybridization (MCH):**

Magnetic beads coated with R3b2-specific DNA probes (30-70 bp sequences) used for hybridization and capture of target sequence

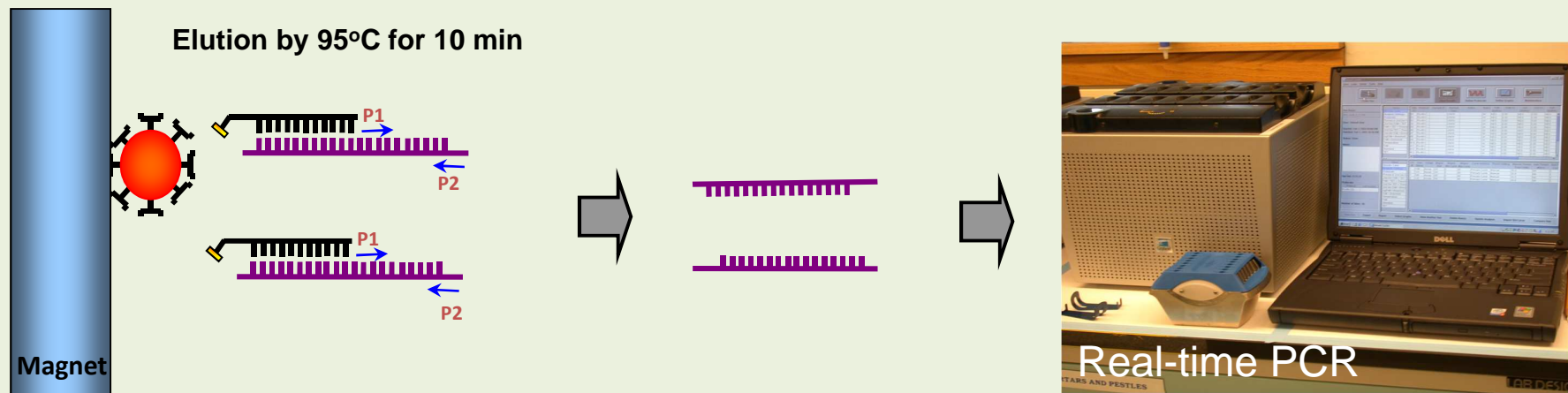
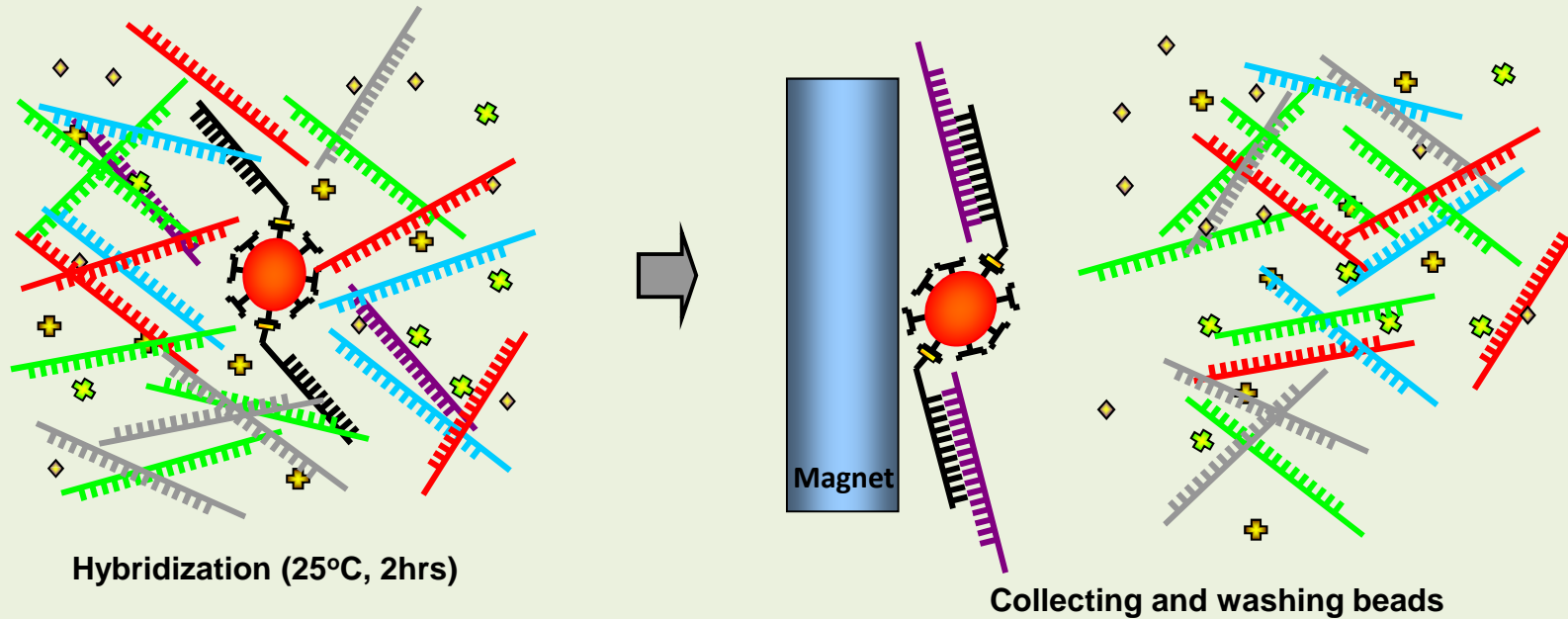


Soil or plant material with possible R3B2



Coming diagnostic tools

- ◆ Magnetic Capture Hybridization (MCH):



Coming diagnostic tools

◆ Coupling IMS AND MCH to Real-Time PCR

IMS-RTPCR preliminary results

R3b2 cell suspensions (pure culture)

~2.5 hr from adding sample to get Ct value

Detection threshold: ~ 50 cells per ml

MCH-RTPCR preliminary results

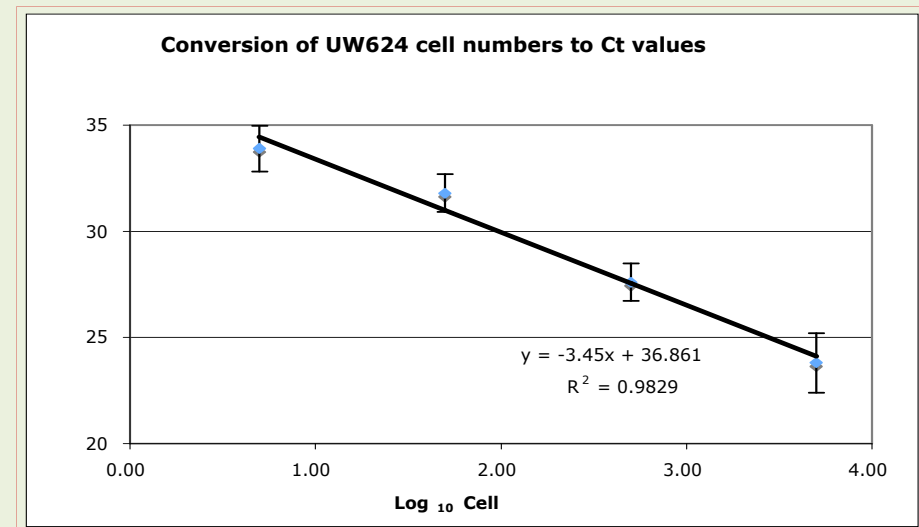
UW624 R3b2 cell suspensions

Detection threshold:

RTPCR: 10^3 CFU / ml

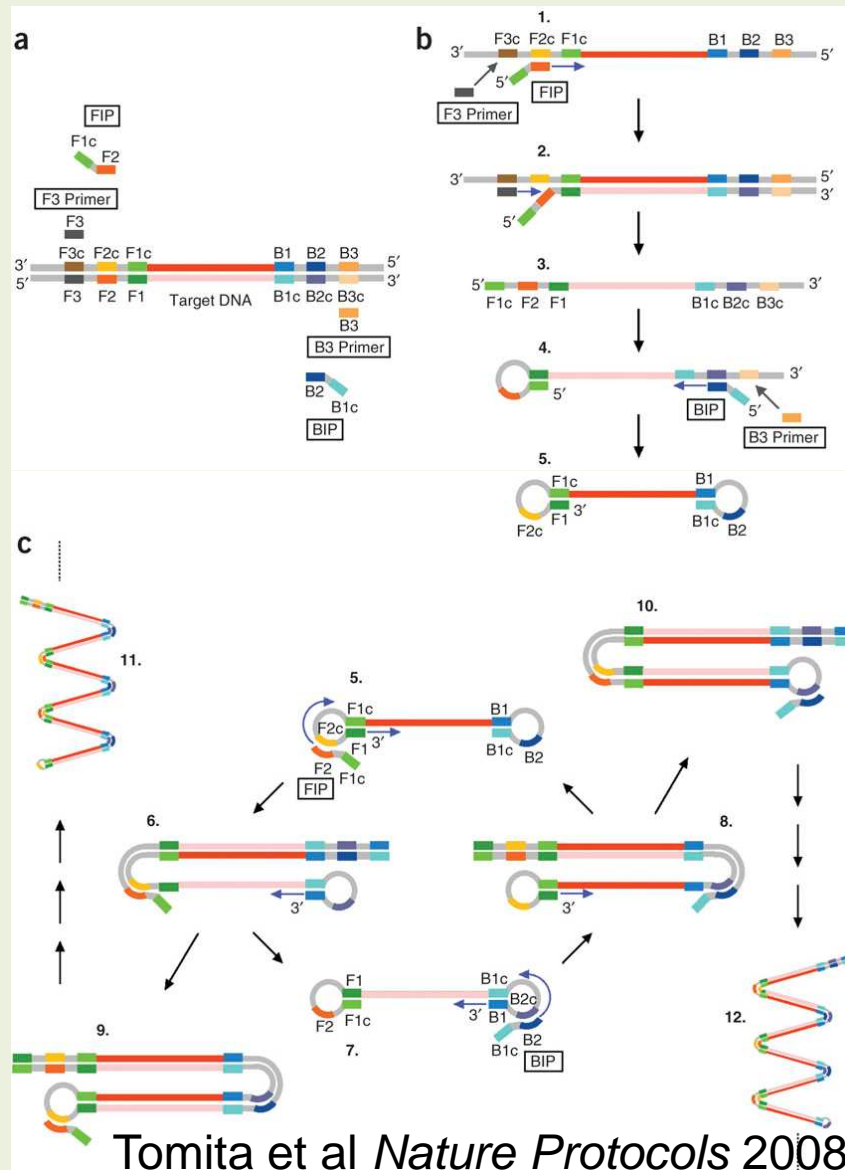
MCH-RTPCR: 10^2 CFU / ml

10-fold enhancement

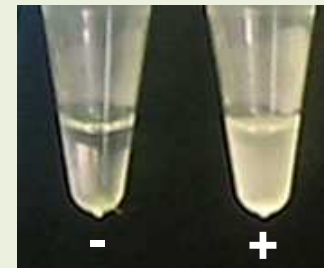


Coming diagnostic tools

◆ LAMP: Loop-mediated isothermal AMPLification



- A simple rapid PCR-like way to generate large quantities of DNA
- Highly specific (3 primer sets)
- Only need 2 temperatures
- No thermocycler required



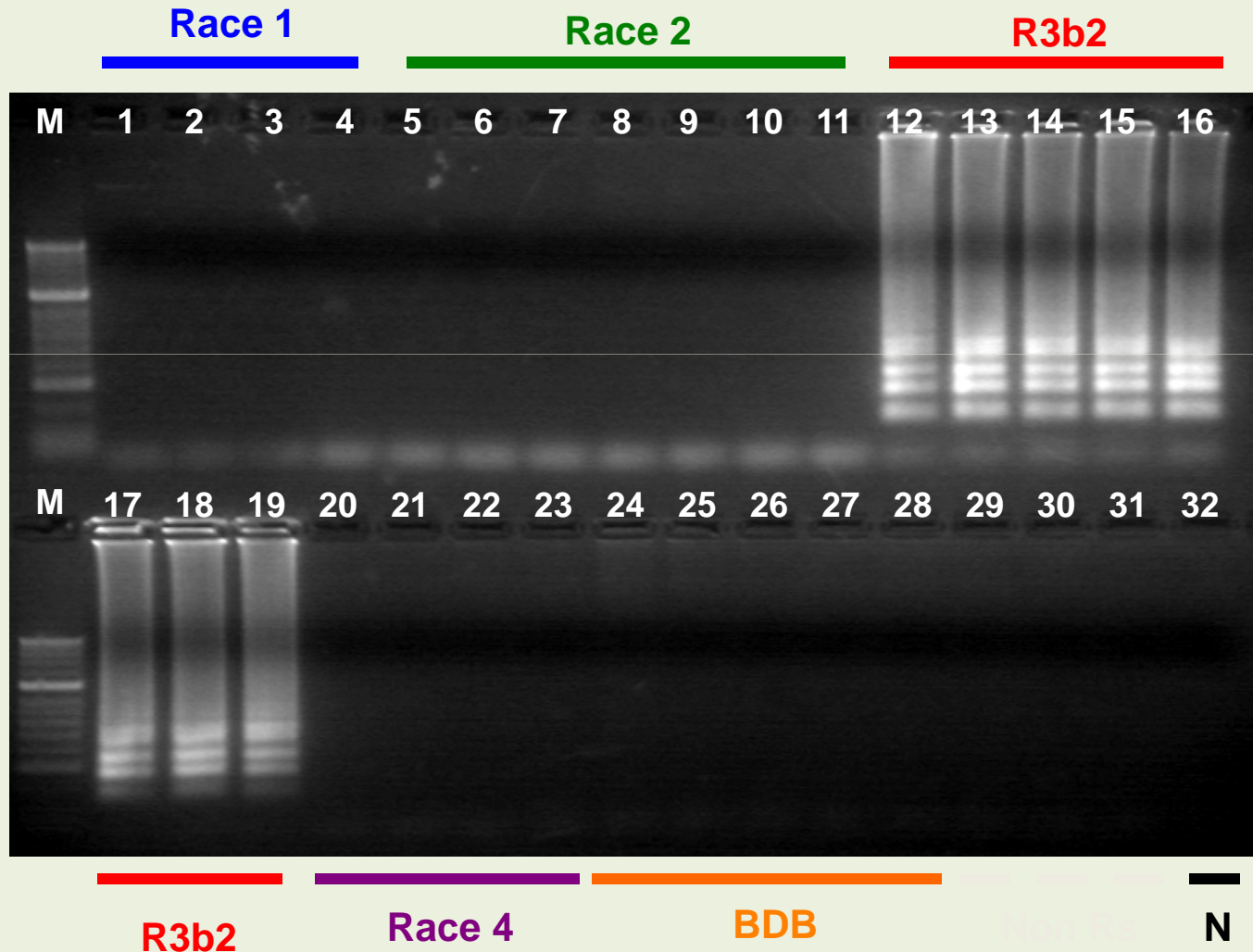
Rapid Visual Assessment

- Sensitive to PCR inhibitors

Coming diagnostic tools

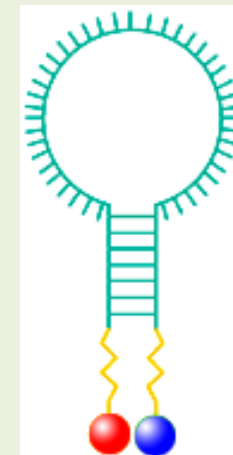
- ◆ LAMP: Loop-mediated isothermal AMPLification

Screening for R3b2-specific LAMP primers



- Designed from R3B2-specific ORFs

- Molecular Beacon technology



Single stranded hairpin shaped oligonucleotide

Coming diagnostic tools

- ◆ Next step for detection group

Optimizing and field-testing in Guatemala:

- LAMP
- IMS plus RT-PCR
- MCH plus RT-PCR



Known brown rot hotspots in potato-growing highlands



Commercial geranium production facilities

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Want to learn
more ?

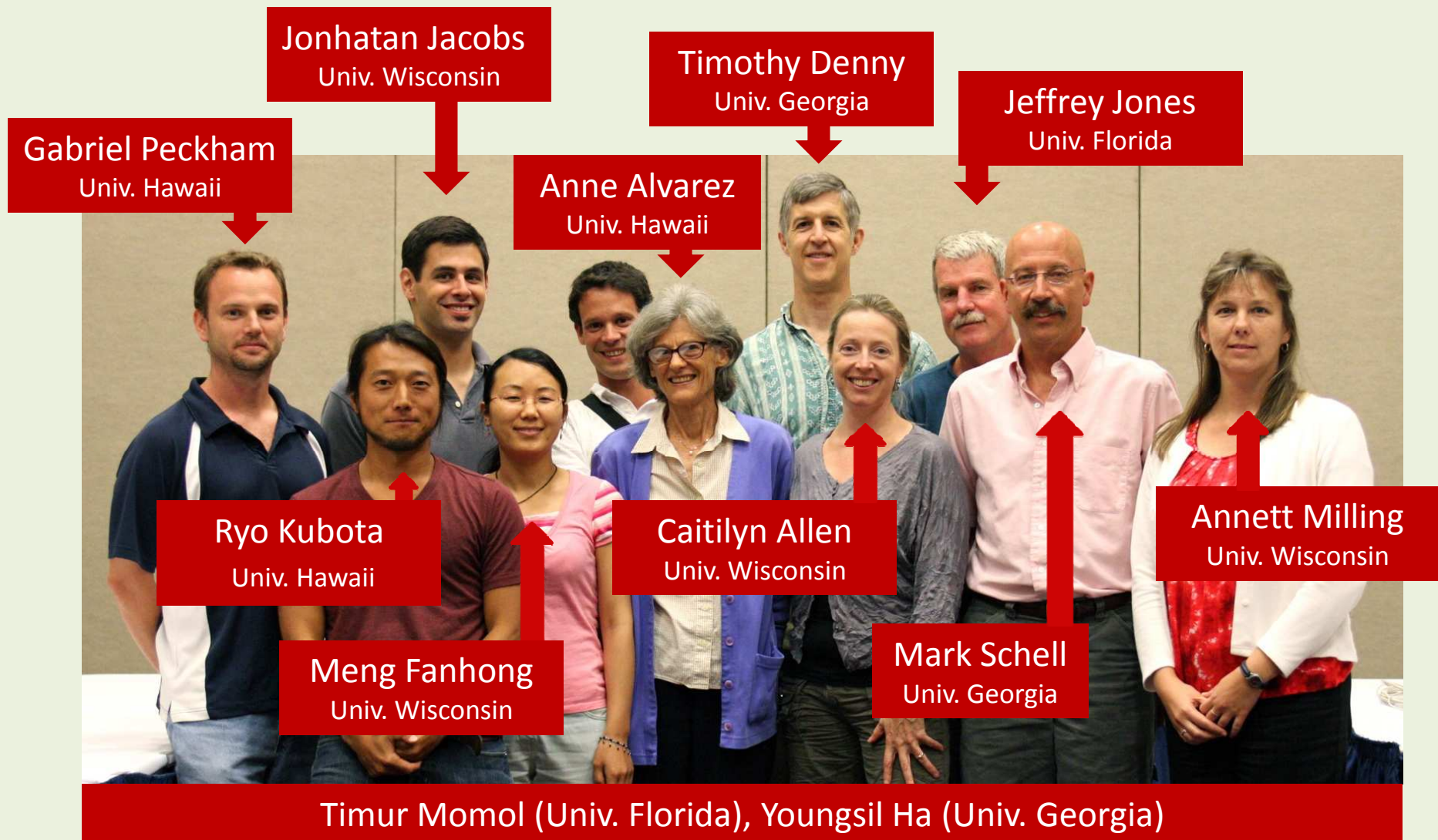
Educational resources

Ralstonia / Bacterial wilt dedicated website: <http://plantpath.ifas.ufl.edu/rsol/>

- ✓ Pest and disease management guides
- ✓ Project description, accomplishments
- ✓ Real time pest alerts and first reports worldwide
- ✓ Protocols, book references and journal articles database
- ✓ Web resources
- ✓ Photo galleries
- ✓ Access to Ralstonia-L mailing list

The screenshot shows the homepage of the Ralstonia solanacearum website. The header includes navigation tabs for 'Bacterial Wilt', 'Ralstonia solanacearum', and 'Brown Rot'. Below this is a secondary navigation bar with 'Research', 'Education', and 'Extension'. A main navigation bar contains 'Home', 'Pathogen and Diseases', 'Alerts', 'Publications', 'Web resources', and 'Photos'. The page is updated as of May 08, 2009, and features logos for the University of Florida, The University of Georgia, and the University of Wisconsin. A feedback survey prompt is visible, along with search bars for the site and Google. The main content area is divided into sections: 'USDA-NRI Project' (Ralstonia solanacearum race 3 biovar 2), 'Hot Topics' (including a Federal Register update, a 6-month post-launch report, management information, a new project publication, symposium abstracts, and an APS Journals update), and 'Recent Alerts - First Reports' (noting an outbreak in Portugal in August 2008). A sidebar on the right provides 'Quick site links' and 'Other site links'.

Acknowledgements



National Research Initiative (NRI) program of the USDA Cooperative State Research, Education, and Extension Services (CSREES).



Thank you for your attention.

