Development of an assay to detect *Xanthomonas oryzae* pathovars from rice seeds

– the application of a molecular tool in facilitating the global movement of "clean" rice seeds

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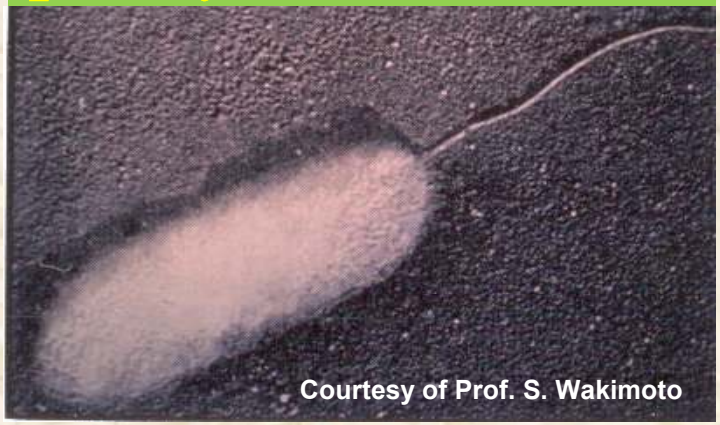
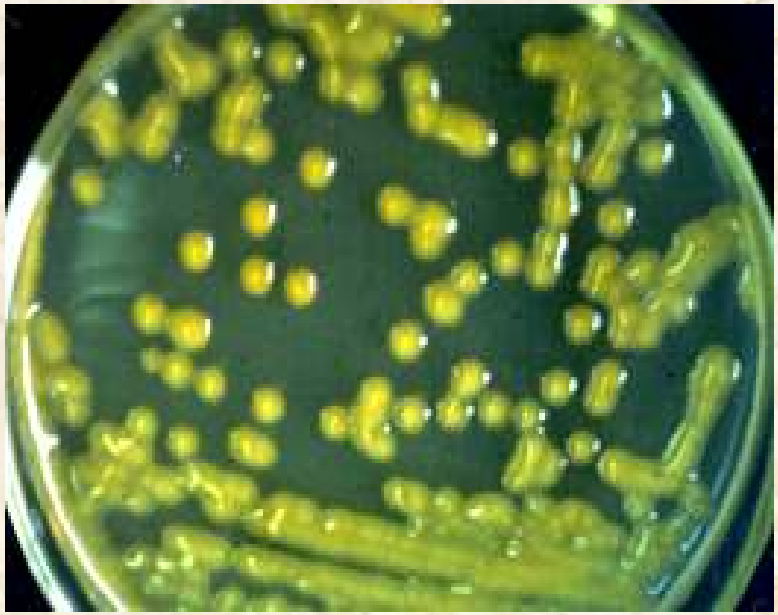
C.M. Vera Cruz, M.H.R. Nguyen, J. Lang, M.R.G. Burgos, B. Cottyn, V. Verdier, D. Mishra, Y. Raj, J.E. Leach

Bacterial Blight of Rice

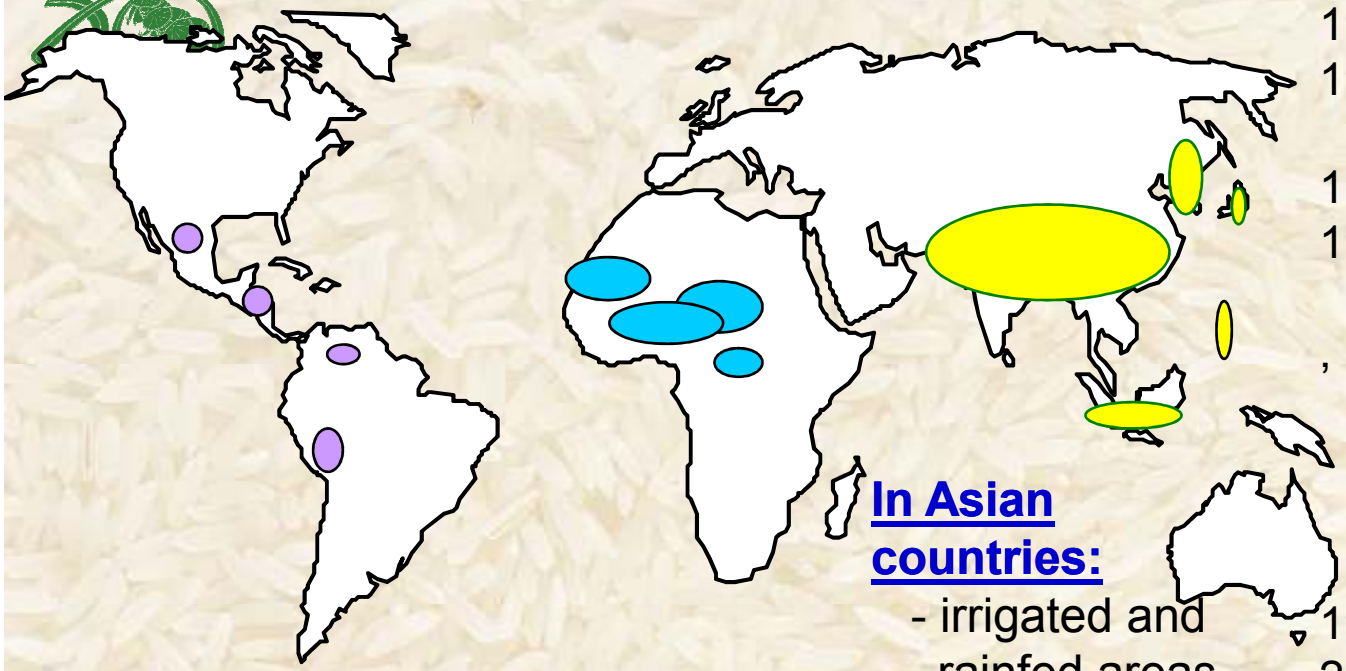
Xanthomonas oryzae pv. *oryzae* (*Xoo*)





- ✓ BB in main rice areas
- ✓ Population structure well known in Asia: races and lineages
- ✓ *Xoo* genomes sequenced (Korea, Japan, China, Phil@ISU, Africa)
- ✓ Oligoarray (UC Davis & ISU)
- ✓ >35 *R* genes identified, 6 cloned, defense genes
- ✓ BB near-isogenic lines (NILs) and pyramids (research tools or donors)

Global distribution



In Latin America:

1977: Colombia?, Venezuela, Panama, Costa Rica, Mexico
1979: Peru
1980: Bolivia
1999: Venezuela, Guarico 35-40% yield losses
2007: 50 to 80% losses

V. Verdier, IRD

In Asian countries:

- irrigated and rainfed areas of South and Southeast Asia

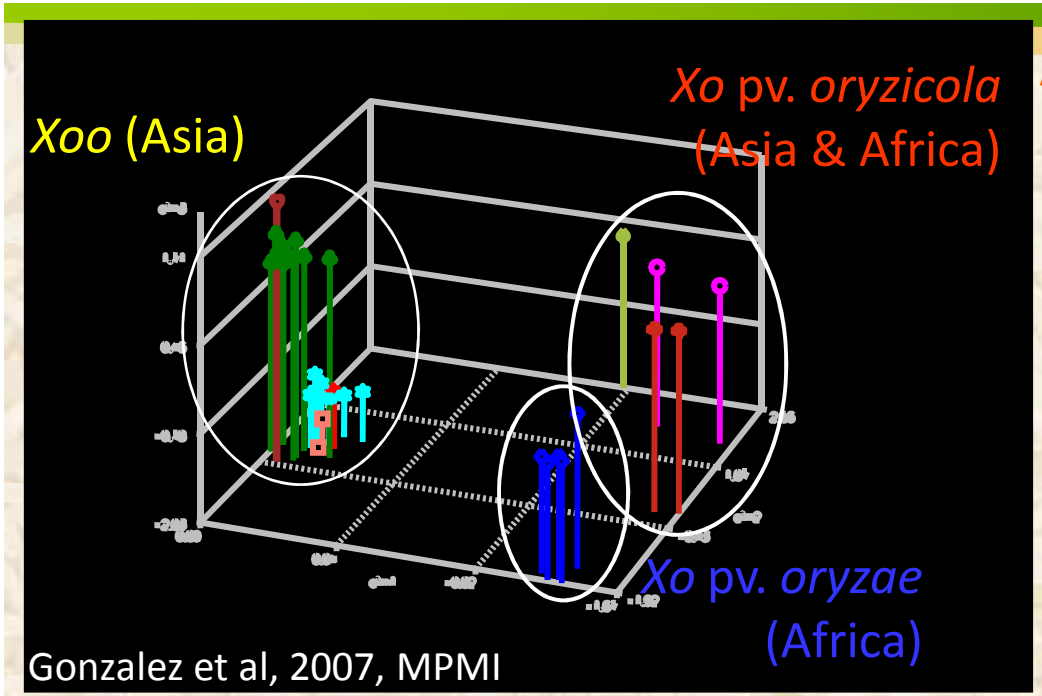
In Africa:

1979: Mali
1980: Sénégal
Cameroon
1983: Niger
1984: Gabon, Burkina, Mauritanie,Gambie
,
Burkina,,Guinée, Guinée Bissau, Ghana, Bénin Nigéria
1985: Madagascar ?
2003: Burkina, Mali, Niger
2012: South and East Africa

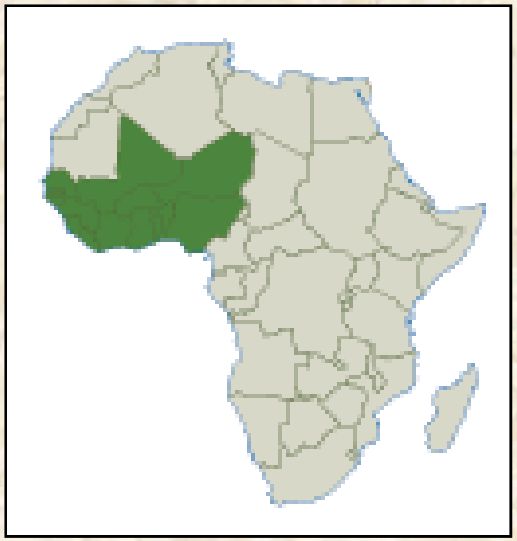
INERA, Burkina
IER, Mali
INRAN, Niger
Nissila, Bigirimana, et al.

Rice Science for a Better World

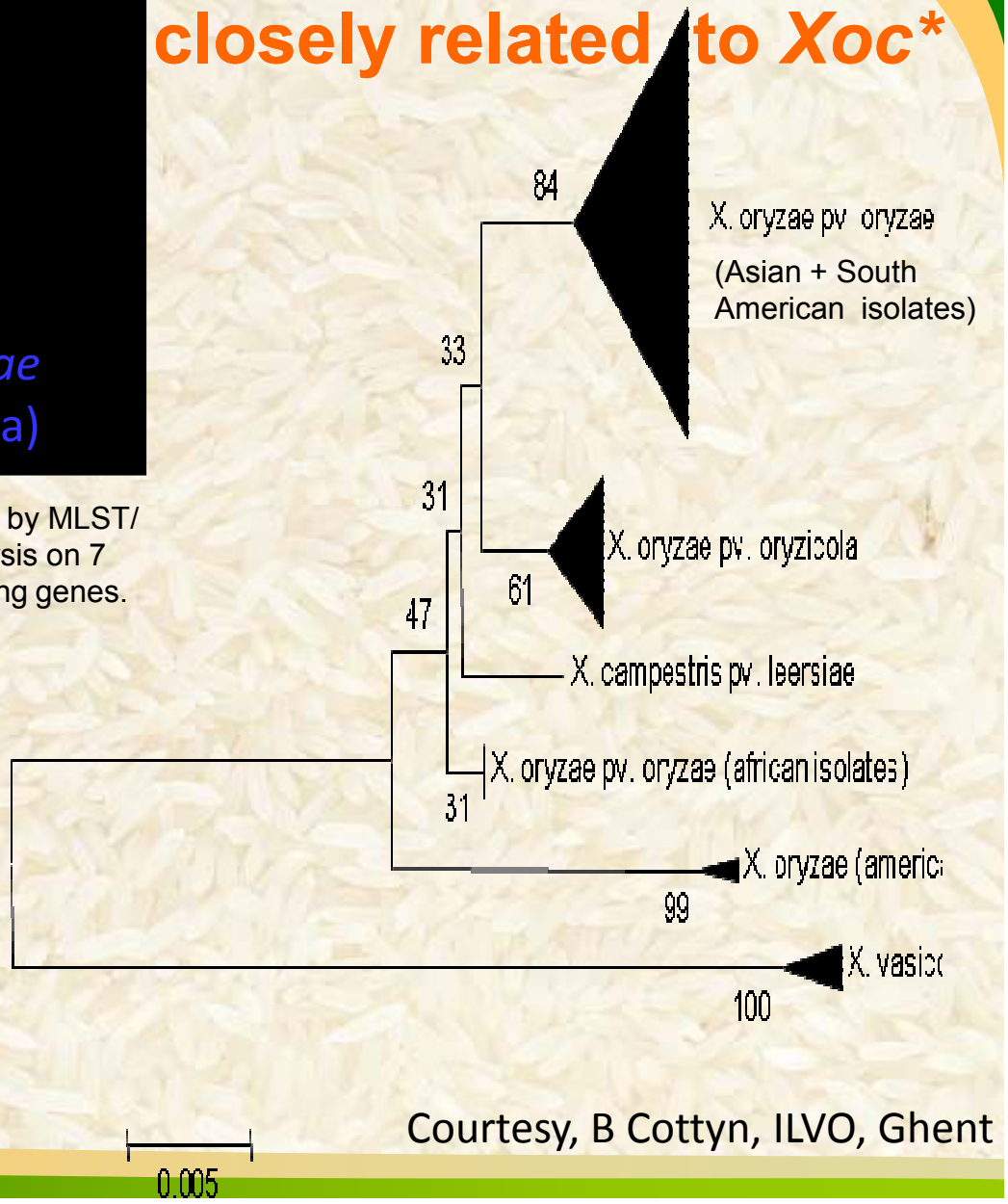
African *Xoo* strains are distant from Asian *Xoo*, closely related to *Xoc**



Gonzalez et al, 2007, MPMI



* Confirmed by MLST/ MLSA analysis on 7 housekeeping genes.



Courtesy, B Cottyn, ILVO, Ghent

Overview

- Increasing demand in agriculture and food production = increased worldwide movement of plant materials or products
- Increasing movement of plant materials also correlates to increased risk of introduction of threatening plant pathogens either intentionally or not.
- *Xanthomonas oryzae* pathovars *oryzae* and *oryzicola*, causal organisms of bacterial blight (BB) and bacterial leaf streak (BLS) are among regulated pathogens (select agents)
- Strict implementation of guidelines and protocols to prevent the spread of these bacterial pathogens
- However, there is still a need for a *standard assay* for these regulated pathogens, a universally accepted protocol for researchers and practitioners from regulatory bodies and quarantine laboratories

Overview

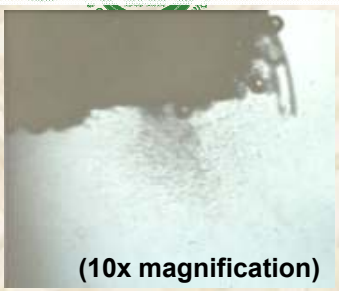
- Here, we are introducing a qualitative assay for the detection of *X. oryzae* pathovars from rice seeds
  - *direct assay for X. oryzae from seeds*
  - *combination of classical and molecular tools for the detection of X. oryzae pathovars from seeds*

- For detection of the pathogen, we use a set of highly specific diagnostic primers for multiplex PCR which can distinguish *X. oryzae* at the pathovar level

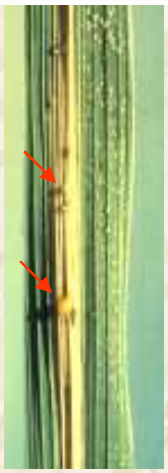
Control strategies

- **Exclusion of the pathogen from the host**
  - ***Quarantine and inspection****
  - ***Pathogen-free seed****
- Eradication or reduction of the inoculum
  - *Field sanitation*
  - *Raising seedbed nursery from inoculum source*
  - *Biological methods*
  - *Physical methods (e.g. heat treatment)*
- Improving resistance of the host
  - *Qualitative and quantitative resistance*
- Direct protection of plants from the pathogen
- Integrated control of plant diseases

Dissemination



Bacterial ooze from infected leaves



Wind-driven rain disseminates *Xoo* in short distances.

Sources of Inoculum

Alternate hosts:
Leersia oryzoides
Zizania latifolia

Xoo may survive in infected rice stubble or plant debris in or on soil as long as the host tissue is not decomposed.

Bacterial blight and bacterial leaf streak at mature crop stage

Note BB lesions with ooze at mature crop stage

Note BLS lesions with ooze

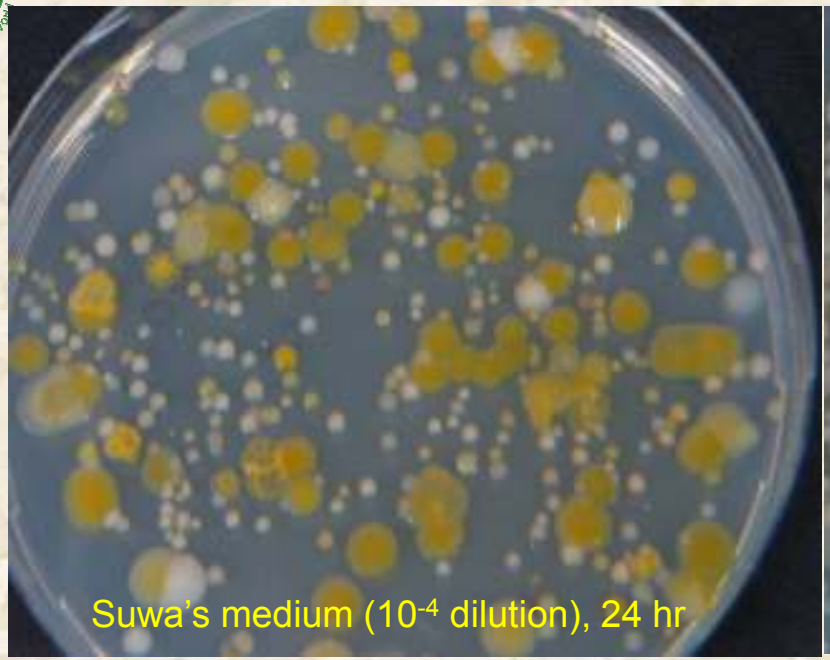
Rice hybrid Er You 838 carrying *Xa21* remained resistant over 3 years of its deployment in a BB hotspot

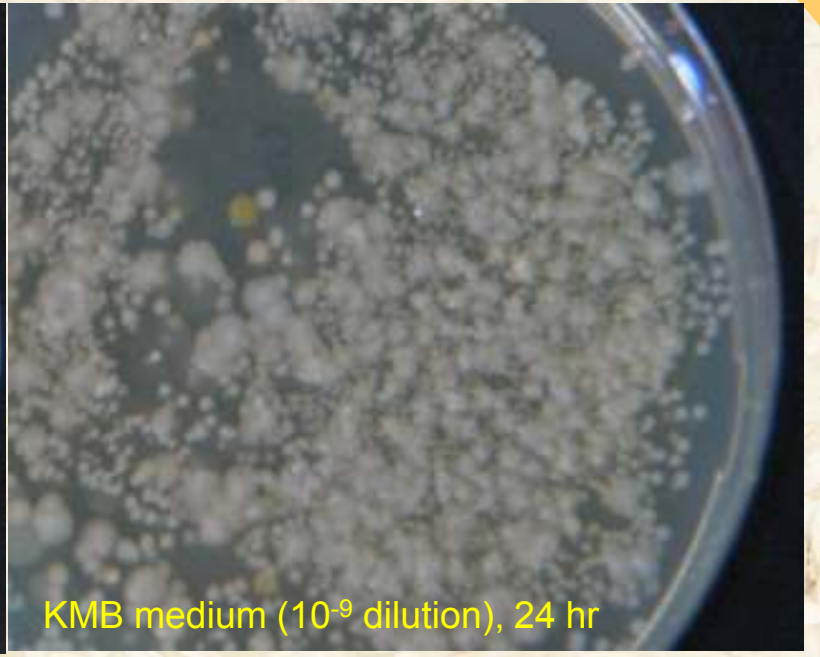
Rice hybrid Er You 838 (*Xa 21*) showed strong resistance to BB in field of Nam Dinh province, North VN, WS 2009

Hybrid ER You 838 *(Xa21)* highly resistant to BB in Thai Binh province, North VN, WS 2011

Photos courtesy of Dr. Duong Thanh Tai, SSJSC

Highly diverse natural microflora from seeds


Suwa's medium (10^{-4} dilution), 24 hr

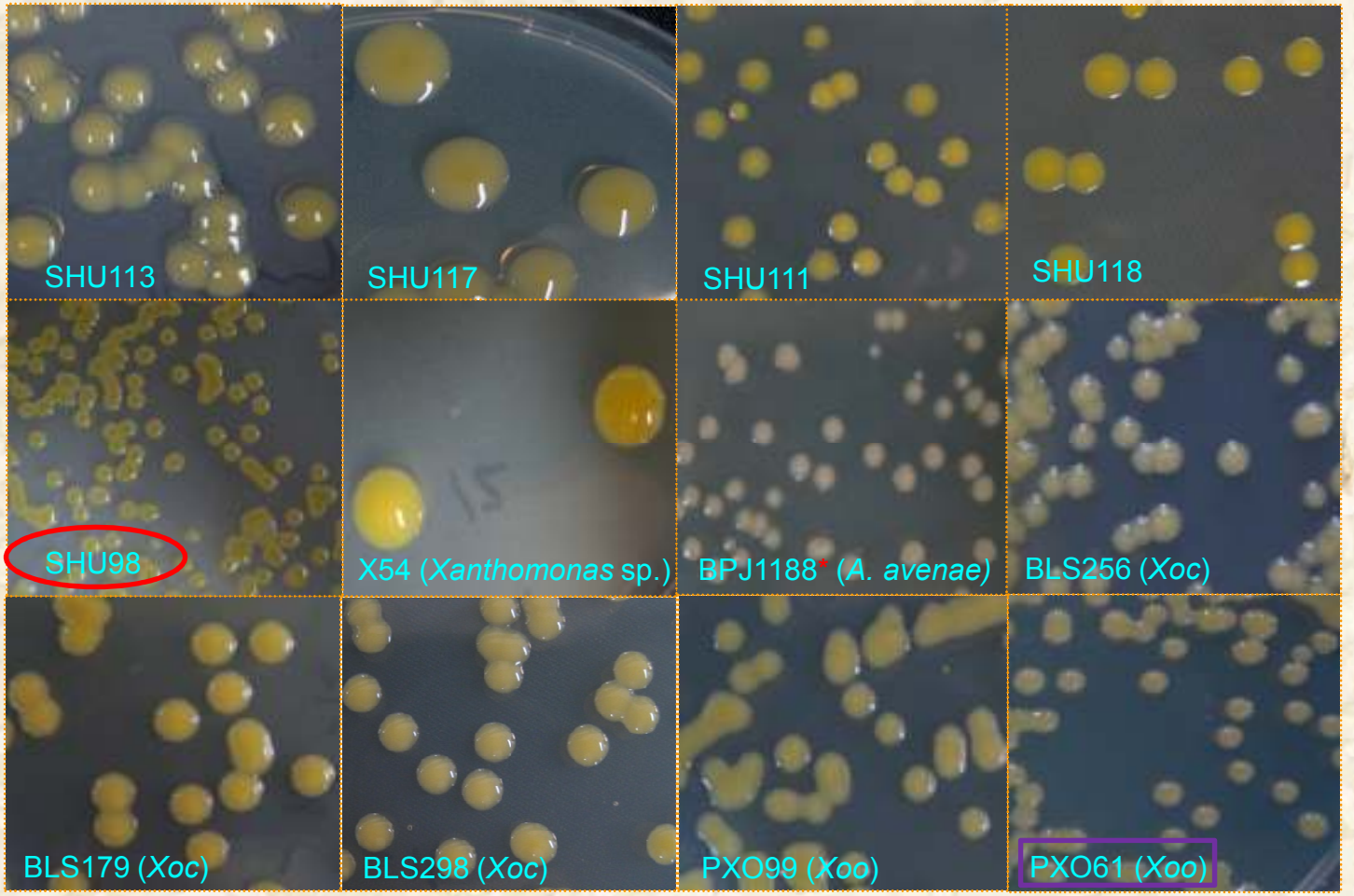

KMB medium (10^{-9} dilution), 24 hr

Isolation of *Xoo* from rice seeds is particularly difficult due to:

- the low population of the pathogen in the seed, if any
- its slow growth in agar medium
- its poor competitive ability relative to other seed-associated bacteria
- the presence of nonpathogenic xanthomonads showing colony appearances similar to those of *Xoo* frequently found in association with rice seeds

Hsieh et al, 1975; Cottyn et al., 2000

Yellow colonies from rice seeds in comparison with *X.o.* pathovars *oryzae* and *oryzicola*



*glistening yellowish gray

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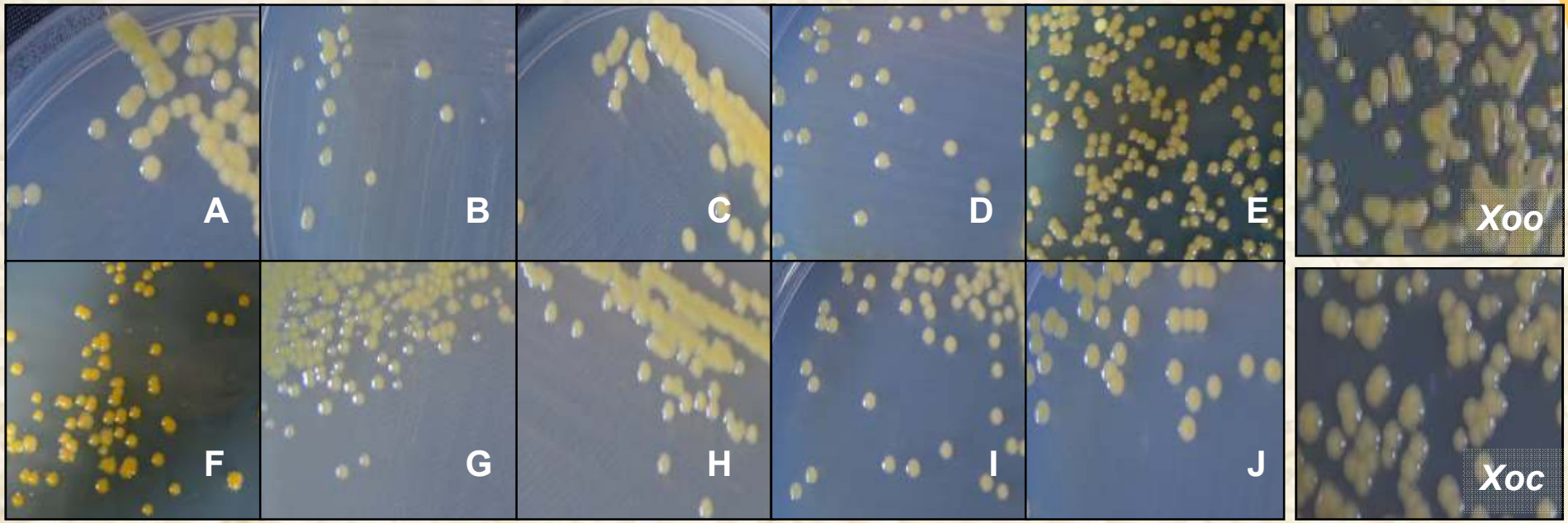
Yellow-colony isolates closely related to *Xoo*/*Xoc* among 277 seed isolates

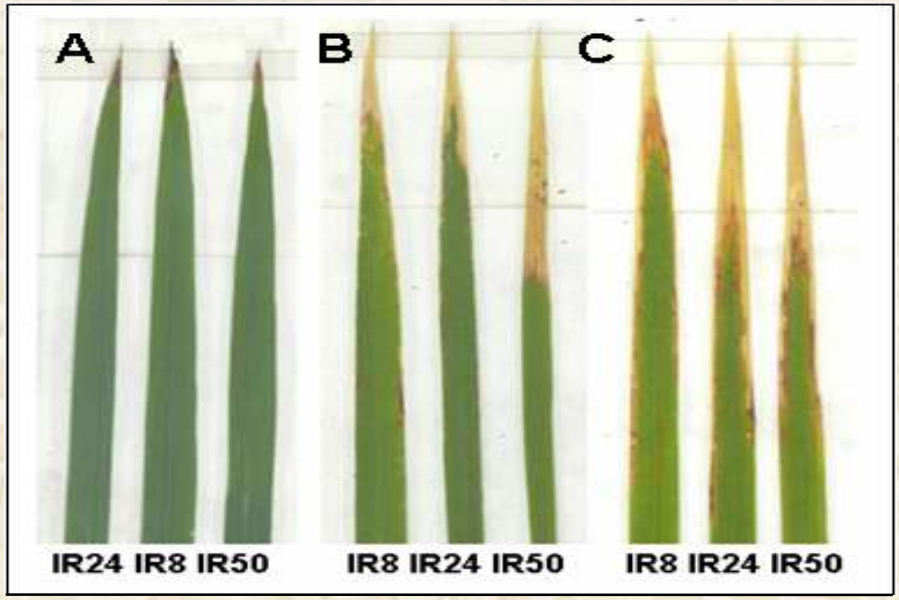


- **None of them clustered with *Xoo* and *Xoc* at the genotypic level (80%), and they are non-pathogenic, however at the morphological level, they are easily misidentified as *Xoo* or *Xoc*.**

- Using MLSA and 'dirty genome sequencing', these yellow *Xoo*-look alikes were identified in Belgium and USA as *Xanthomonas* sp. (Bart Cottyn, Jan E. Leach, 2011)

Pathogenicity of *Xoo/Xoc* look-alikes on susceptible rice

Spray-inoculated plants



Xoo look-alike (SHU83) | *Xoo* (PXO86) | *Xoc* (BLS256)

Clip-inoculated plants

Xoo look-alike (SHU83) | *Xoo* (PXO86) | *Xoc* (BLS256)

The SHU isolates produced negative results when inoculated into IR24, IR8, and IR50 plants.

Detection methods for plant pathogenic bacteria

- Immunodiagnosis
  - *Antigenic molecules from bacterial cell surfaces, react with specific antibodies*
  - *ELISA, flow cytometry*
- Genotypic approaches
  - *PCR, multiplex PCR*
- Integration of several methods for detection and identification
  - *Short culturing step preceding an immunodiagnostic or DNA-based assay*

www.cpgr.plantbiology.msu.edu

CPGR - Home Page - Mozilla Firefox

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http://cpgr.plantbiology.msu.edu/index.html

CPGR - Home Page

Comprehensive Phytopathogen Genomics Resource

Home | Resources | Diagnostics | BLAST | Download | Info

CPGR Overview

Transcript Assemblies(TAs):

Group	Species	TAs
Fungi	37	210,718
Nematode	15	66,891
Stramenopile	5	69,353
Total	57	346,962

For more information about the CPGR TAs, see the CPGR TA Overview

Genomes

- 21 Plant Pathogen Genomes
- 45 Annotated Molecules
- Genome Browser

rDNA Database

- 22,099 Plant Pathogen rDNA seqs
- 48,840 related rDNA seqs

Genome Warehouse

	Status			
Group	F	D	IP	EST
Bacteria	28	10	17	0
Fungi	5	10	11	19
Nematode	0	1	0	14
Stramenopile	0	4	2	5
Virus	623	0	0	0
Viroids	36	0	0	0
Total	692	25	20	38

Status: F - Finished, D - Draft, IP - In progress, EST - EST Project

Introduction to the Comprehensive Phytopathogen Genomics Resource

Welcome to the Comprehensive Phytopathogen Genomics Resource(CPGR). The CPGR aims to provide a comprehensive plant pathogen genomics and annotation resource. A major part of the CPGR is the development of diagnostic molecular markers and robust diagnostic protocols for plant pathogens. To keep informed of developments on the CPGR site, please sign up on the CPGR mailing list.

Comprehensive Plant Pathogen Genomic Warehouse

The Comprehensive Plant Pathogen Genomic Warehouse is a database of finished, draft and in progress genome sequencing projects and EST projects for viral, bacterial, stramenopile, fungal, and nematode plant pathogens. It will be updated on a frequent basis and and will be integrated into the main CPGR annotation database and comparative analysis database serving as a portal for the database.

Plant Pathogen Ribosomal DNA (rDNA) Database

The CPGR Plant Pathogen Ribosomal DNA (rDNA) Database contains all the ribosomal DNA in GenBank for Bacterial, Stramenopile, Nematode and Fungal plant pathogens. The rDNA sequences for other species in the genus of each pathogen are also stored in the database. The web interface for the rDNA database allows searching to find the genera and species of interest, selection between nuclear and mitochondrial rDNA for eukaryotes, display of the defintion lines of the rDNA sequences to allow fine control over selecting the sequences, and the choice displaying the sequence in the browser or sequence file download.

Plant Pathogen Transcript Assemblies

An objective of the CPGR is the clustering and assembling of ESTs generated from plant pathogen EST projects. For this project, we have developed an automated EST clustering and assembly pipeline based on the tgicl package. The clustering and assembly process forms sets of unique transcipts or transcript assemblies. Basic search, report, flat file data and a plant pathogen BLAST server are now available and we will implementing enhanced annotation of the transcript assemblies and search tools in the near future.

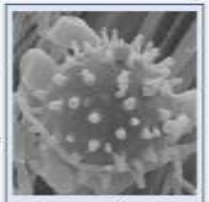
Under Development

CPGR web site: The Genomes section is being completed, and new pages are being added daily.

Diagnostics: Work on the *Xanthomonas Oryzae* pathovars is being completed and will be available soon. Work on Pythium diagnostic markers is starting soon

CPGR News

- CPGR Warehouse Updated - April 2008 - 4 new finished genomes
- CPGR Warehouse Updated - March 2008
- CPGR Workshop at CSU, Feb 2008 - Lecture Slides and Tutorials Available
- The CPGR Transcript Assemblies have been updated! 8 new species added, 4 existing TAs updated.
- Sign up for CPGR mailing list to recieve updates by email



An oospore of *Pythium jasmonium*.

Marker development from *Xoo* and *Xoc* genome sequences

http://cpgr.plantbiology.msu.edu/index.html

Align available locus sequences for *X. oryzae pv. oryzae* and *X. oryzae pv. oryzicola* to identify conserved and divergent loci

Synthesize primer pairs that computationally distinguish *X. oryzae pv. oryzae* and *X. oryzae pv. oryzicola* from each other and from other bacteria

Validate primers by ePCR and BLAST searches against all sequenced *Xanthomonas* genomes

Evaluate primer pairs against a subset of *X. oryzae pv. oryzae* and *X. oryzae pv. oryzicola* strains for specificity and robustness of amplification

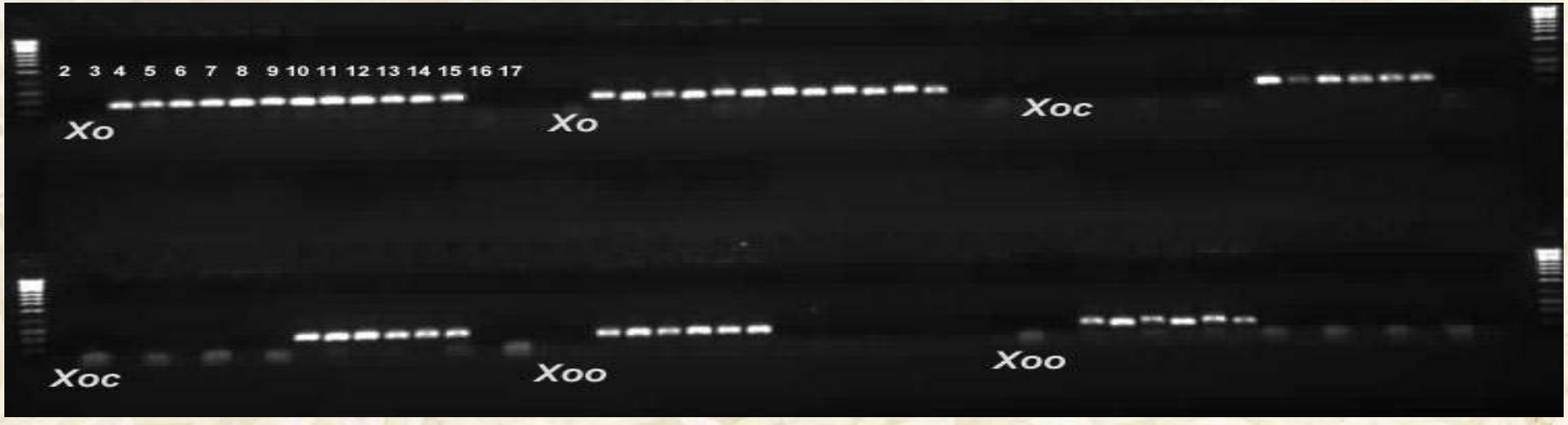
Screen advanced sets of primers against a geographically and genetically diverse collection of *X. oryzae pv. oryzae* and *X. oryzae pv. oryzicola* strains and other phytopathogenic bacteria

Redesign most robust primer pairs into varying sized amplicons for a multiplex PCR to distinguish *X. oryzae pv. oryzae* from *X. oryzae pv. oryzicola* and other bacteria in one reaction

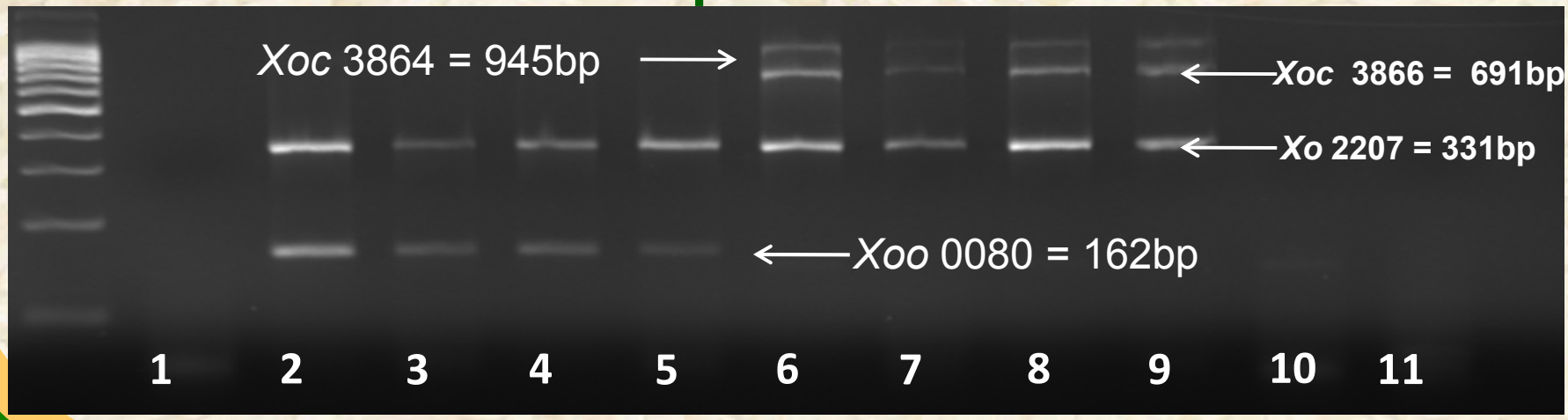
Lang et al, 2010

Detection Methods: Single and multiplex primers

Conventional PCR



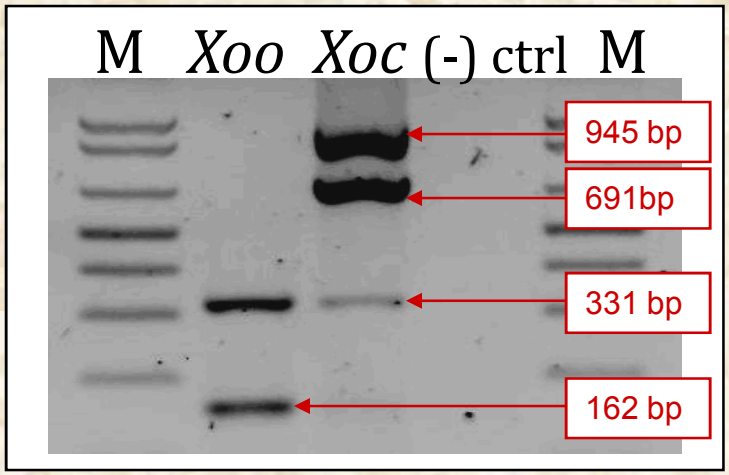
Multiplex PCR



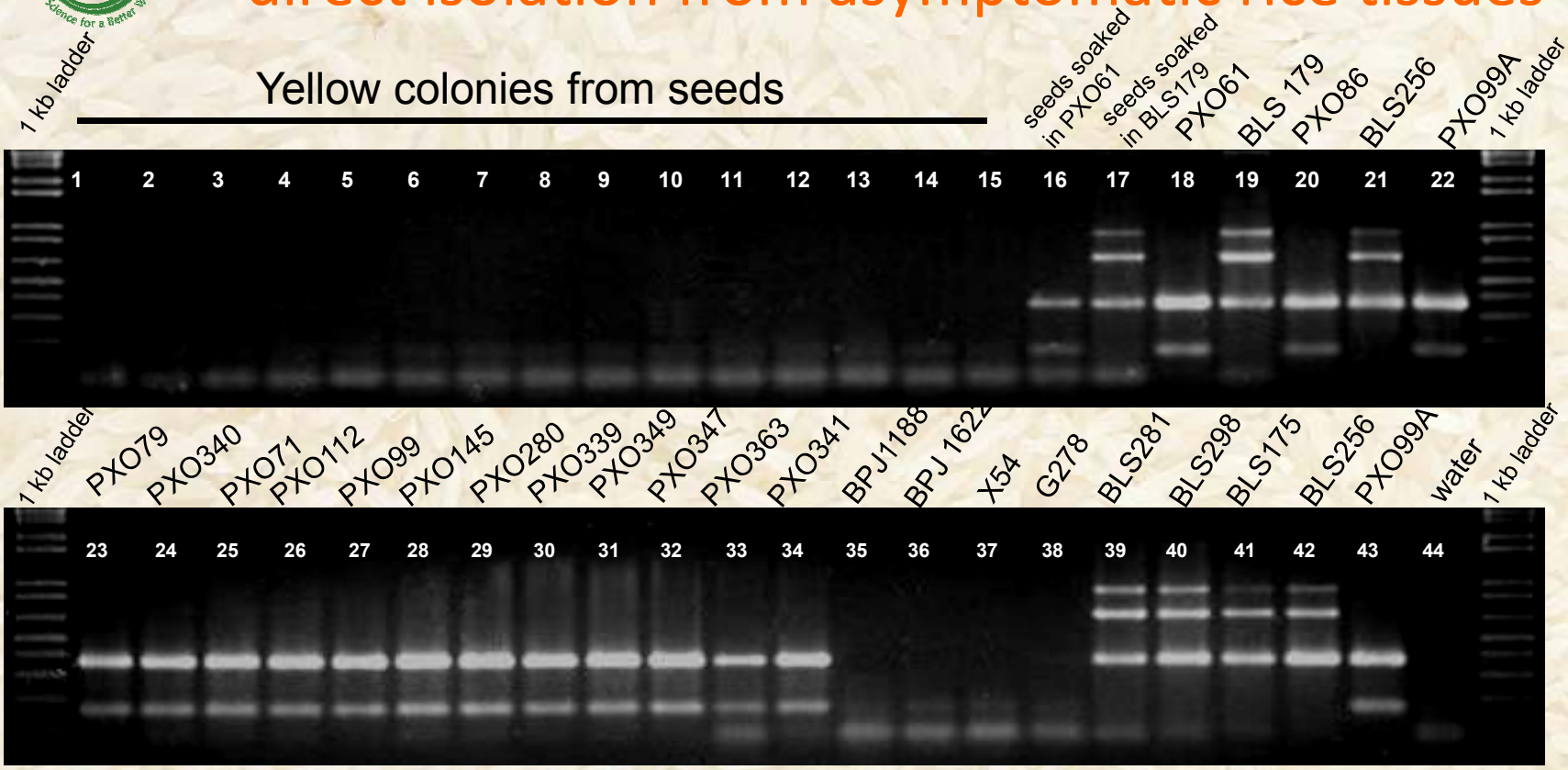
Lang et al, 2010

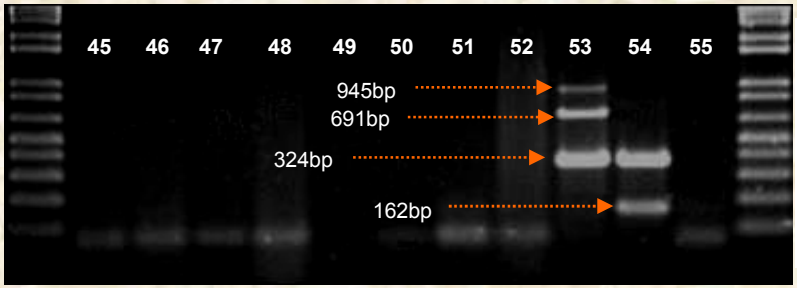
Diagnostic primers for *Xoo* and *Xoc*

Target	Name	Sequence (5'-3')	Product size (bp)
X. oryzae	Xo3756F	CATCGTTAGGACTGCCAGAAG	331
	Xo3756R	GTGAGAACCACCGCCATCT	
X. oryzae pv. *oryzae*	Xoo281-8F	GCCGCTAGGAATGAGCAAT	162
	Xoo281-8R	GCGTCCTCGTCTAAGCGATA	
X. oryzae pv. *oryzicola*	Xoc3866F	ATCTCCCAGCATGTTGATCG	691
	Xoc3866R	GCGTTCAATCTCCTCCATGT	
	Xoc3864F	GTGCGTGAAAATGTCGGTTA	945
	Xoc3864R	GGGATGGATGAATACGGATG	



Multiplex PCR of yellow colonies from rice seeds and direct isolation from asymptomatic rice tissues





(45) asymptomatic BB-filled grains 2 h, (46) asymptomatic BB-filled grains 24 h, (47) aBB-unfilled grains 2 h, (48) aBB-unfilled grains 24 h, (49) white colony isolate - filled seeds 2h, (50) white colony isolate - filled seeds 24 h, (51) white colony isolate from sheath 2 h, (52) white colony isolate from sheath 24 h, (53) BLS256, (54) PXO99A, (55) water.

Lang et al 2010

Assay development

- Target users
- Sampling protocol
- Detection approach
- Sample preparation
- Specificity
- Reliability
- Limit of detection
- Controls
- Validation

Photos courtesy of Dr. E. Roumen

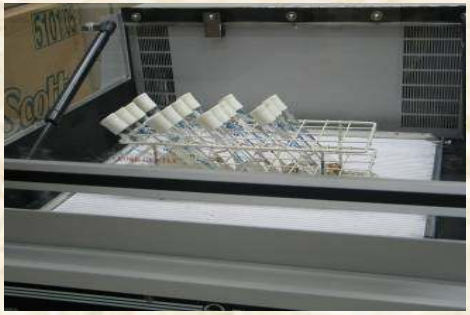
Extraction and isolation of bacteria from seeds

Pre-washed seeds (5 g each sample) under running water (30-60 min)

Seeds (5 g) in sterile mortar and pestle

Place 10 ml PBS (pH7.4) with 0.025% Tween 20 per sample

Macerate the seeds in 10 ml PBS



Shake samples for 2 h or 24 h (for enrichment using 10 ml extract in 900 ml NB) at 130 rpm

Isolation using serial dilution

Purification

Identification

Pathogenicity test

Direct PCR

Comparing sonication and vortexing of samples for recovery of bacterial cells from rice seeds suspended in PBS

Soak seeds in inoculum
(PXO 99, PXO 339, BLS 256, BLS 157)

Dry seeds in laminar flowhood

Vortex

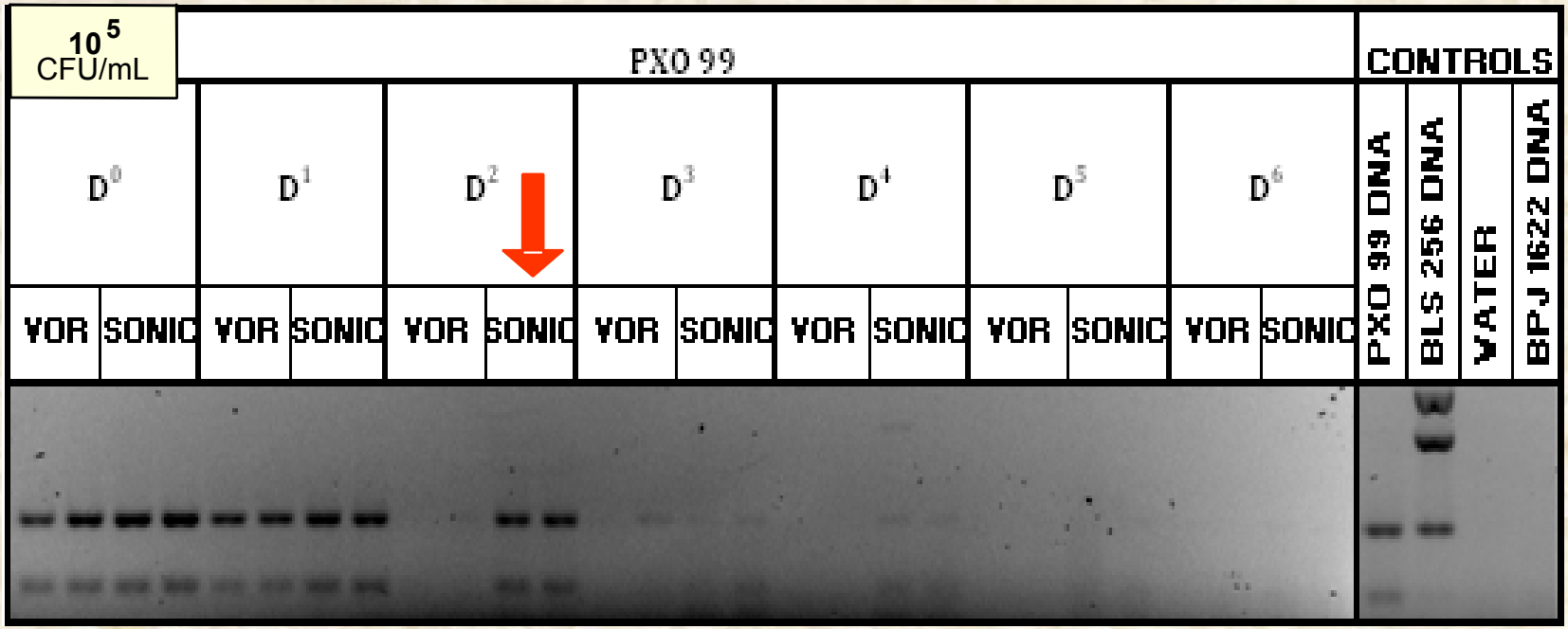
Perform Direct Assay

Sonicate

Serial dilution and plating

Multiplex PCR

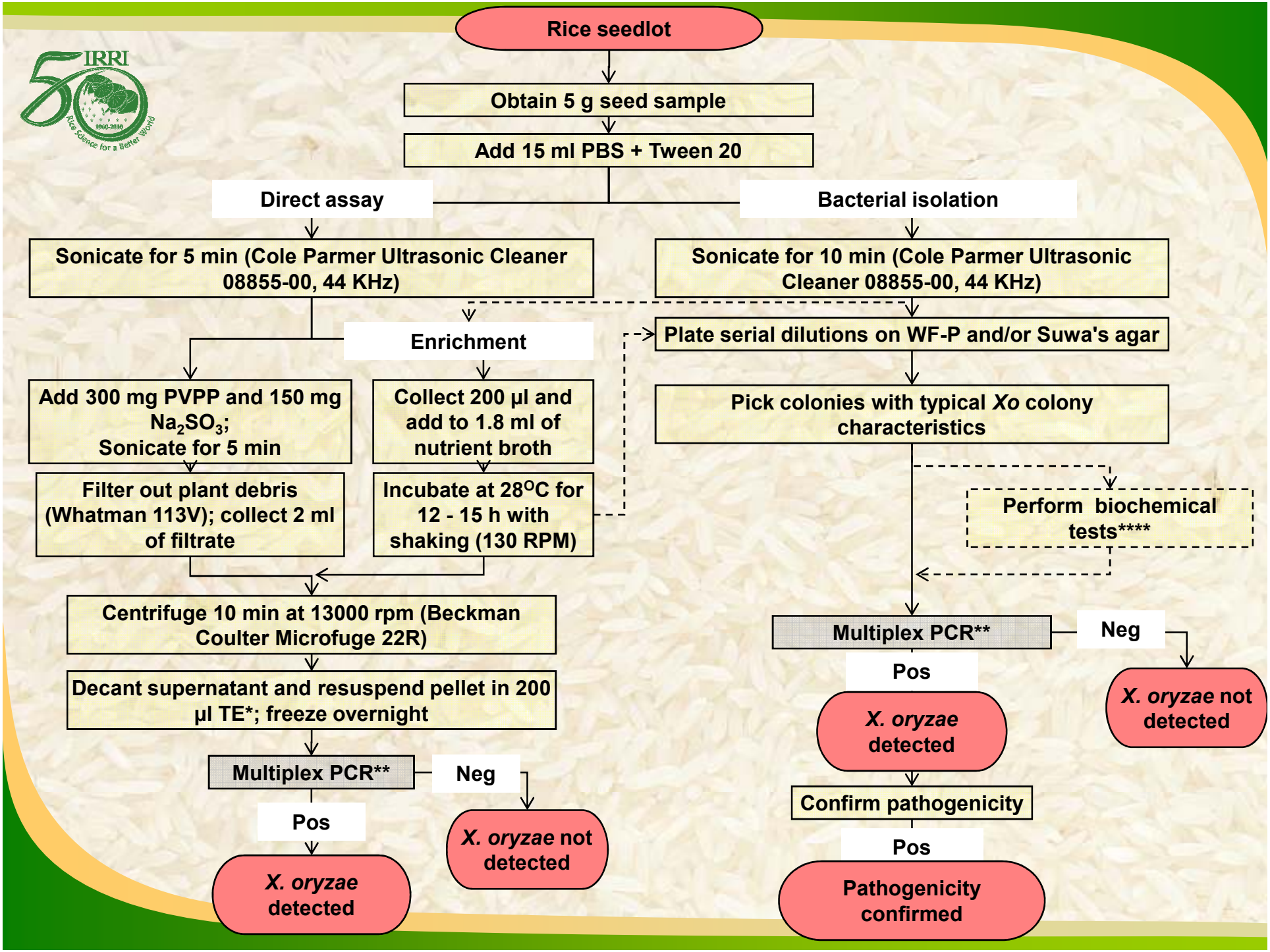
Comparison using sonication and vortexing for dislodging bacterial cells from seed samples



✓ The use of sonication (10 min) for dislodging bacterial cells from seeds resulted in a tenfold increase in assay **sensitivity** than vortexing (10 min).

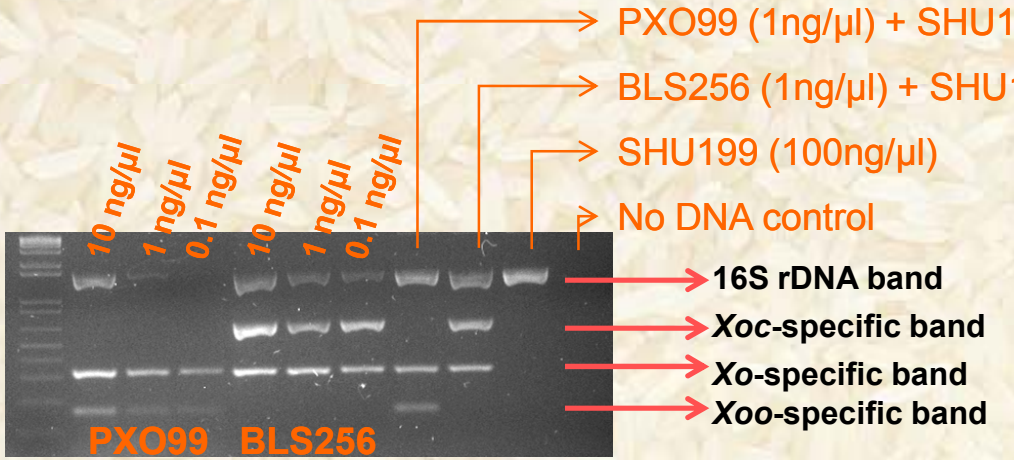
✓ Based on cell count, there was no difference between the number of cells dislodged by sonication and vortexing.

✓ Sonication allows high-throughput sample analysis → **convenience and efficiency**


Rice seedlot
Obtain 5 g seed sample
Add 15 ml PBS + Tween 20
Direct assay
Bacterial isolation
Sonicate for 5 min (Cole Parmer Ultrasonic Cleaner 08855-00, 44 KHz)
Sonicate for 10 min (Cole Parmer Ultrasonic Cleaner 08855-00, 44 KHz)
Enrichment
Add 300 mg PVPP and 150 mg Na_2SO_3; Sonicate for 5 min
Collect 200 µl and add to 1.8 ml of nutrient broth
Filter out plant debris (Whatman 113V); collect 2 ml of filtrate
Incubate at 28°C for 12 - 15 h with shaking (130 RPM)
Centrifuge 10 min at 13000 rpm (Beckman Coulter Microfuge 22R)
Decant supernatant and resuspend pellet in 200 µl TE*; freeze overnight
Multiplex PCR**
Neg
Pos
X. oryzae not detected
X. oryzae detected
Plate serial dilutions on WF-P and/or Suwa's agar
Pick colonies with typical Xo colony characteristics
Perform biochemical tests****
Multiplex PCR**
Neg
Pos
X. oryzae not detected
X. oryzae detected
Confirm pathogenicity
Pos
Pathogenicity confirmed

Addition of 16S ribosomal DNA primers to the multiplex primer set

- **As internal control**
  - ***Indicator of PCR assay efficiency and sample quality***
- **16S rDNA replaced 945 bp *Xoc* fragment**
- **Target sequences (*Xo*, *Xoo*, *Xoc*) and intrinsic DNA are simultaneously detected**



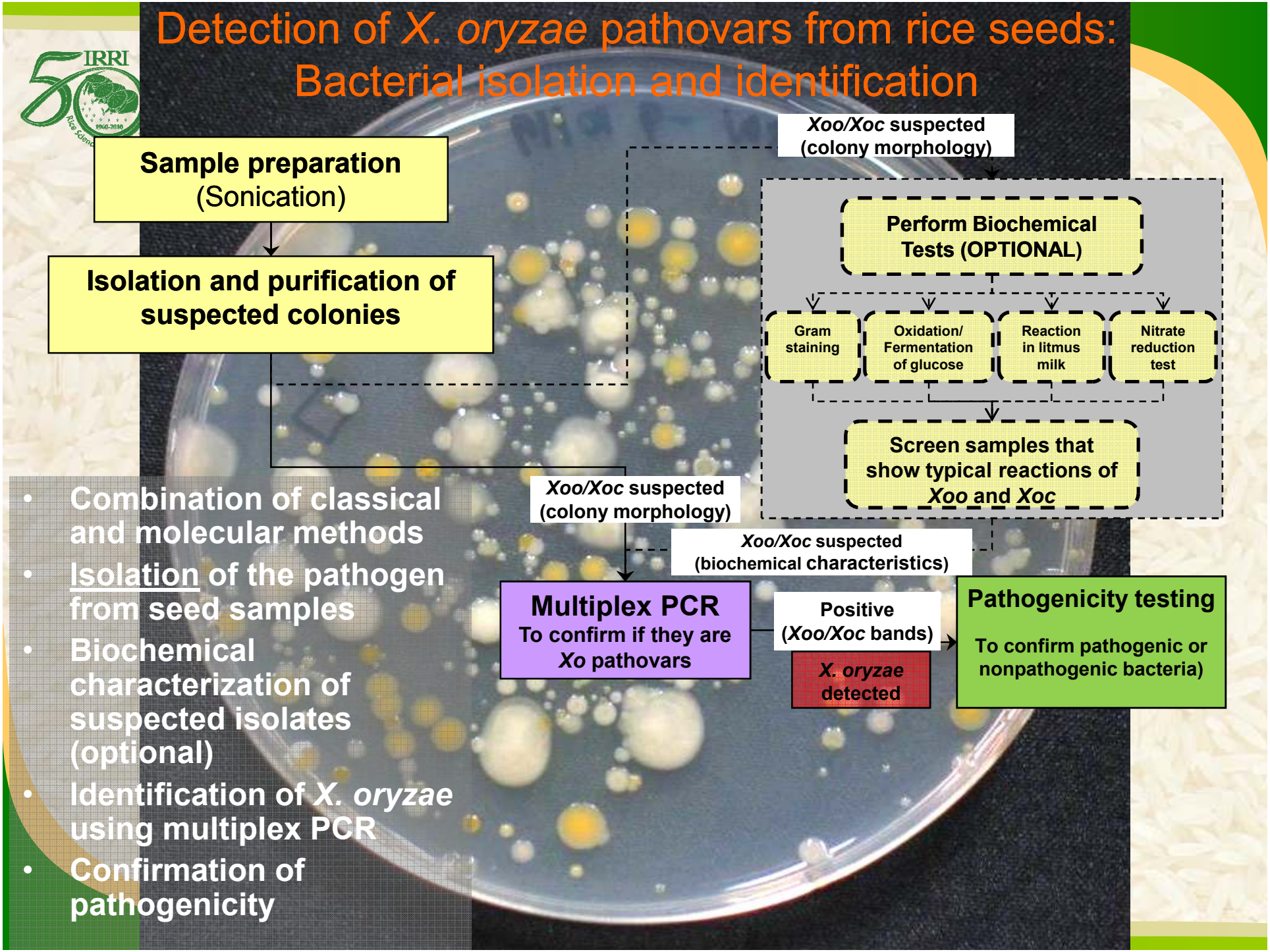
Training-Workshop on Harmonizing detection of *Xanthomonas oryzae* pathovars

- ✓ Validated and refined the latest protocols in detection of *Xanthomonas oryzae* pathovars (X. *oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*)
- ✓ Twenty three (23) participants from 14 countries agreed and harmonized the use and application of a robust and inexpensive diagnostic tools for identification and classification of *Xanthomonas oryzae* pathovars

Samples tested for the presence of *Xoo* and *Xoc* using the direct assay

Sample #	Sample Code	*Xoo*	*Xoc*	Run date
1	8	+	-	11032010
2	AC-1 1	+	+	
3	AC-14 61	+	-	
4	AC-1 1	+	+	11182010
5	AC-9 9	+	-	
6	8	+	-	
7	90	+	+	
8	111	+	+	
9	614	+	-	
10	#1	+	+	
11	#2	+	-	
12	#3	+	+	
13	AC-3 58	+	-	09032010
14	AC-17 59	-	+	
15	AC-11 46	+	+	
16	AC-18 62	+	-	
17	AC-23 60	-	+	
18	AC-10 56	-	+	09222010
19	AC-9 47	+	-	
20	AC-17 59	-	+	
21	AC-33 126	-	+	09302010
22	AC-7 48	-	+	
23	AC-33 83	-	+	
24	AC-11 46	-	+	
25	AC-11 70	+	-	
26	AC-30 123	+	-	

Detection of *X. oryzae* pathovars from rice seeds: Bacterial isolation and identification



- Combination of classical and molecular methods
- Isolation of the pathogen from seed samples
- Biochemical characterization of suspected isolates (optional)
- Identification of *X. oryzae* using multiplex PCR
- Confirmation of pathogenicity

Summary: Detection protocol for *X. oryzae* pathovars from rice seeds

- **Qualitative assay** for the detection of *X. oryzae* pathovars from rice seeds

- Direct assay:
  - *enables the **rapid** detection of these pathovars from rice seeds, no need to plate and isolate the bacteria*
  - *data available on the same day (within 8 hrs, from sampling to PCR results, and 24 hrs with enrichment step)*
- Combination of classical and molecular methods – **isolation** of the pathogen from seeds, optional biochemical characterization of suspected isolates, determining if they are indeed *X. oryzae* using **multiplex PCR**, and confirming their pathogenicity by **inoculation into susceptible plants**
- Other application: population biology and epidemiology

Key issues remaining …

- Acceptability of the assay for wide adoption in progress
- Viability of *Xoo/Xoc* diagnostic kit as a product that can sustain itself

Thank you!

CGIAR Research Program on Rice
Global Rice Science Partnership

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