Polyclonal Antiserum Specificity against *Leifsonia xyli* subsp. *Xyli*, Causal Agent of Sugarcane Ratoon Stunting Disease

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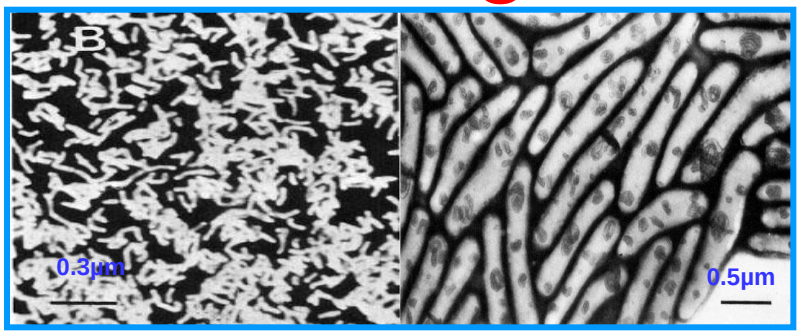
Ratoon Stunting Disease (RSD)

- **RSD is one of the most important diseases affecting sugarcane production worldwide.**
- **RSD produces no easily visible symptoms other than stunting, especially in ratoon crops.**
- **RSD causes yield losses from 10-60% depending on the susceptibility of sugarcane variety and weather conditions.**



Sugarcane Stunting Disease (RSD)

Pathogen



Vector



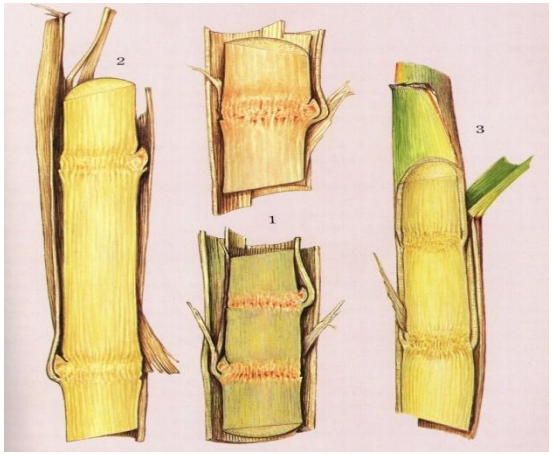
Host

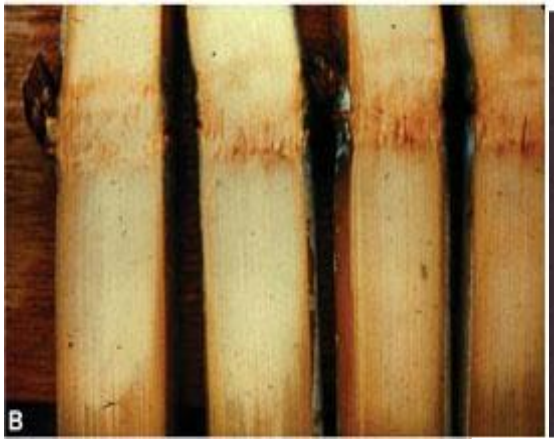


RSD Diagnosis

1. Observation (heavily infected)

RSD causes no obvious external symptoms other than stunting, involving reduced vigor and tillering. However, such stunting growth may also result from other stress. Internal symptoms in heavily infected RSD include a salmon pink discoloration just below the growing point of young canes and an orange-red discoloration of the nodal vascular bundles in mature stalks, but these symptoms are unreliable as they vary within and among cultivars.



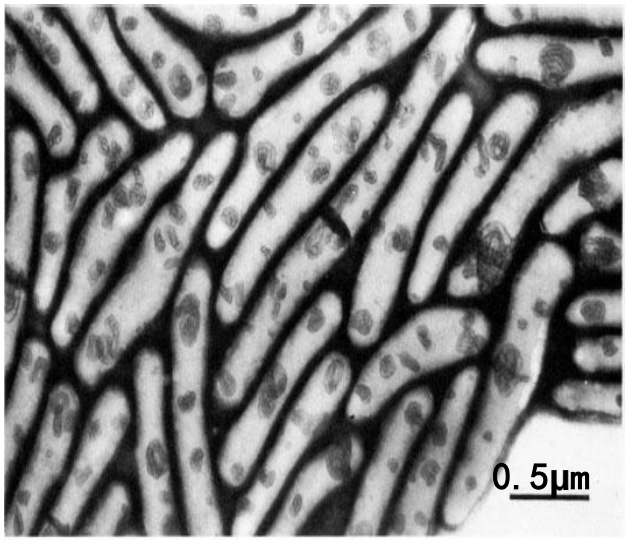




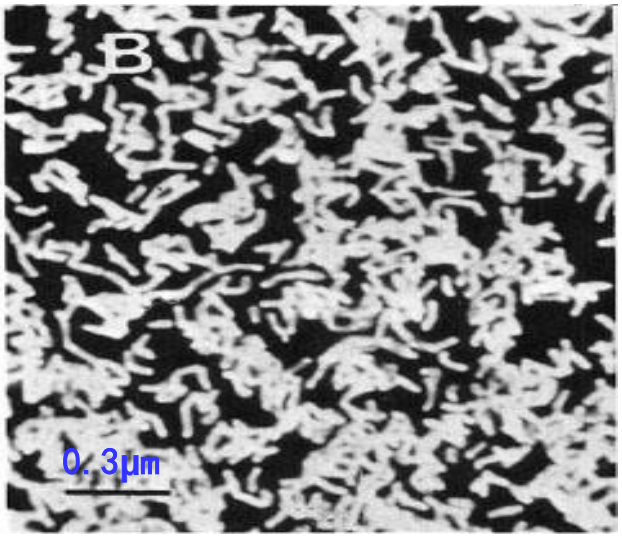
ised by RSD

healthy infected healthy **healthy infected** **infected**

2. Light microscopy technique



Electron microscopy

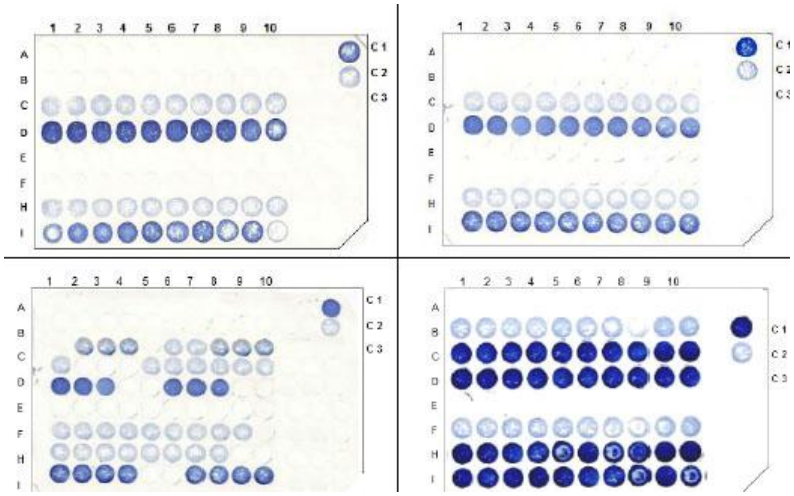


Dark-field microscopy

Lxx is a gram-positive, coryneform, non-motile, and obligatory aerobic bacterium. Bacteria cells are pleomorphic rods of 0.25-0.5 µm by 1-4 µm with straight or slightly curve shapes. They infect the xylem vessels of the sugarcane plant.

3. Serological Test

The serological assays include dot blot, evaporative-binding enzyme immunoassay (EB-EIA) and tissue blot immunoassay (TBIA). In these assays, ***antibody*** specific to the bacterium is combined with other ingredients to give a colour reaction when bacteria are present.





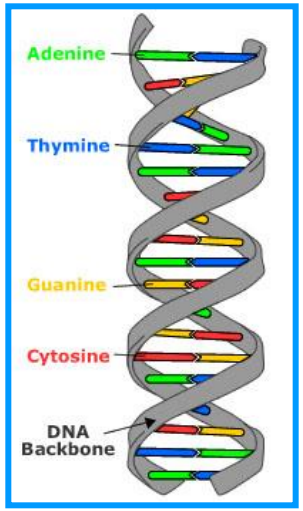
Detect Lxx populations of 10^5 cells/ml

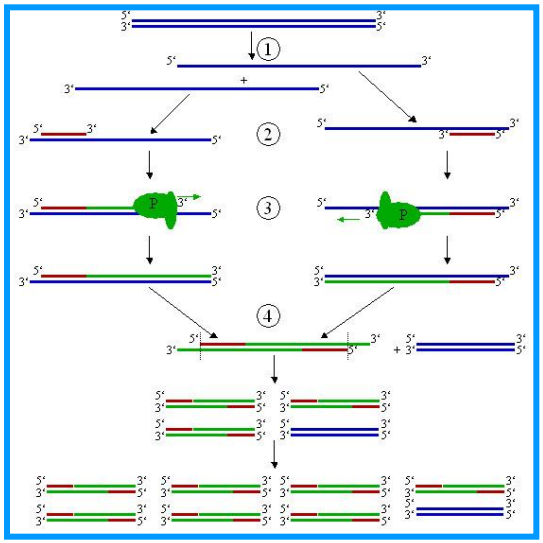
4. Polymerase Chain Reaction (PCR)

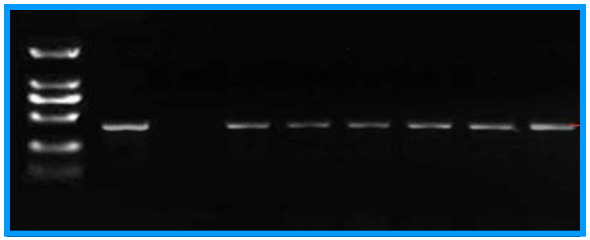
PCR-based test is the most powerful method as it can detect as few as 1-10 bacteria. It was developed by ***Dr. Pan et al.*** (1998)



Real Time PCR has been developed recently.





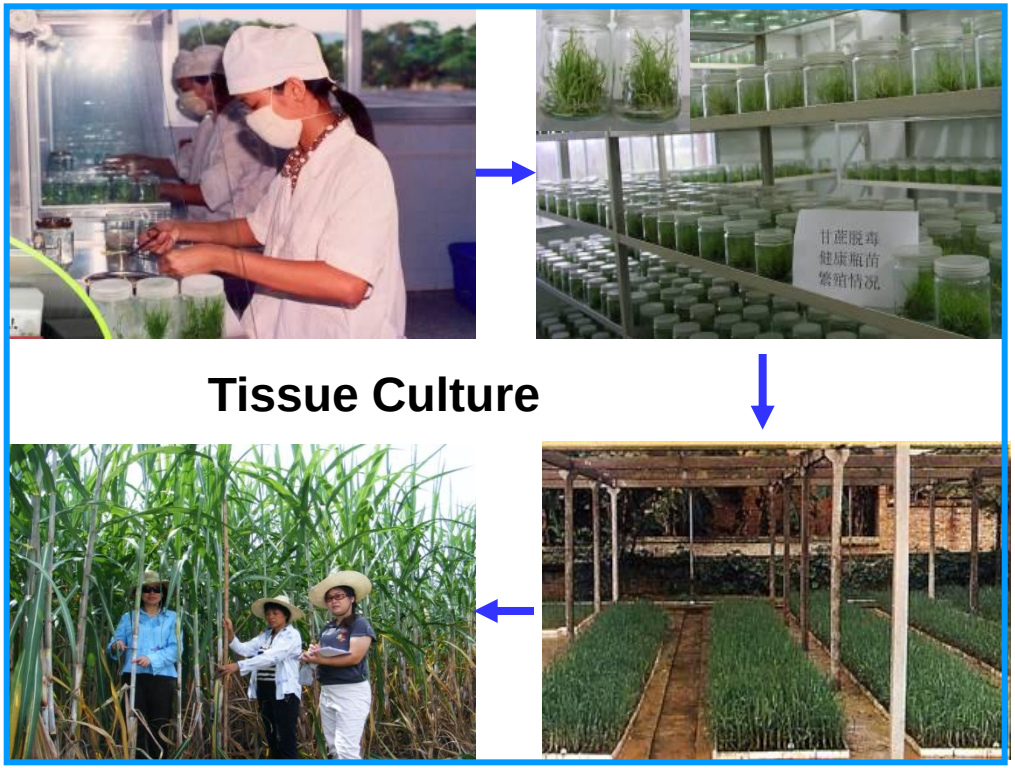


RSD Control: Healthy Seedcane



Hot water treatment





Tissue Culture

Genetic Resistance

Development of cultivars resistant to RSD provides the best approach to assist in RSD control and offers a long-term solution.

To find RSD resistant clone and understand the interaction between pathogen and host is important for breeding for RSD resistance.

Research Funding

- National R & D Research Program (2007BAD30B00)
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- Guangxi R & D Research Program (GK1123008-1)
- Guangxi Natural Science Foundation (2011GXNSFF018002)
- Guangxi Academy of Agricultural Sciences Research Fund (GNK2011YT01)

Long-Term Goal

- **Select RSD resistant clones or cultivars**
- **Find RSD resistant genes by genomic and proteomic techniques**
- **Understand physiological and biochemical responses of sugarcane to RSD**
- **Understand molecular response of sugarcane to RSD**

Objectives

- **Isolation and culture of RSD bacteria *in vitro***
- **RSD bacterium protein isolation and purification**
- **Polyclonal RSD antiserum**

Sugarcane Lxx Isolation and Culture



Infected Sugarcane



sample from basal stalk



Centrifugation



PCR detection

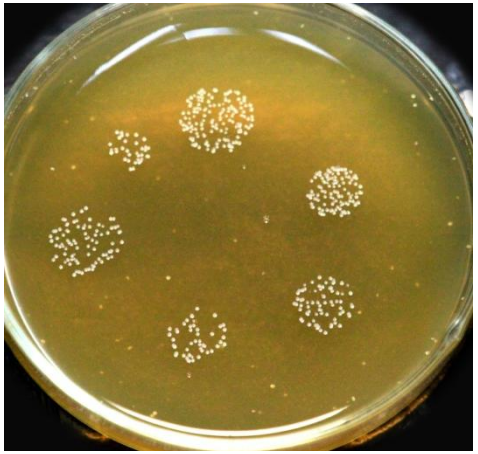


culture in SC mediun

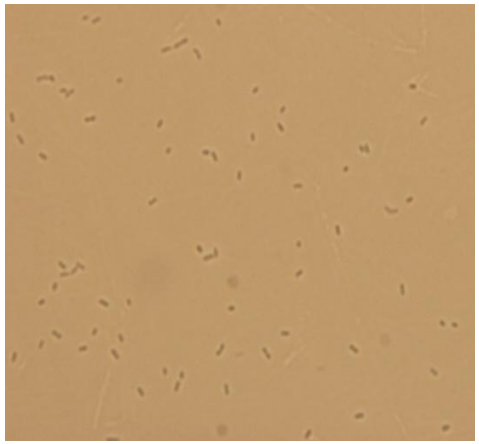


Incubated @ 28^{o}C

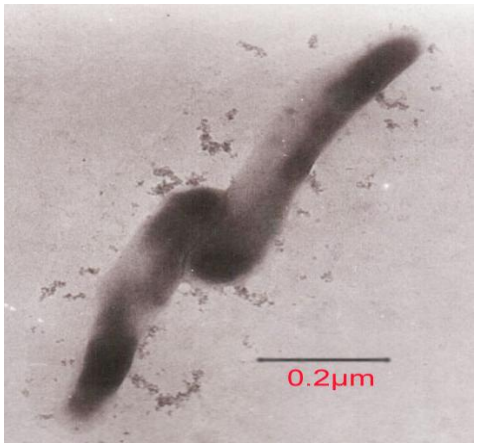
RSD Pathogen



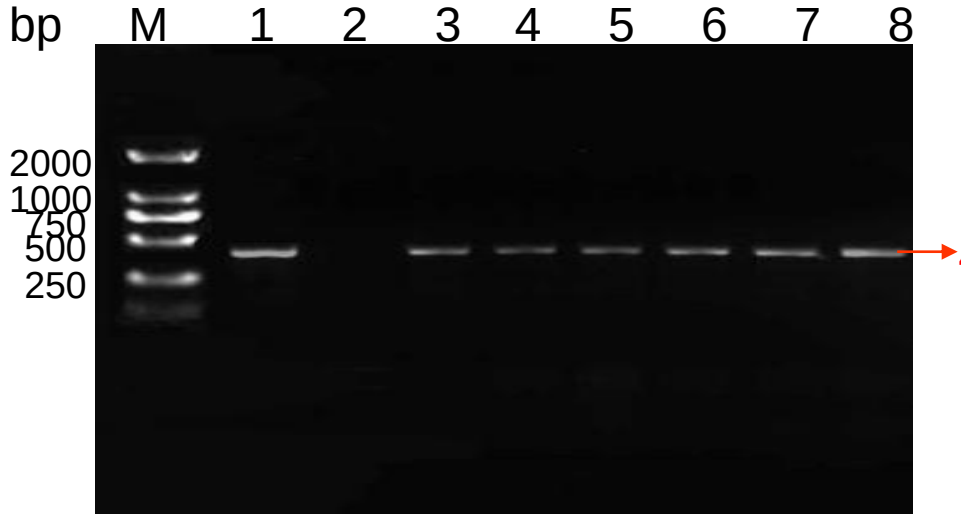
Colony



RSD pathogen (×1000)

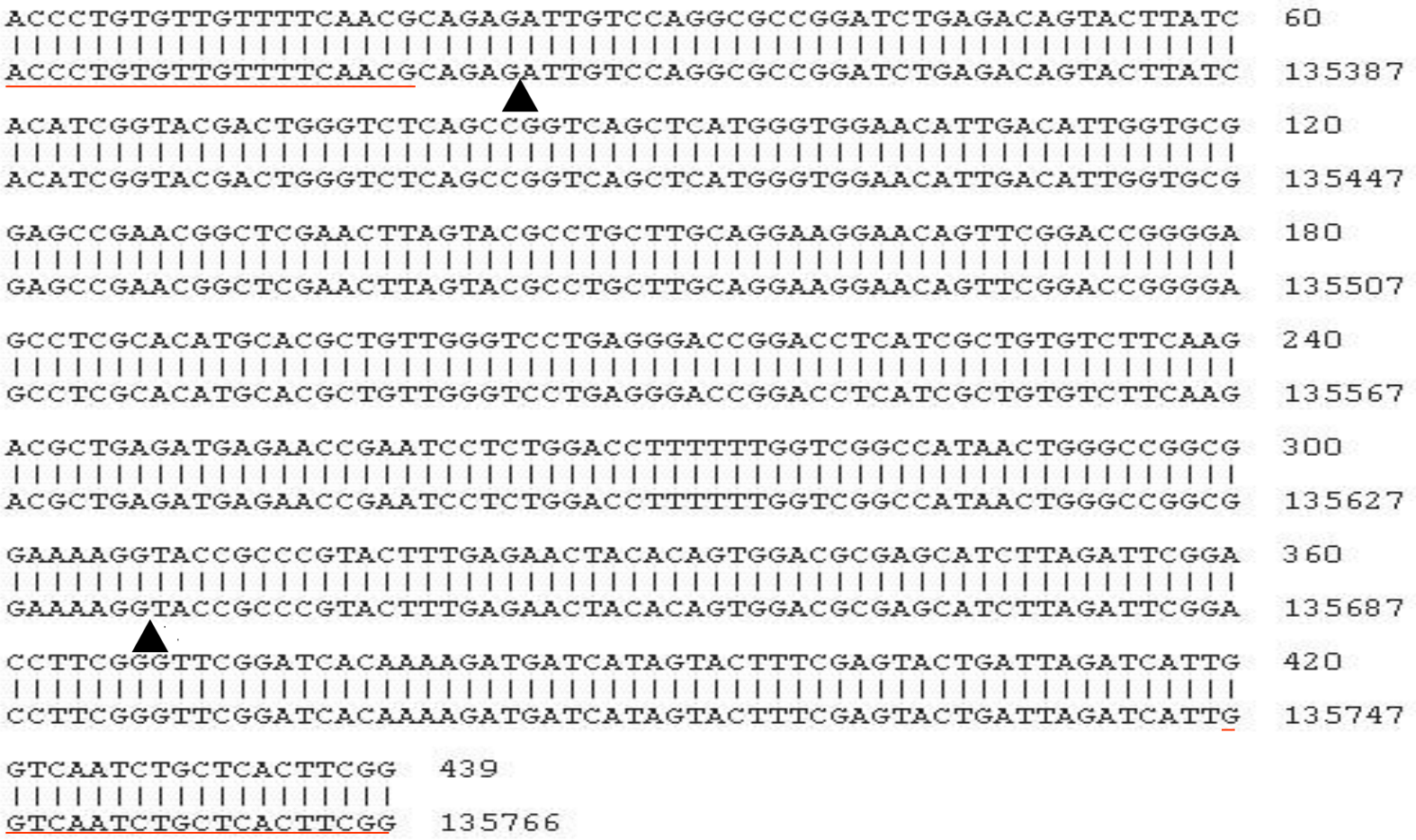


RSD pathogen (×40000)



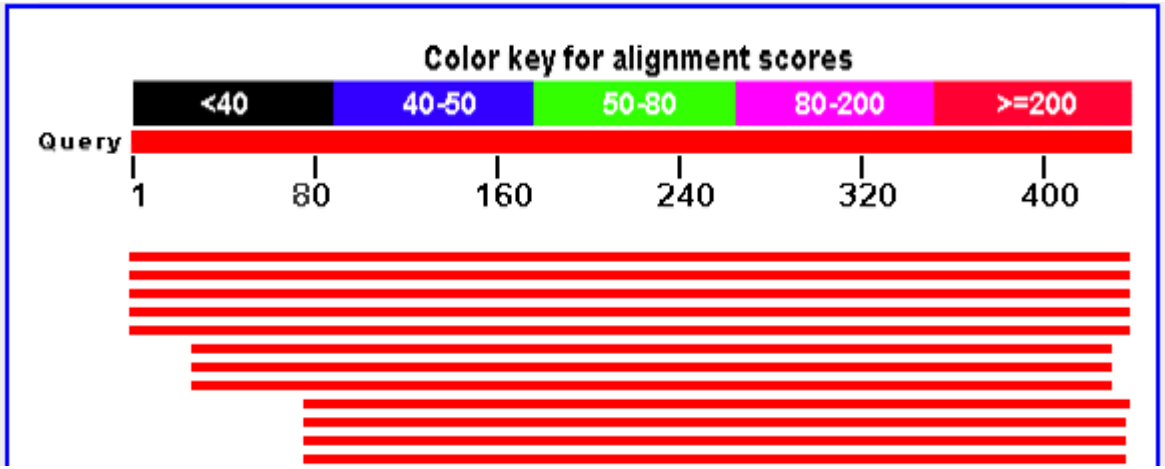
1：positive
2：negative
3-8：
randomly selected colonies

DNA sequenc of PCR product



The DNA sequence is 100% similar to those of AE016822 isolated from Lxx rDNA ITS, Brazil.

Sequencs Comparison



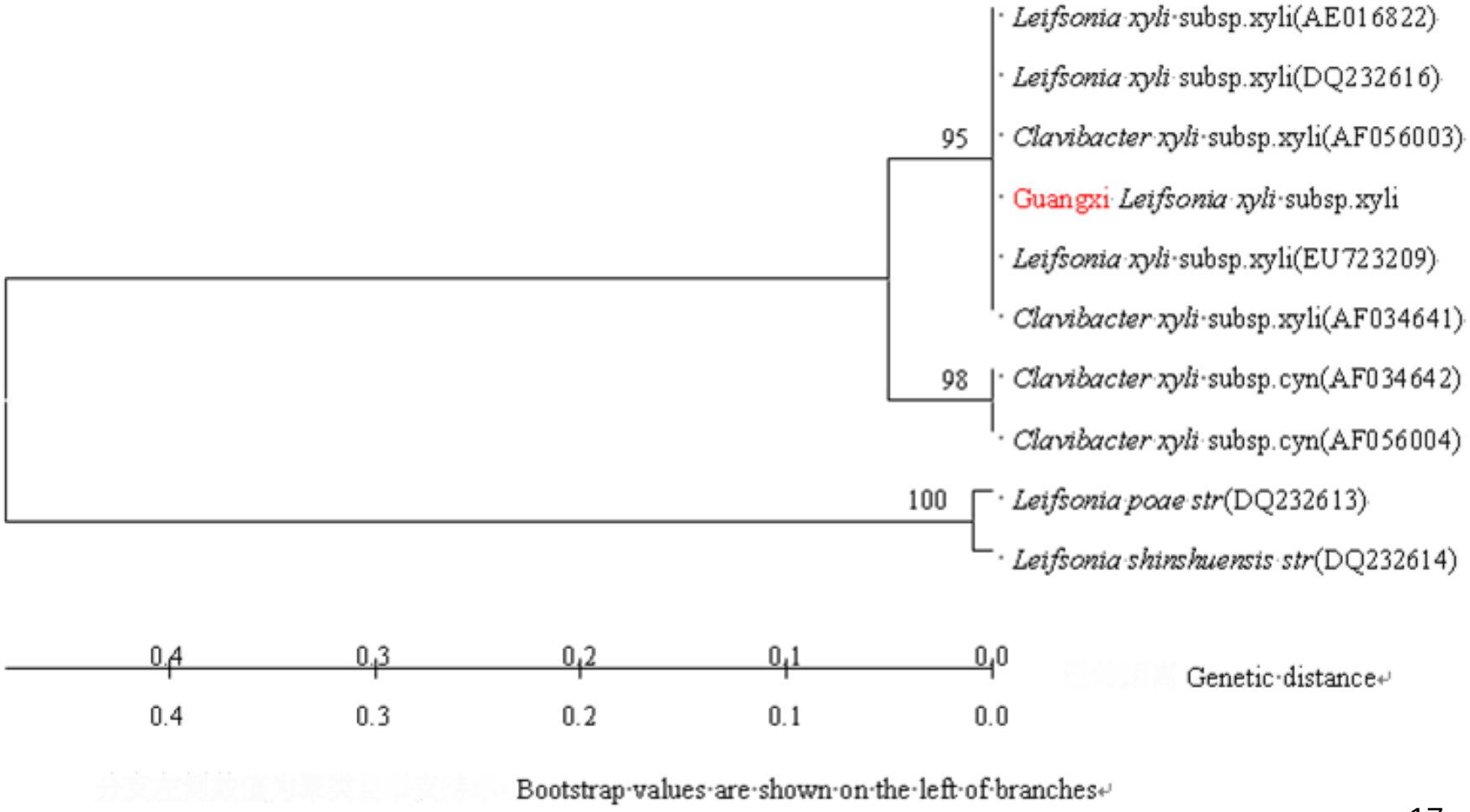
Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer PubChem BioAssay

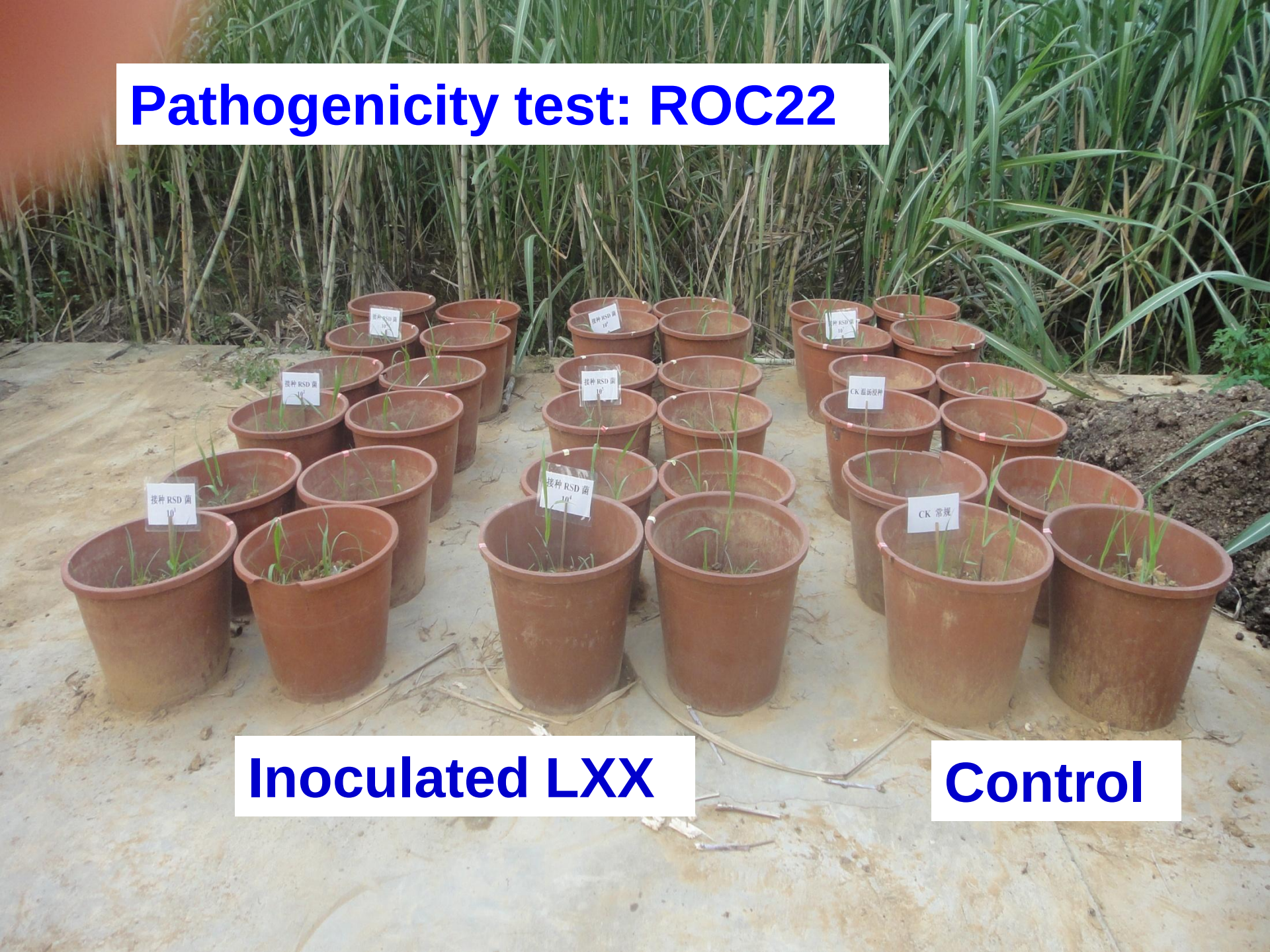
Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
EU723209.1	Leifsonia xyli subsp. xyli 16S-23S ribosomal RNA intergenic spacer, p	809	809	100%	0.0	100%	
DQ232616.2	Leifsonia xyli subsp. xyli strain DNPRC108 16S ribosomal RNA gene, p	809	809	100%	0.0	100%	
AE016822.1	Leifsonia xyli subsp. xyli str. CTCB07, complete genome	809	809	100%	0.0	100%	
AF034641.1	Clavibacter xyli subsp. xyli 16S-23S intergenic spacer region	809	809	100%	0.0	100%	
AF056003.1	Clavibacter xyli subsp. xyli 16S ribosomal RNA gene, partial sequence	797	797	100%	0.0	99%	
DQ232615.2	Leifsonia xyli subsp. cynodontis strain DNPRC039 16S ribosomal RNA	455	455	92%	1e-124	87%	
AF034642.1	Clavibacter xyli subsp. cynodontis 16S-23S intergenic spacer region	455	455	92%	1e-124	87%	
AF056004.1	Clavibacter xyli subsp. cynodontis 16S ribosomal RNA gene, partial se	455	455	92%	1e-124	87%	
DQ232614.2	Leifsonia shinshuensis strain DB102 16S ribosomal RNA gene, partial s	412	412	82%	8e-112	88%	
DQ232618.2	Leifsonia aquatica strain VKM Ac-1400 16S ribosomal RNA gene, part	366	366	82%	6e-98	86%	
DQ232613.2	Leifsonia poae strain VKM Ac-1401 16S ribosomal RNA gene, partial s	366	366	82%	6e-98	86%	
DQ232612.2	Leifsonia naganoensis strain DB103 16S ribosomal RNA gene, partial s	361	361	82%	3e-96	86%	
GQ907162.1	Sphingomonas sp. SLAU-13 16S ribosomal RNA gene, partial sequenc	132	132	62%	2e-27	77%	

Phylogenetic tree analysis of RSD pathogen and some highly homologous strains based on rDNA-ITS



Pathogenicity test: ROC22

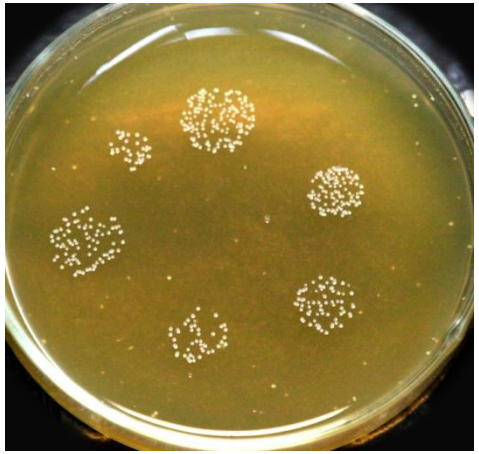


RSD was detected in three pre-mature canes

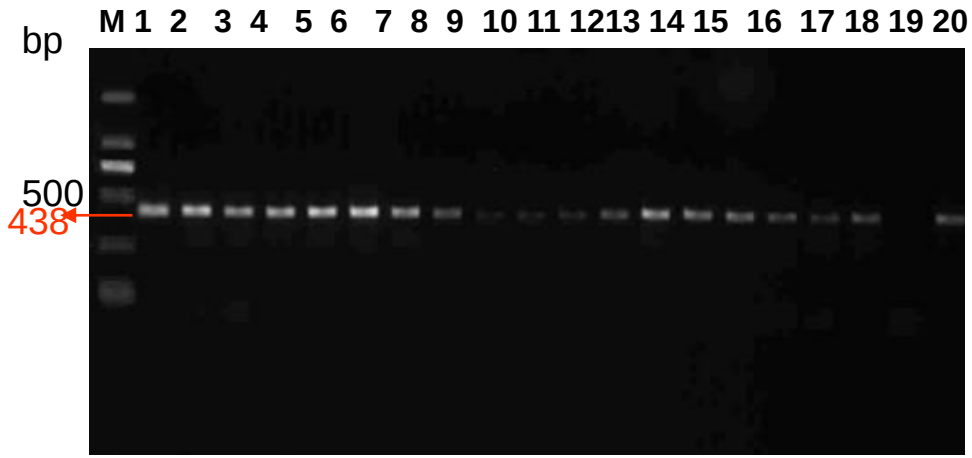


Inoculated LXX Healthy

Lxx protein extraction



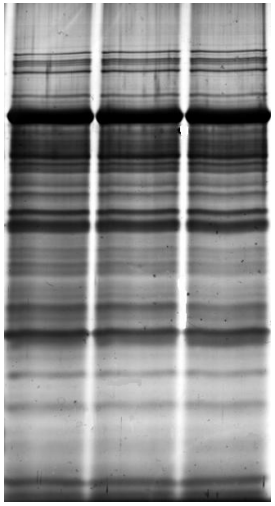
Colony



1-18: Lxx culture; 19: negative; 20: positive



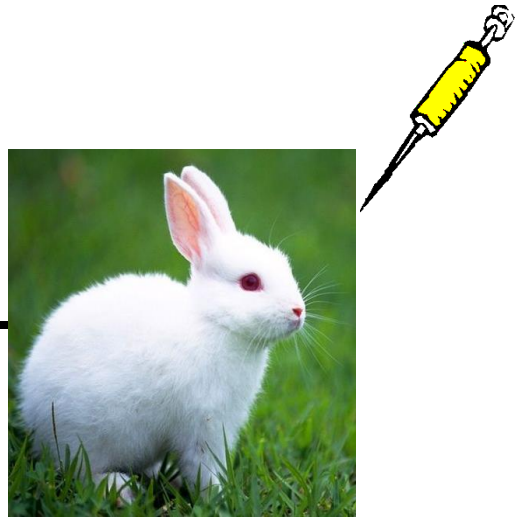
Lxx liquid culture



Lxx total protein (3μg)

Preparation of polyclonal antibody

Bacterial liquid → Centrifugation（12000 rpm, 10 min） → Collect precipitation → Add Freund's complete adjuvant (FCA) into precipitation



antiserum → Purification of polyclonal antibody

Potency Determination for Lxx: ELISA

Sample	Diluted multiple ($\times 10^3$)									
	1	2	4	8	16	32	64	128	256	512
Immune serum	2.091	1.979	1.794	1.406	1.202	0.918	0.466	0.336	0.209	0.151
Normal serum	0.109	0.085	0.073	0.060	0.075	0.066	0.065	0.072	0.071	0.075
Blank	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091
P/N	19.183	23.282	24.575	23.433	16.027	13.909	7.169	4.667	2.943	2.013

Note: The data are optical density values at 450 nm

The highest dilution of antiserum (1:256000) presented strong positive reaction and high specificity against *Lxx*.

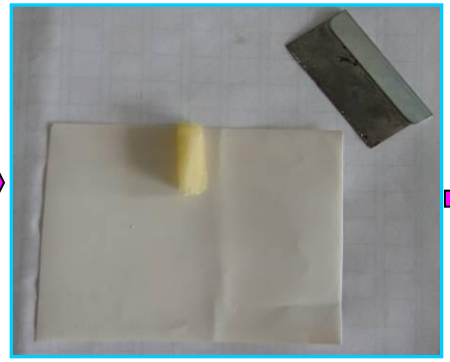
Tissue Blot Immunoassay (TBIA)



Cane stalk



Cut to small pieces by a perforator or knife



Print juice in cellulose nitrate membranes



@70ºC for 30-60 min



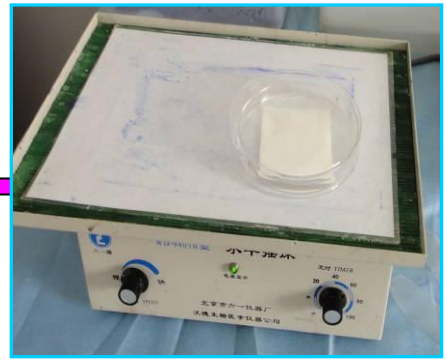
Coloration



Reaction

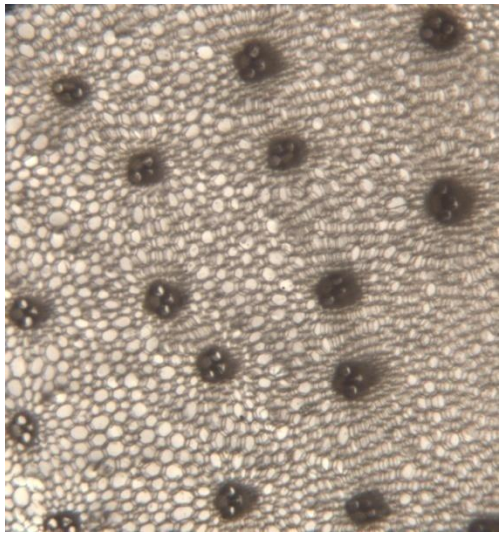


1st antibody and 2nd antibody

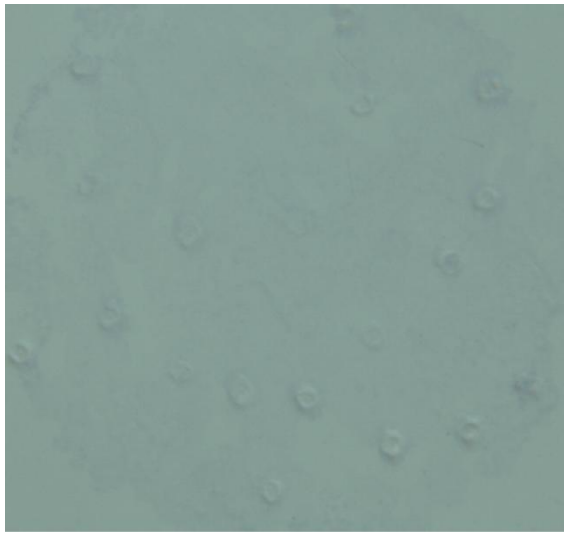


Block

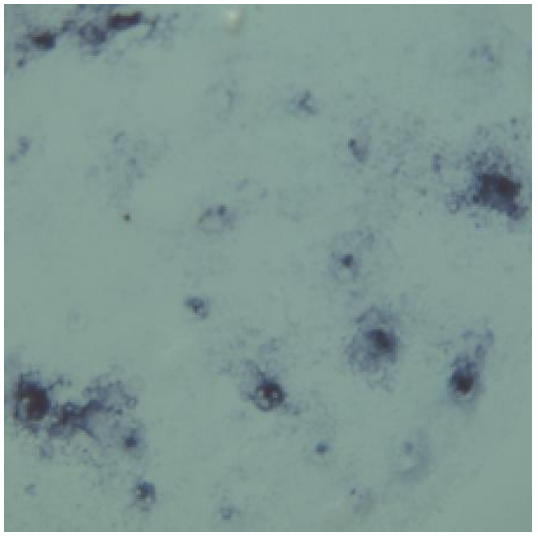
Evaluation the Results of TBIA



Cross-section
of stalk (20 ×)

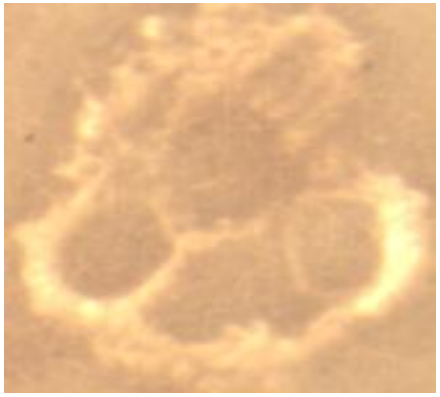


Healthy (10 ×)

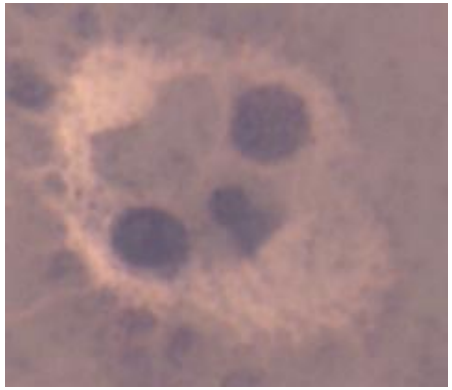


Infected (20×)

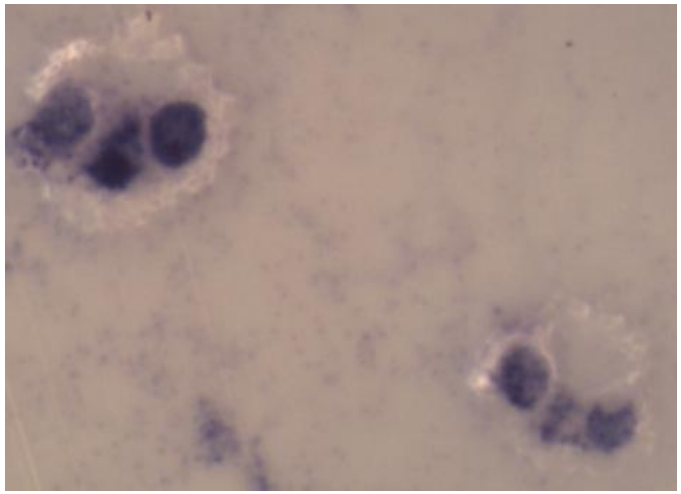
Single vascular bundle (40×)



Healthy



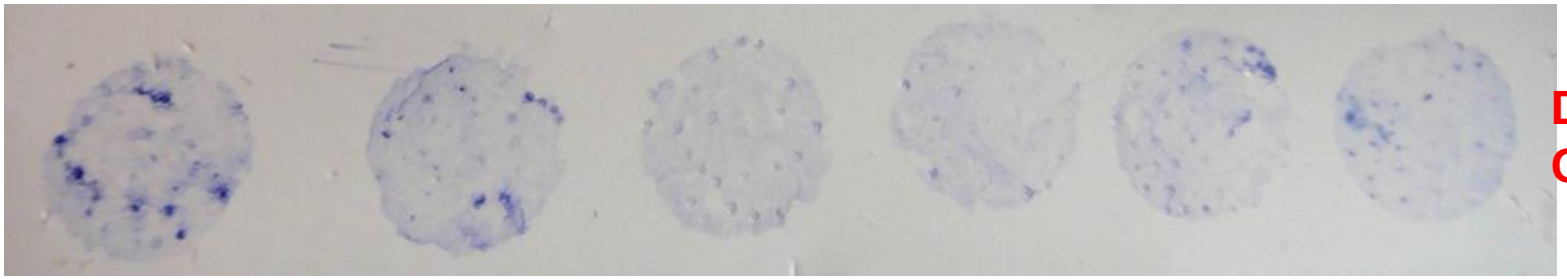
infected



infected

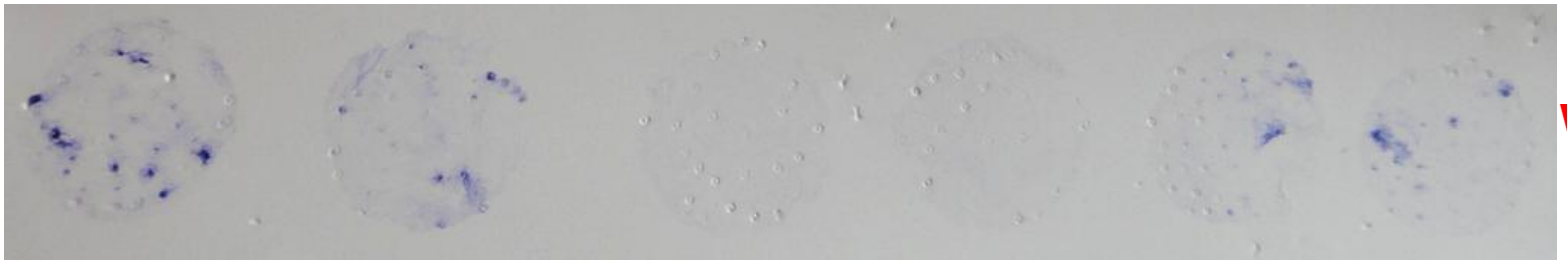
Anti-Lxx Comparison

1 2 3 4 5 6



Dr. J. Comstock

1 2 3 4 5 6



We made

1st antibody：1:3000 2nd antibody：1:5000

1,2,5,6: infected RSD 3,4: healthy

Summary

The isolated pathogen from sugarcane is bacterium Lxx based on morphology, PCR detection, rDNA - ITS sequence comparison and pathogenicity test.

The polyclonal antiserum against Lxx is reliable to detect sugarcane RSD.

Acknowledgements



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GXU



Mr. Ming-Hui Chen
GXU



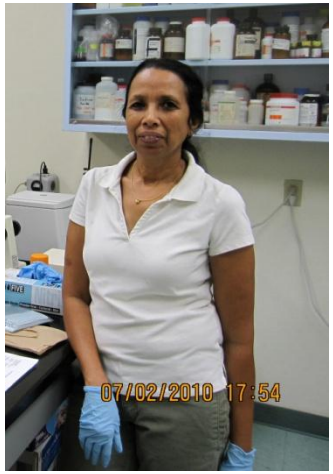
Dr. Jack Comstock
USDA-ARS



Dr. Rong-Bao Pan
USDA-ARS



Ms. Dan Zhou
GXU



Dr. Sushma Sood
USDA-ARS


Thank You !
西校园大礼堂鸟瞰