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BioScience

Ed Roumen

Tomorrow's Rice

Testing seed for the presence of the bacterial leaf blight pathogen *Xanthomonas oryzae* pv *oryzae*

Bacterial leaf blight and seed transmission

- The scientific literature is divided on the topic if seed transmission of the BLB pathogen (*Xanthomonas oryzae* pv *oryzae*) is likely to occur. However, seed transmission by heavily (artificially) infected samples has been reported.
- Unlike for fungal infections, chemical treatment to effectively cure infection is not available.
- As a result, the bacterial leaf blight pathogen is considered a quarantine pathogen in many countries, resulting in serious transnational shipping barriers for hybrid rice seed.
- A number of scientific papers have published results aimed at being rapid, specific tests for the bacterial leaf blight pathogen. And these are being used as QC type test by authorities
  - ELISA kit by AGDIA inc
  - Various DNA primers
- Non-pathogenic Xanthomonads commonly occur as epiphytes and saphrophytes

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Plant damage by the BLB pathogen

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Detection methods for the BLB pathogen

- Appearance of colonies in culture
- Clip-inoculation
- ELISA kit
- DNA primers

The basis for validating all the other methods

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Detection methods for the BLB pathogen

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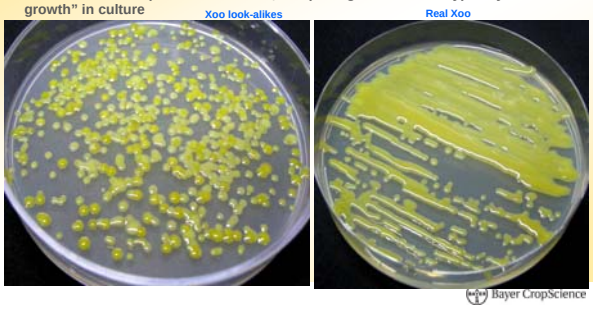
The BLB pathogen cannot reliably be distinguished from its look alike non- pathogenic relatives

- **Using Xanthomonas selective culture medium, the real BLB pathogen is obtained along with look-alike Xanthomonads and Pseudomonads**
- **Reliable separation of the rice-pathogenic from the non-pathogenic species by visual observation is not possible. However, rice pathogenic colonies typically show "slow growth" in culture**

Xoo look-alikes | Real Xoo



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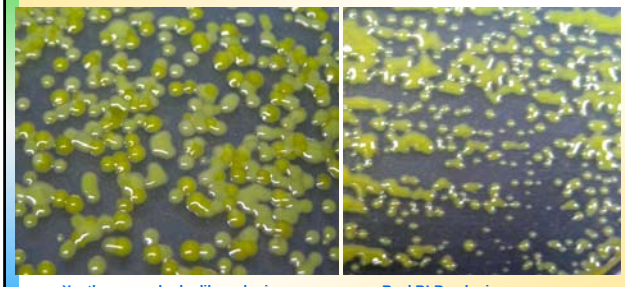
A close up of look alike non- pathogenic Xanthomonads and real BLB pathogen colonies



Xanthomonas look-alike colonies | Real BLB colonies

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Detection methods for the BLB pathogen

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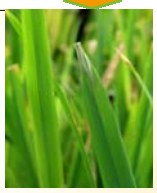
Clip inoculation

- (left) Non-pathogenic isolates induce dark brown reaction at the inoculation site
- (right) pathogenic isolates induce a grey, whitish lesion that elongates >2 cm from the inoculation site



Non-Xoo

Xoo

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Clip inoculation: Xoo symptom development in susceptible rice plants

Xoo

Xoo

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Evaluation of AGDIA kit

Explanation of table contents in slides that follow

- Column 2 — 15-8031013, etc = Xoo-resembling colonies isolated from the seed samples
  Positive Control = Real Xoo, pathogenic as verified by rice plant inoculation test
  Negative Control = Water
- Column 3 — Optical density (OD) of the liquid culture used in wells (different concentrations)
- Column 4 — Optical density of the liquid in the wells after test is completed
- Column 5 — ELISA results (positive or negative) at different concentrations of sample
- Column 6 — Clip inoculation results (positive = disease appeared; negative = no disease)

Note: Rows in green color show the ELISA reaction results at 0.01 OD, the recommended concentration of bacteria.

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Evaluation of AGDIA kit (ELISA) for diagnosis of Xoo

Well No.	Sample ID	Sample OD at 600 nm	Test color OD (at 405 nm)	ELISA Result	Inoculation Result
1	15-8031013	0.68	0.976	False Positive	Negative
2	15-8031013	0.1	0.593	False Positive	Negative
3	15-8031013	0.01	0.210	Negative	Negative
4	15-8031013	0.001	0.173	Negative	Negative
5	16-802059/18	0.604	0.971	False Positive	Negative
6	16-802059/18	0.1	0.734	False Positive	Negative
7	16-802059/18	0.01	0.231	Negative	Negative
8	16-802059/18	0.001	0.168	Negative	Negative
9	15-8031009	0.44	0.976	False Positive	Negative
10	15-8031009	0.1	0.506	False Positive	Negative
11	15-8031009	0.01	0.203	Negative	Negative
12	15-8031009	0.001	0.182	Negative	Negative
13	16-80562/16	0.72	0.975	False Positive	Negative
14	16-80562/16	0.1	0.437	False Positive	Negative
15	16-80562/16	0.01	0.213	Negative	Negative
16	16-80562/16	0.001	0.158	Negative	Negative
17	Positive chk1	0.324	0.977	Positive	Positive
18	Positive chk1	0.1	0.978	Positive	Positive
19	Positive chk1	0.01	0.974	Positive	Positive
20	Positive chk1	0.001	0.962	Positive	Positive

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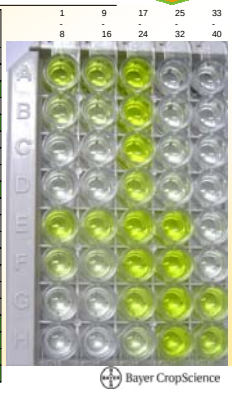
Evaluation of AGDIA kit .... cont'd

Well No.	Sample ID	Sample OD at 600 nm	Test color OD (at 405 nm)	ELISA Result	Inoculation Result
21	Positive chk 2	0.368	0.974	Positive	Positive
22	Positive chk2	0.1	0.973	Positive	Positive
23	Positive chk2	0.01	0.985	Positive	Positive
24	Positive chk2	0.001	0.460	Positive	Positive
25	15-8031013	0.01	0.161	Negative	Negative
26	15-8031013	0.02	0.240	Negative	Negative
27	15-8031013	0.03	0.282	Negative	Negative
28	15-8031013	0.05	0.322	Negative	Negative
29	Positive control 1	0.01	0.973	Positive	Positive
30	Positive control 1	0.02	0.973	Positive	Positive
31	Positive control 1	0.03	0.985	Positive	Positive
32	Positive control 1	0.05	0.970	Positive	Positive
33	Negative control	0	0.155	Negative	Negative
34	Negative control	0	0.144	Negative	Negative
35	Negative control	0	0.154	Negative	Negative
36	Negative control	0	0.166	Negative	Negative
37	16-802059/18	0.01	0.201	Negative	Negative
38	16-802059/18	0.01	0.211	Negative	Negative
39	Positive control 2	0.01	0.983	Positive	Positive
40	Positive control 2	0.01	0.968	Positive	Positive



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AGDIA kit (ELISA) conclusions

- This ELISA kit is meant for testing pure bacterial cultures only !
- The kit can differentiate between Xoo and non-Xoo if test sample is having a concentration of bacterial cells ranging from 10^5-10^7 cfu/ml.
  - Above 10^7 cfu/ml, it will give false positive results for related but non-pathogenic Xanthomonad species.
  - If sample concentration is less than 10^5cfu/ml, it will give false negative results
- Sampling issue: When crushing rice seed in sterilized water and then spreading on nutrient media (including on Xanthomonas specific culture media),a large no. of bacterial colonies appear; it is not possible to run ELISA test on each and every one of them.
- A time consuming and tedious process: Analysing a sample takes approx 10 days :
  - isolation of bacterial colonies on culture media
  - picking single colonies followed by multiplication in liquid culture
  - ELISA test

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Lane 1991 - 16sRNA primer

- primers 27f & 1525r.
  - 5'-AGA GTT TGA TCM TGG CTC AG -3'
  - 5'-AAG GAG GTG WTC CAR CC -3'
- As per "in silico", same fragment "103aa" is amplified on various species
  - 1 Xanthomonas oryzae pv oryzae — strain PXO99A
  - 2 Xanthomonas oryzae pv oryzae — strain MAFF311018
  - 3 Xanthomonas oryzae pv oryzae — strain KACC10331
  - 4 Xanthomonas axonopodis pv citri — strain 306
  - 5 Xanthomonas oryzae pv oryzicola — strain BLS256
  - 6 Xanthomonas campestris pv campestris — strain 8004
  - 7 Xanthomonas campestris pv campestris — strain ATCC33913
  - 8 Xanthomonas campestis pv vesicatoria
  - 9 Xanthomonas campestris pv amoraciae
- but not on
  - 7 Pseudomonas syringae pv syringae — strain DC3000
  - 8 Erwinia carotovora pv atroseptica
- Conclusion: These primers are not specific to X. oryzae pv. oryzae and cannot be used as diagnostic kit.

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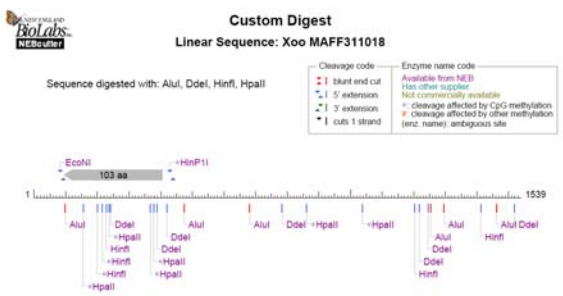
Lane 1991; in silico illustration for Xanthomonas Oryzae pv oryzae MAFF311018



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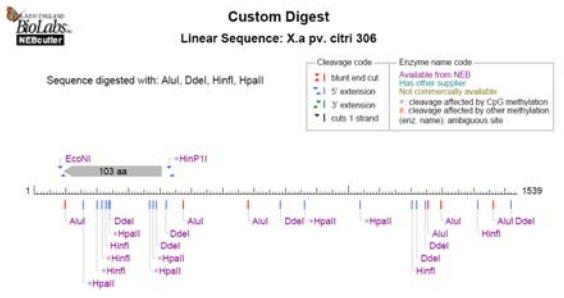
Lane 1991; in silico illustration for Xanthomonas albinians pv citri 306



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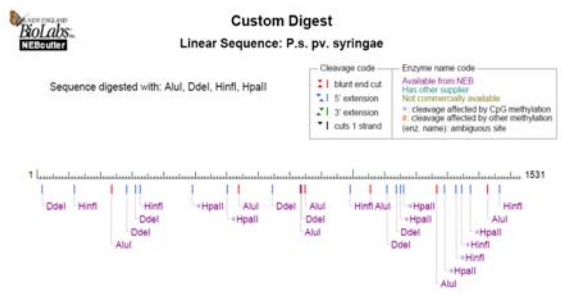
Lane 1991; in silico illustration for Pseudomonas s. pv syringae



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  - **Adachi, Nato and Oku, Takashi 2000. PCR-mediated detection of *Xanthomonas oryzae* pv. *oryzae* by amplification of the 16S-23S rDNA spacer region sequence. Journal of General Plant Pathology, 66 : 303-309.**

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Adachi et al, 2000 16S-23S rDNA Primers

- **Primers and their sequence**
  - XOR-F (5'-GCATGACGTCATCGTCCTGT-3')
  - XOR-R2 (5'-CTCGGAGCTATATGCCGTGC-3')
- **Specificity**
  - As per the paper, these primers were able to amplify a 470-bp fragment from all strains of *Xanthomonas oryzae* pv. *oryzae* isolated from Japan but, was also able to amplify same region in *X. axonopodis* pv *citri*, X. *Campestris*. pv. *zinniae* and X. C. pv, *incanae*.
  - As per "in silico" analysis, these primers are also amplifying the same fragment in *X. axonopodis pv. citri* 306, X. *campestris* pv. *armoraciae*, X. *campestris* pv. *vesicatoria* and X. *campestris* pv. *campestris* 8004 and ATCC33913.
- **Conclusion: These primers are not specific to X. oryzae pv. oryzae and cannot be used as diagnostic kit.**

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  - **Sakthivel et al, 2001. Applied Microbiology and Biotechnology 56: 435-441**

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Sakthivel et al, 2001. DNA primer test "TXT"

- TXT primer sequence
  - TXT 5'-GTCAAGCCAACTGTGTA-3'
  - TXT4R 5'-CGTTCGCGCCACAGTTG-3'
- Based on the insertion sequence IS1113, which was detected by hybridization in both *X. oryzae* pv. *oryzae* and *oryzicola*, but **rarely** in other *Xanthomonas* (Jan Leach, pers. comm)
- Amplifies a 964 bp fragment
- Might be the best diagnostic primer among tests published.
  - Additional validation is ongoing

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  - Sakthivel et al, 2001. Applied Microbiology and Biotechnology 56: 435-441
  - **New primers under validation in collaboration with Colorado State and with IRRI**

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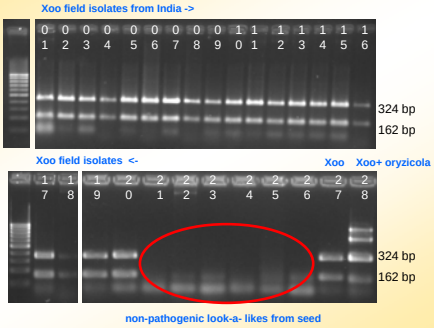
New primers under validation in collaboration with Colorado state University and IRRI

- Validate new primers by testing them on a large number of isolates from different origin and countries



- New primers show promise to differentiate real Xoo from look-a-likes

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Request/proposal

- Pls share your Xoo with Bayer Cropscience / Colorado State University
  - Xoo pv oryzae
  - Xoo pv oryzicola
  - Xoo "look-a-like"
- We shall use these to further validate new primers in collaboration with Colorado state and IRRI
- And lobby that such validated primers be implemented as reliable diagnostic tool kit to detect Xoo on seed by phytosanitary authorities

Contributors to this presentation:

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Thank you for your kind attention