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Detection of *Clavibacter michiganensis* subsp. *michiganensis* in seeds of tomato

EPPO Panel for Diagnostics in Bacteriology

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Outline of presentation

- General information Cmm
- Seed assay protocol
- Evolution of seed assay to ISHI 4.1
- Validation
- Comparative testing
- New developments
- Conclusions

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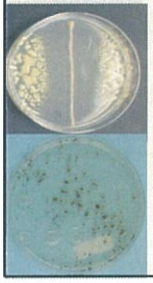
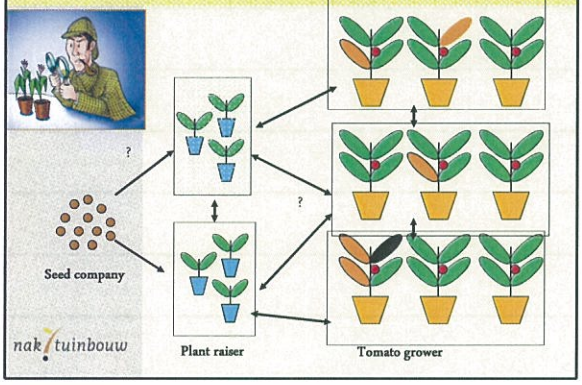
Detection of Cmm in tomato seeds



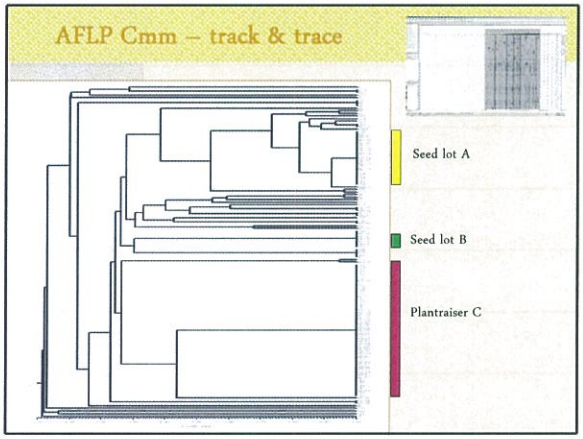
Detection of Cmm is rather difficult

- Tomato seed lots are <u>treated</u> (HCl) therefore only internal contamination is detectable
- Cmm is a relatively slow grower and is easily out competed by saprophytes
- Cmm colonies are difficult to recognise on semi-selective media due to variation in colonies morphology and presence of "lookalikes"
- Presence of antagonistic bacteria and fungi that hamper detection Cmm

Source of the primary inoculum outbreaks 2007?



AFLP Cmm – track & trace



AFLP Cmm – track & trace

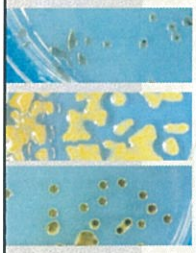
- Claims of 2007 were traced back to 3 clusters of strains
- Two tomato seed lots were primary source
- Third cluster traced back to one plantraiser (no contaminated seeds recovered)
- AFLP analysis basis for International Cmm reference collection and sequencing project

Emphasis should be on prevention of claims that is having a more reliable seed assay!!

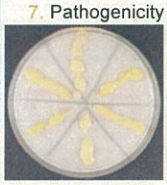
Detection of Cmm on tomato seeds ISHI 4

1. Overnight soaking of seeds in PB
2. Stomacher extraction (no dry grinding)
3. Concentration of bacteria by centrifugation
4. Dilution plating on 2 semi-selective media and compulsory spiking of extract

Detection of Cmm on tomato seeds (2)



5. Transfer suspected colonies to YDC
6. PCR identification PTSSK Taqman and improved Pastrik PCR
7. Pathogenicity assay



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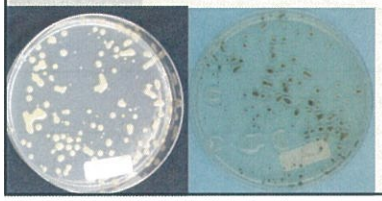
ISHI 4.1 Protocol on ISF website

Evolution of Cmm seed assay

- Use of thiosulphate to neutralise hypochlorite residue
- Increase of number of seeds
- Centrifugation (slow speed and high speed)
- Improved complementary media Cmm1T and SCMF
  - Cmm1T: Suppression antagonists
  - SCMF: Typical Cmm morphology & fast growth
- Transfer more suspects



Evolution of Cmm seed assay

- Recovery of spike rule in concentrated extract
- Improved Pastrik primers (Bert Woudt, Syngenta)

False positives PSA-4!!

```
PSA-4        ATC TTG GTC AAT TCT GTC TCC C
Cm non-mich  ... ... ... ... ... ... TC. A

"PSA-8"          TTG GTC AAT TCT GTC TCC CTT C
```

- Development PTSSK Taqman (Berendsen et al. APS poster 2011)

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Validation ISHI 4.0

EPPO & Dutch guideline for the validation

- Detection limit
- Measuring range
- Analytical specificity
- Selectivity
- Reproducibility / Repeatability
- Robustness

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Validation of ISHI 4.0 protocol

- Dilution plating on SCMF and CMMT in tomato seed (Validation report 1)
- Molecular characterisation (Validation report 2)
  - Improved Pastrik PSA08
  - RZ PTSSK Taqman
- Validation data available for EPPO
- ISO17025 approval for Cmm assay of Naktuinbouw

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NAL Cmm comparative testing 2012

- Cmm proficiency test (PT*) was organized test in framework of NAL (Naktuinbouw accredited laboratories)

* Proficiency test means that laboratories should use their own in house Cmm protocol for testing the seeds. Previously some degree of freedom in f.i. extraction method (Ultra turax, dry grinding, stomaching) were allowed

** For a comparative test (CT) laboratories should use a well defined fixed protocol (no degree of freedom)

Set up of PT

- 22 samples under code for each participant
- Some laboratories used extra sets for additional testing
- Data sheets to participants to fill out data
- Some extra participants (non NAL laboratories)

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Set up of PT

Table 1. Composition of the sample set and expected results for the PT Cmm 2012.

Sample type	Contamination level	number of samples	Seed lot
Healthy seed lot	No Cmm	5	ZZB 15
Cmm contaminated	Medium A (high Cmm load)	4	ZZB 377/ZZB 15*
Naturally contaminated	Medium B (low Cmm load)	5	ZZB 390/ZZB 15*
Naturally contaminated	Medium C (low Cmm load)	6	ZZB 391/ZZB 15*
Naturally contaminated	high	2	ZZB 391

* Seed lots were blended in a ratio of 500/4500 seeds per seed lot

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Analysis of PT data

- NAL office process data to retain anonymity of laboratory (coding of labs)
- Data transferred to Naktuinbouw R&D
- R&D uses binomial approach (sample is positive or negative) to analyse data
  - No Cmm colonies identified in sample = negative
  - ≥1 Cmm colonies identified = positive
- Individual laboratories are compared with average of all laboratories
- Underperformance could lead to yellow or red card
- No insight in processing details of laboratories because of anonymity

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Actions after sending out NAL report

- Ask permission from participants to share data and release information on extraction
- Extend PT data analysis beyond previous binomial approach
- Send out extra sample sets to investigate extraction parameters in one laboratory

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Additional work in one laboratory

- Reason for reduced sensitivity for Cmm
  - Extraction buffer composition (PBST* versus ISHI 4.0)
  - Seed extraction method (dry grinding** versus stomaching)
  - Direct plating versus extra cold incubation

* 8, 5 gr NaCl/L and thiosulphate (0,5 gram/L)
** Retsch grinding device (15 sec, 10,000 rpm)
*** extra 2 hrs at 4°C

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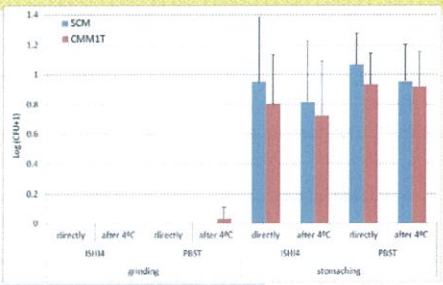
Dry grinding versus stomaching



Cmm	grinding	Stomaching
positives	1	10
negative	9	0

Conclusions PT

- PT showed that most laboratories were able to detect and identify Cmm with their "in house" Cmm protocol
  - ISHI 4.0 or "ISHI4.0 lookalike" protocol
- Grinding is very critical parameter and current stomacher fixation in ISHI 4.0 is justified by this PT
- ISHI 4.1 (definition of CCP and more explict about extraction method)

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New developments

- Availability of relatively high amounts of naturally contaminated seed lots
- Characterisation of 11 Cmm's and 9 lookalike bacteria by next generation sequencing data
- Objective is new Taqmans for replacing modified Pastrik PCR
- Several new Taqmans have been validated for colony identification leading to ISHI 4.3.1.

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New developments

- EU TESTA: Investigate whether seed extract PCR and or French enrichment PCR (3 days shaking at 28°C according to EPPO protocol) can be used to detects Cmm in seed extract (prescreening method)
- Challenge is sensitivity (low numbers of Cmm and/or difficult lysis of gram positive bacteria)
- No isolation of pure cultures*
- Internal amplification control for monitoring PCR inhibition and sensitivity (Cmtes)

* Treatment tomato seeds compromise isolation of Cmm cultures in case of complaints

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Conclusions

- Seed assay based on dilution plating has strongly evolved especially in the last 5 years: Media,PCR's were strongly improved
- At least 10,000 seeds per sample
- Compulsory spiking crucial for monitoring inhibition growth of Cmm (ISHI 4.0/ISHI 4.1)
- Detection of Cmm in dirty seed lots is difficult
- Seed processing very critical for detectability Cmm
- ISHI 4.0 officially validated
- Strongly improved new EPPO protocol

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Acknowledgements

- Anne Alvarez for providing Cmm and lookalike strains.
- Naktuinbouw routine laboratory and René Dekter for validation experiments

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Trueness

Strain designation	Taqman PTSSK	patho assay	AFLP	Identity	IF prime diagnostics	Immunostrip Cmm Agdia
ALV4588	17,6	pos	Cmm	Cmm	pos	pos
ALV4690	19	pos	Cmm	Cmm	pos	pos
ALV4763	22,6	pos	Cmm	Cmm	pos	pos
ALV2701	18,6	pos	Cmm	Cmm	pos	pos
ALV4004	23,1	pos	Cmm	Cmm	pos	pos
ALV4868	38,6	neg	no Cmm	Cm?	pos	pos
ALV4877	19,6	pos	Cmm	Cmm	pos	pos
ZUM3059	17,3	pos	Cmm	Cmm	pos	pos
NBC 987	38,6	neg	no Cmm	Cm?	neg	pos
NBC 1235	40	neg	no Cmm	M. testaceum	neg	pos
NCPPB 382	24,6	pos	Cmm	Cmm	pos	pos
PD520	17,8	pos	Cmm	Cmm	pos	pos
NBC 1344	40	neg	no Cmm	Cm?	pos	pos
NBC 1495	35,7	neg	no Cmm	Cm?	pos	neg
LMG7294	40	neg	no Cmm	Cmt	pos	pos
LMG3663	40	neg	no Cmm	Cmi	pos	neg
PD 5752	40	neg	no Cmm	Cm?	pos	pos
NBC 1783	16	pos	Cmm	Cmm	pos	pos
NBC1340	38,3	neg	no Cmm	Cm?	neg	neg
NBC 251	16,1	pos	Cmm	Cmm	pos	pos
					6 FP	6 FP

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