Culture-free rapid molecular detection of *Clavibacter michiganensis* subsp. *michiganensis* in seeds of tomato

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EPPO conference on diagnostics for plant pests
December 1st , Angers, France

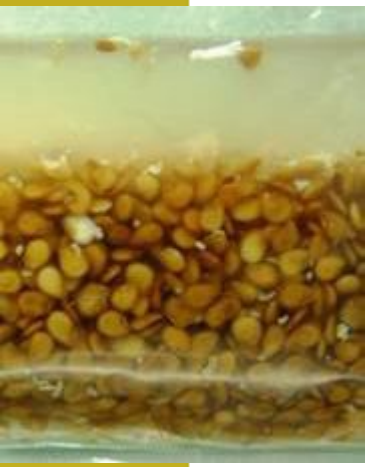
Testa

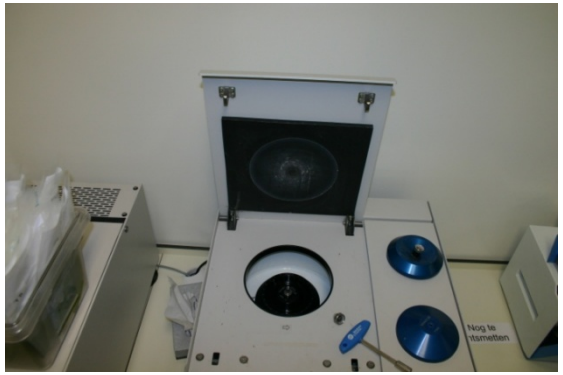
General information Cmm

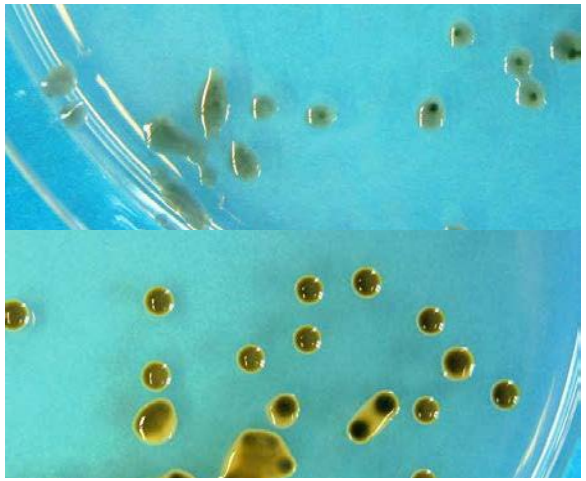
- Cmm is causal organism of bacterial wilt and canker of tomato
- “Limited” number of closely related subspecies
  - *sepedonicus (potato)*
  - *insidiosus (alfalfa)*
  - *nebraskensis (maize)*
  - *tessellarius (wheat)*
  - californiensis (tomato)/chilensis (pepper)/phaseoli (bean)
- Several large outbreaks

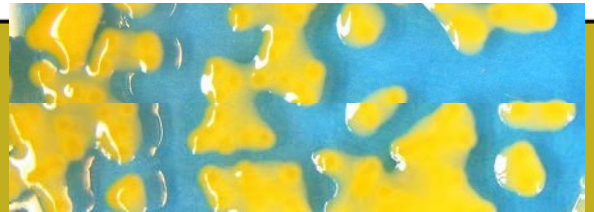
Detection of Cmm in tomato seeds (ISHI 4.3)

1. Overnight soaking of ≥10,000 seeds in PB
2. Bagmixer extraction
3. Centrifugation
4. „Concentration“ plating (CP) on complementary semi-selective media including Cmm spiking

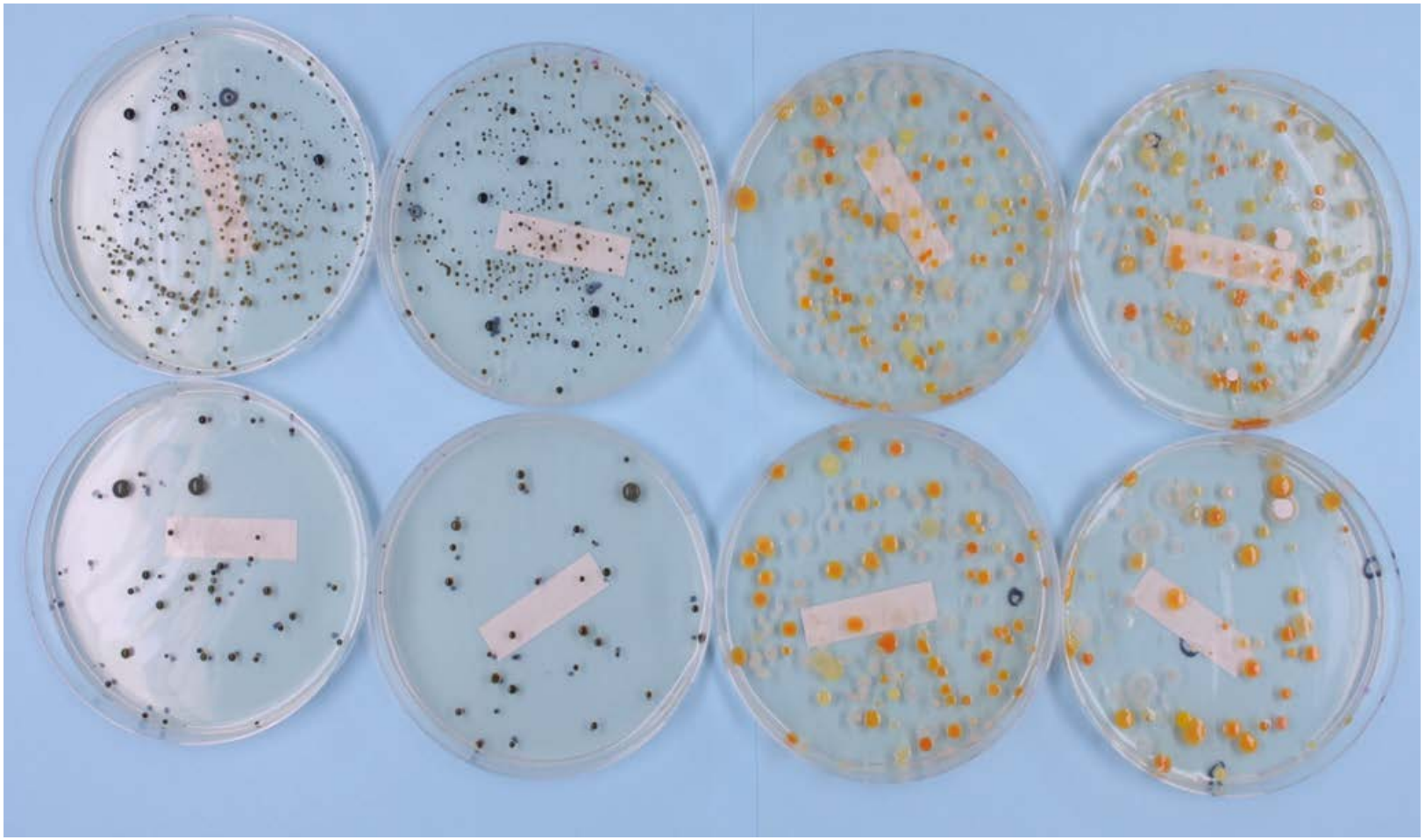




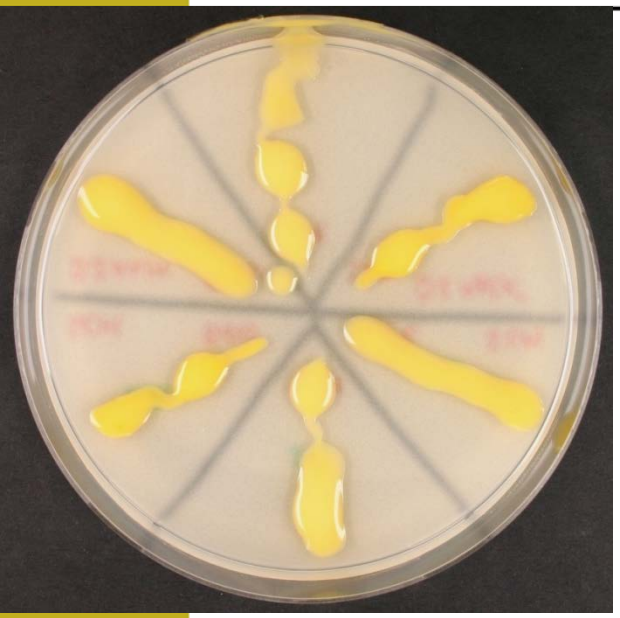




Recognition Cmm colonies difficult



Detection of Cmm in tomato seeds



5. Transfer suspected colonies to YDC
6. PCR identification using independent targets
7. Pathogenicity assay

Adapted objective in EU TESTA

- “Culture-free” detection of Cmm in seed extracts such as for Acit/Xcc?

Objectives

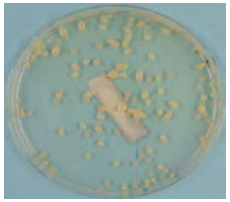
- Develop & validate high throughput seed extract PCR
- Process control based on gram positive IEC
- Broad application from early to late stages in tomato seed processing
  - Young “fresh biological” seeds to old museum samples

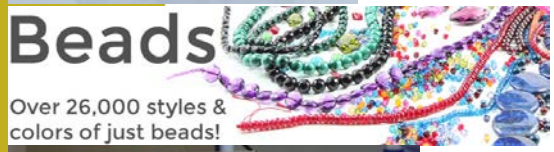
Analytical specificity Cmm Taqmans

	PTSSK	MVS15	MVS 21	ClvA	PycA
Number of Cmm with Ct ≤32	577	577	577	576	575
Number of Cmm with Ct Ct >32 and ≤35	1	0	0	1	1
Number of Cmm with Ct Ct >35 and ≤37	0	0	0	0	0
Number of Cmm with Ct >37 and <40	0	0	0	0	1
Number of Cmm with no amplification	0	1	0	1	1
Total Cmm	578	578	577	578	578
Number of lal with Ct ≤32	0	0	1	0	0
Number of lal with Ct Ct >32 and ≤35	5	6	7	4	5
Number of lal with Ct Ct >35 and ≤37	8	12	15	9	10
Number of lal with Ct >37 and <40	5	9	3	9	9
Number of lal with no amplification	55	46	47	51	49
Total lal	73	73	73	73	73

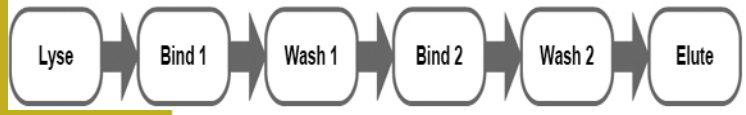
PTSSK and MVS21 good candidates
PCR's at 60 ° C!

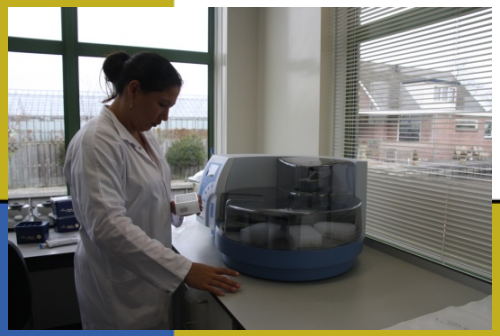
Optimised seed extract PCR

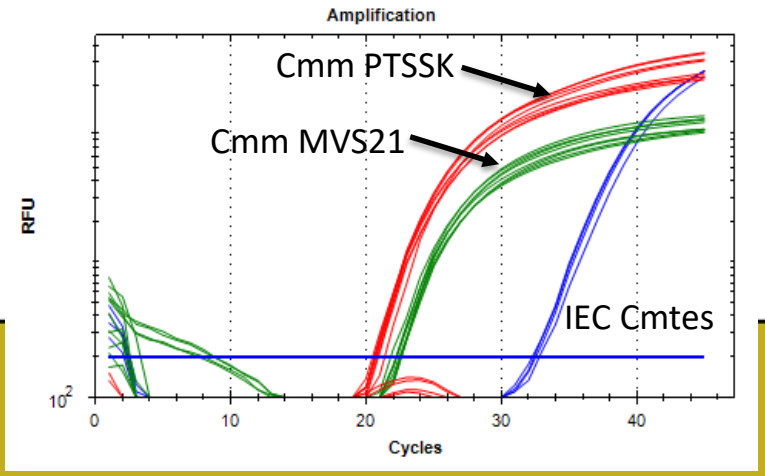




1. Overnight soaking of ≥10,000 seeds in PB
2. Bagmixer extraction
3. Addition of Cms spike (IEC)
4. Centrifugation
5. Bead beating and lysis of bacteria
6. DNA purification with Sbeadex kit (LGC)
7. Triplex Taqman PCR using PTSSK, MVS21 and NAKT21 (IEC)







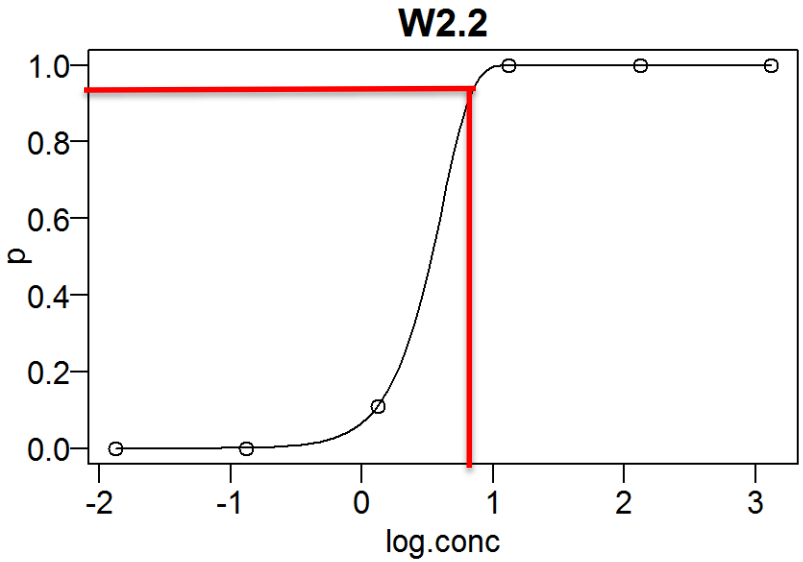
Analytical sensitivity

Approach:

1. 3 times 10 fold dilution series in seed extract and buffer
2. Dilution plating and DNA measurement
3. Positive or negative with dilution plating (3 reps)/Taqman for PTSSK and MVS21 (9 reps)

PTSSK	cells/ml					
Taqman	22100	2210	221	22,1	2,21	0,221
1A	27,1	30,78	33,54	45	45	45
1B	27,47	30,57	33,79	45	45	45
1C	27,47	30,56	33,05	35,71	45	45
2A	27,18	30,55	34,37	45	45	45
2B	27,24	30,19	33,42	41,97	45	45
2C	26,89	30,04	33,5	44,63	45	45
3A	27,12	30,4	33,23	45	45	45
3B	28,17	30,68	35,13	45	45	45
3C	27,26	30,9	34,22	45	45	45
# positives	9	9	9	1	0	0

Analytical sensitivity



PTSSK Taqman		
		Weibull
Best fitting model:		2.2
no concentration		
log p95	0,87	
p95	8	cells/ml
after concentration		
log p95	2,09	
p95	130	cells/ml

MVS 21 Taqman		
no concentration		
log p95	0,70	
p95	5	cells/ml
After concentration		
log p95	1,92	
p95	75	cells/ml

Analytical specificty Cmm Taqmans

	Real Cmm	Lookalike
PTSSK Taqman positive	True positive 53	False positive 0
PTSSK Taqman negative	False negative 0	True negative 24

	Real Cmm	Lookalike
MVS21 Taqman positive	True Positive 53	False positive 0
MVS21 Taqman negative	False negative 0	True negative 24

67 ° C!

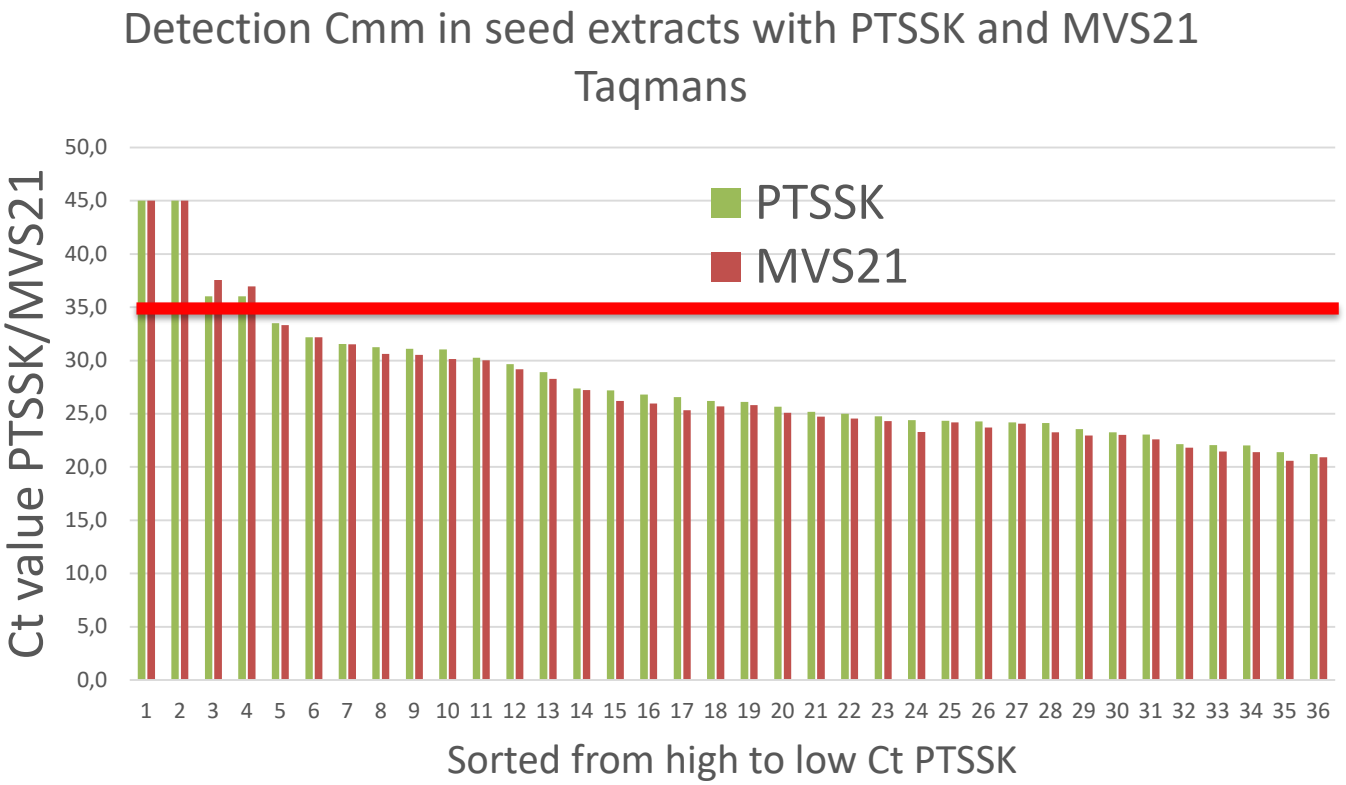
Repeatability data

Sample	Composition
1	10000 seeds of positive seed lot ZZB134
2	10000 seeds of positive seed lot ZZB471
3	1000 seeds of positive seed lot ZZB390 and 9000 seeds of negative seed lot ZZB3

Average Ct-value per sample and standard deviation

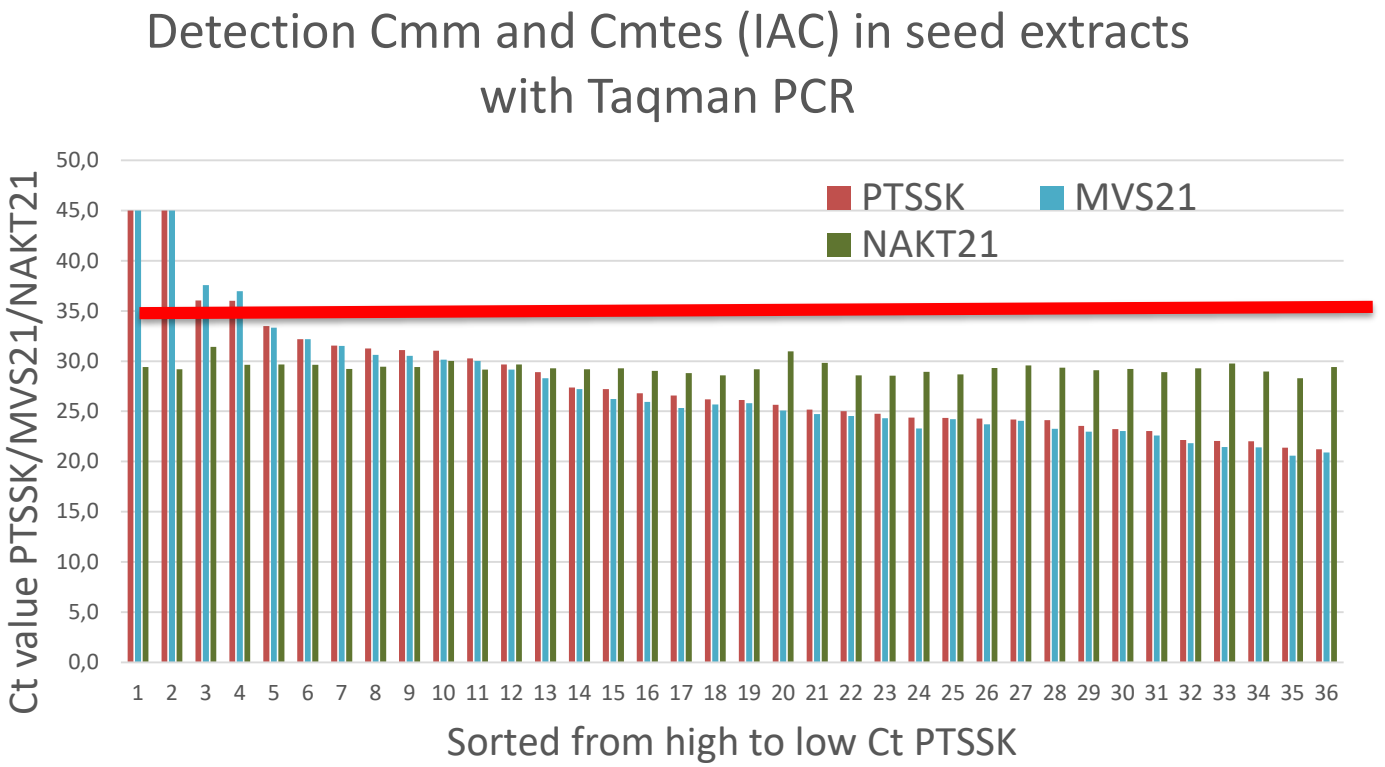
	Sample		
	1	2	3
PTSSK	28.80 ± 0.28	23.57 ± 0.18	26.17 ± 0.18
MVS21	29.01 ± 0.38	23.46 ± 0.16	26.38 ± 0.27
NAKT21 (IAC)	31.78 ± 0.26	32.32 ± 0.79	31.47 ± 0.59

Trueness seed extract Taqmqn PCR



No false negatives with seed extract PCR (“CP reference”)

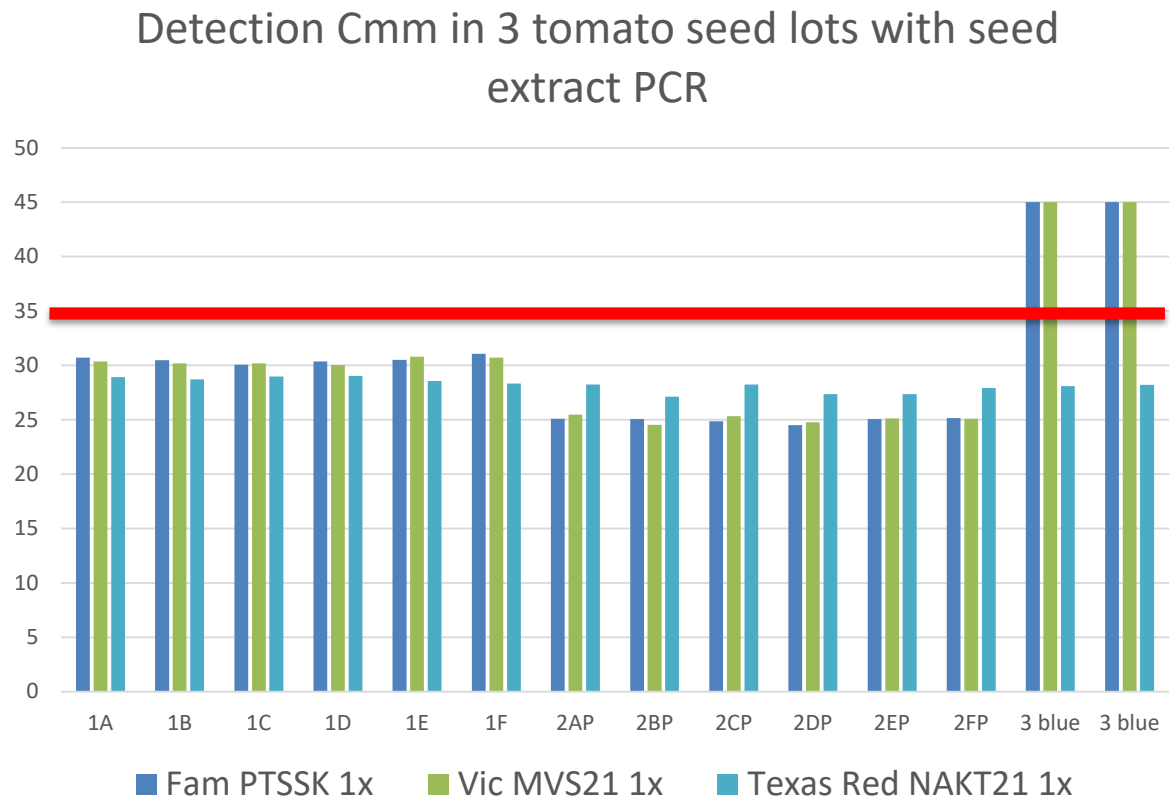
Trueness seed extract Taqman PCR



180 CFU Cmtes/ml

IAC Cmtes suitable

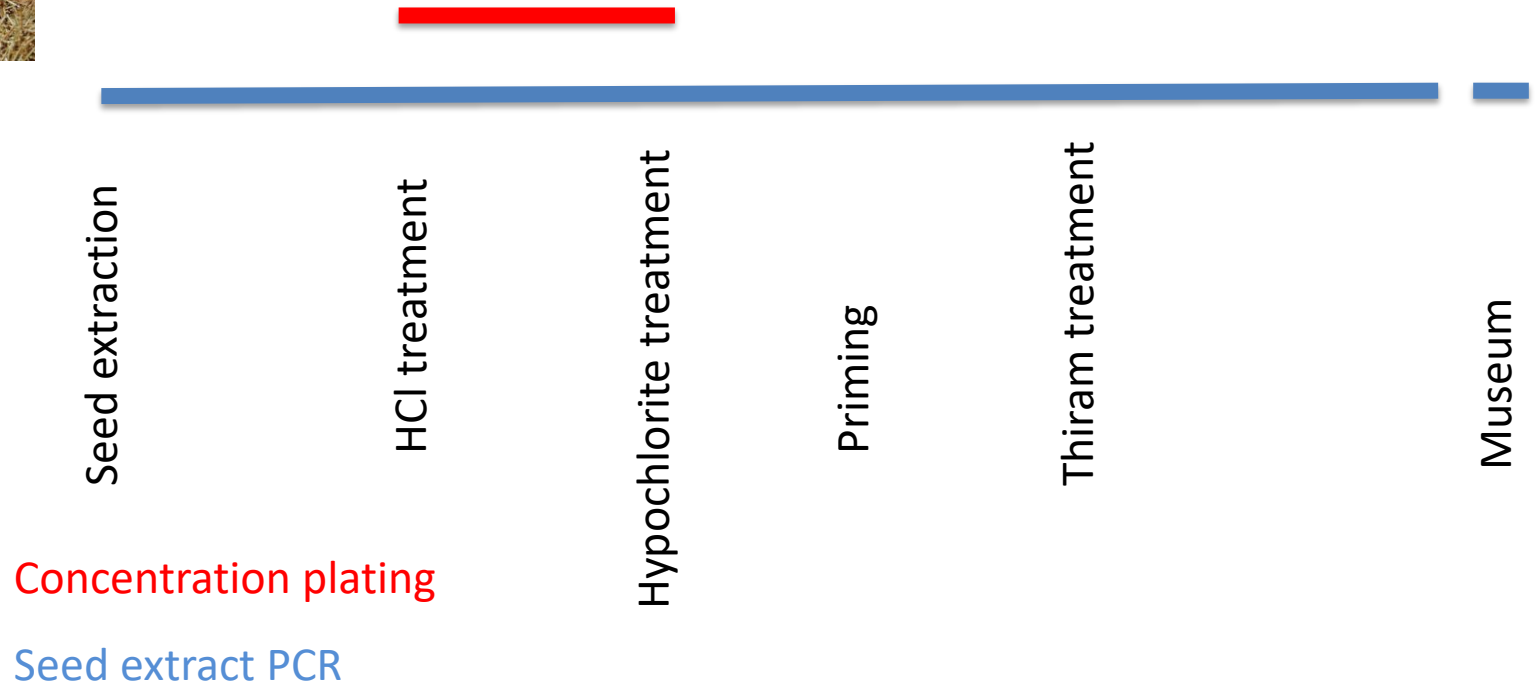
Detection Cmm in seed lots with variable treatments



Conclusions

- High throughput seed extract PCR developed and validated
  - All dilution plate positive samples detected
  - Kingfisher platform compatible with GP bacteria
  - Robust assay irrespective of age/treatment seeds
- Complementary Cmm Taqmans available
- Cmtes IEC available for seed extract PCR

Wide window for seed extract PCR



Pros and cons

	Concentration plating	Seed extract PCR
Speed	2-5 weeks	2 days
Costs	high	“low”
High through put	no	yes
Dead or Alive	Only viable cells	Dead and alive
Treatment interference	strong	weak
Process control	Partly (“late spike)	Yes (early spike)
Colony isolation	yes	no
Validation	yes	yes

Acknowledgements

Seed companies for providing seeds
Debby Beugelsdijk for work on Cmm Taqmans

Thank you! Any questions?

Testa

Quality in Horticulture