Enrichment procedures to improve detection of *Clavibacter michiganensis* subsp. *michiganensis* in seed extracts with a dilution plating, a TaqMan PCR and a LAMP assay

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SEVENTH FRAMEWORK PROGRAMME

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Introduction

- *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is a seed-borne pathogen, the causal agent of bacterial canker of tomato
- This harmful bacterial is present in most production areas around the world
- There is no commercial resistant cultivar
- Testing of seed and planting material is important in the disease management

Aim of the study

- To improve the sensitivity of the detection assay for Cmm in seed by an enrichment procedure (i.e. enrichment of the pathogen in the tomato seed extracts prior to detection)

Research strategy

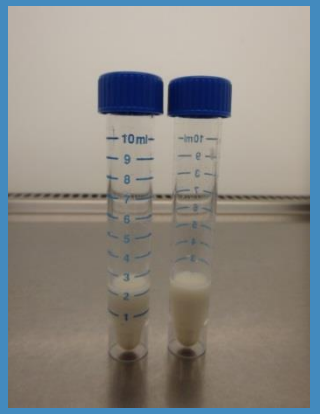
- Find conditions that favour growth of Cmm spiked to a tomato seed extract
    - Diluent, antibiotics
    - Different strains
- Generate tomato seeds (internally) infected with GFP-tagged strain of Cmm
    - Scarification, imbibition, vacuum infiltration, incubation, storage
    - Evaluation by dilution plating, microscopic studies
- Development of enrichment procedure using spiked seed lots
- Evaluation of the enrichment with naturally-infected seed lots

Finding conditions for selective growth of Cmm in seed extracts

Tomato seeds
+ PBST

Incubation
overnight
4°C

Bag Mixer ®(400cc) Lab
Blender
7 min - speed 4



Finding conditions for selective growth of Cmm in seed extracts

Trimethoprim (80 mg/l),
Nalidixic acid (20 mg/l)
Nystatin (100 mg/l)

Spiked with Cmm
or infected seeds

Incubation at
25°C /5d
with agitation

Plating on
SCM fast

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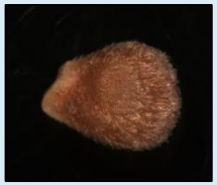
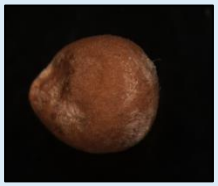
Conclusions

- Cmm is resistant to the concentrations of antibiotics in an enrichment broth commonly used in semi-selective agar media for Cmm
- No growth of Cmm was found in seed extracts prepared with PBST buffer without the added antibiotics
- No growth was found in seed extracts prepared with 0.1XTBY broth plus antibiotics
- No difference in growth between 3 Cmm strains in seed extracts with antibiotics
- The extension of the incubation period to 5 days did not further increase densities

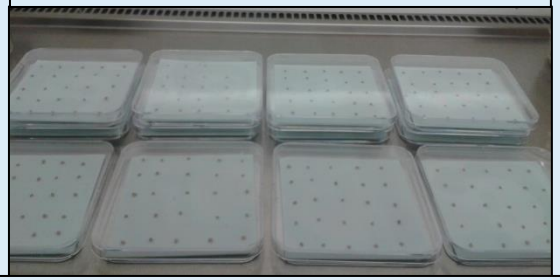
Production of internally infected seeds



Scarification
silversand+stones
vortex/10 min



Imbibition



blotter wetted with Ringer
16h/25°C

Vacuum infiltration

* IPO3525 (GFP)
* water

Disinfection

Ethanol 70% /1min
washed in sterile water/1min

Drying

Low Cmm concentration

Stored (4°C)

Incubation on blotter wetted with TBY 10X diluted +
(Trimethoprim, Nalidixic acid, Cycloheximide, kanamycin and rifampicin)
3 days at 25ºC

High Cmm concentration

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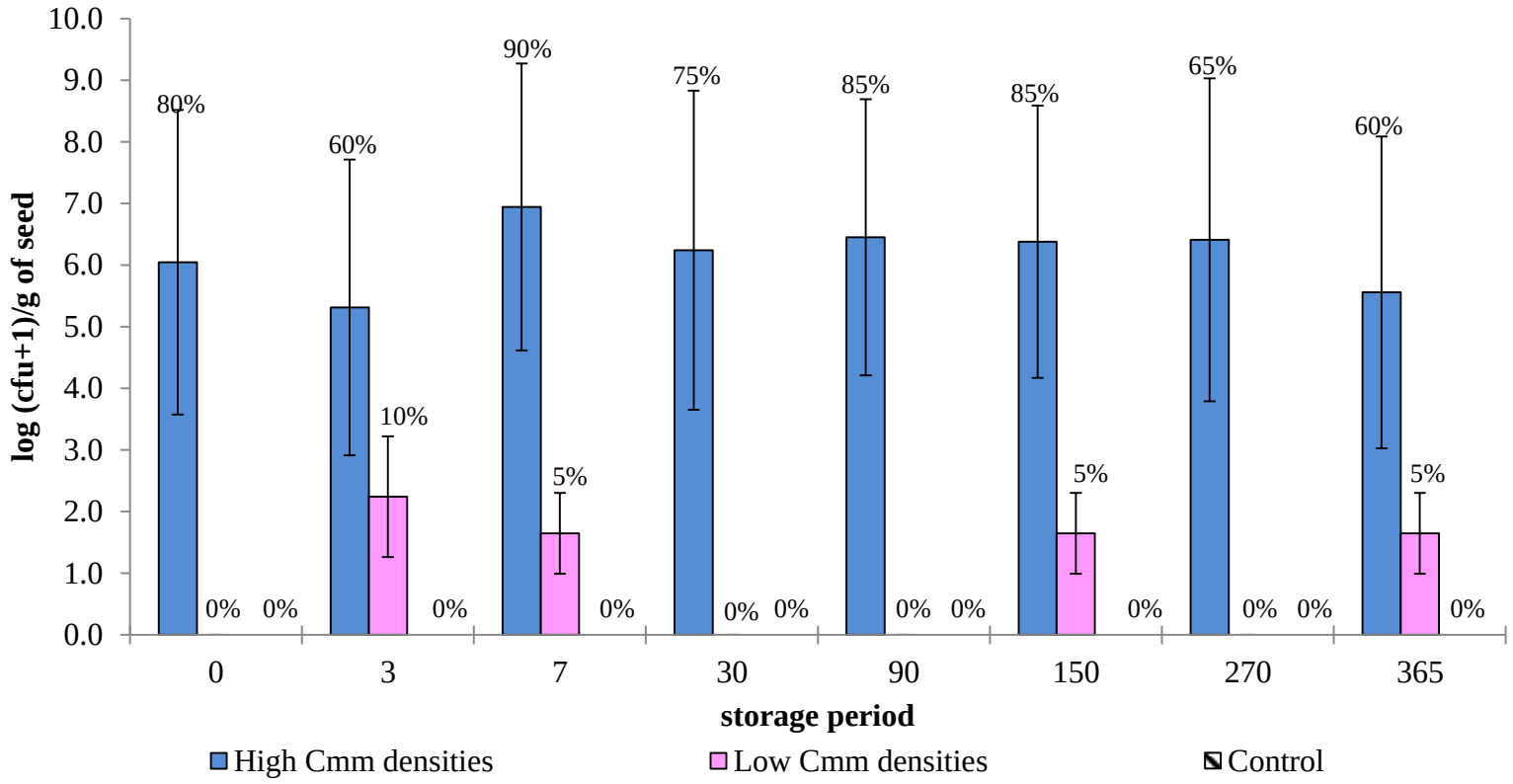
The experiment was repeated

Analysis of infected seeds

- Stored seeds were analysed 8 times in the first repetition and 6 times in the second repetition over a period of maximally one year
- 20 seeds were incubated for 1-5 days on TBY medium supplemented with Rifampicin and Kanamycin (TBY^{ab}) at 25°C and analysed individually by
  - pour-plating (GFP positive colonies per seed were counted using an epifluorescence stereomicroscopy (ESM))
  - ESM: the number of ESM positive seeds were counted
  - Confocal Laser stereomicroscopy: to look for internal infections

Storability artificially contaminated seed lots (storage at 4°C - n=20)



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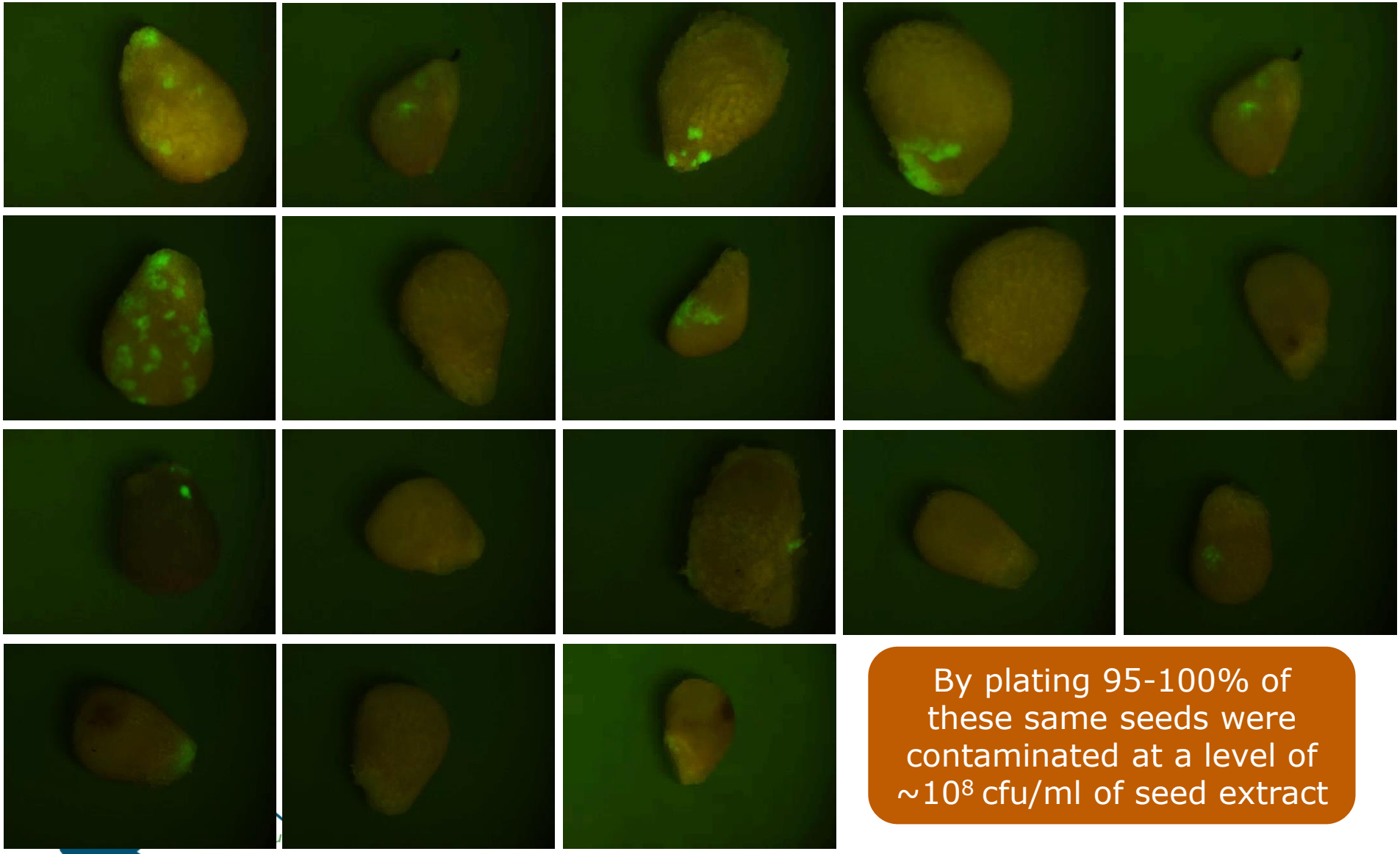
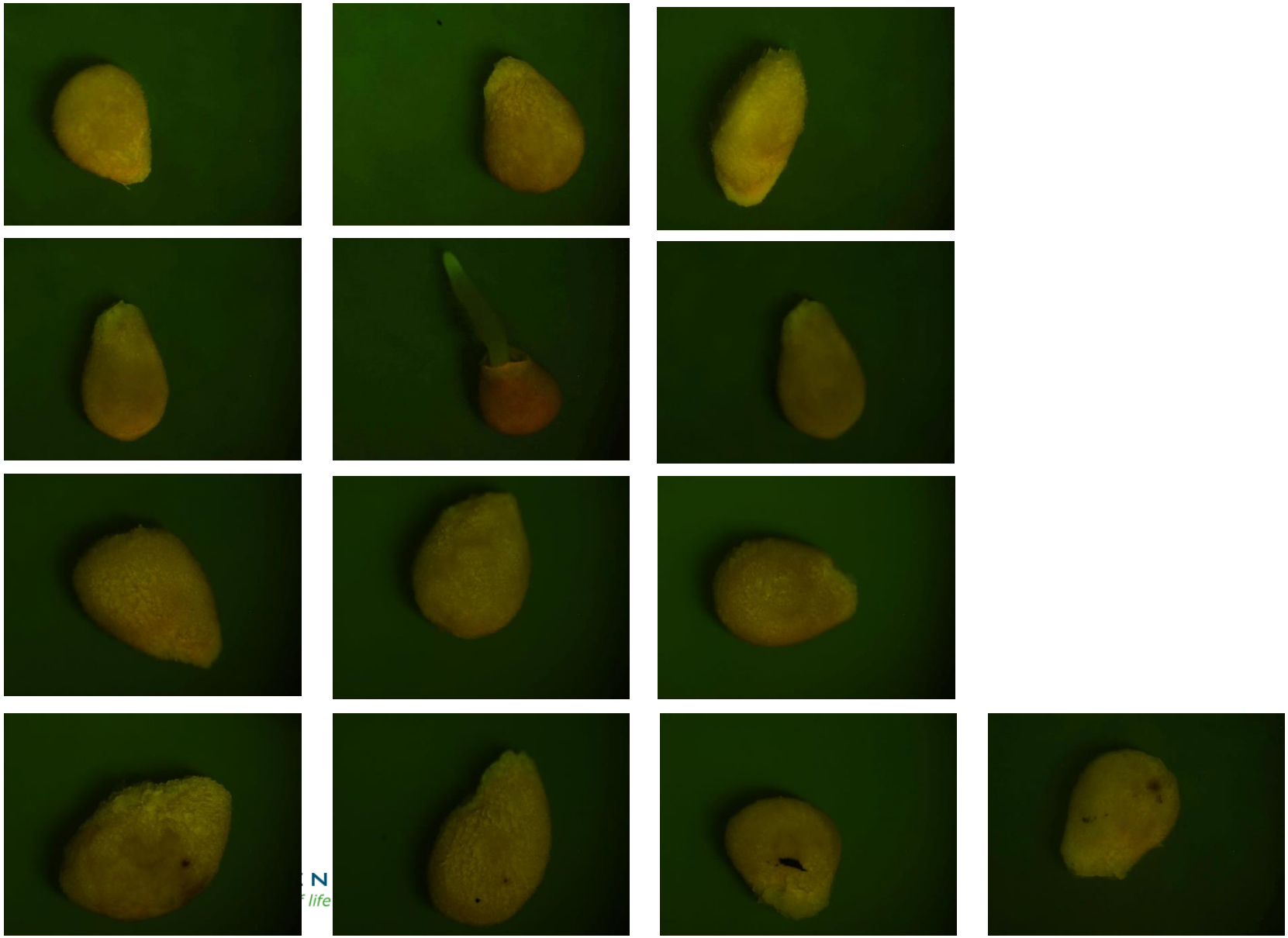
Seeds with low densities, stored for 30 d at 4°C and analysed 3 days after incubation at 25°C on TBY[ab]

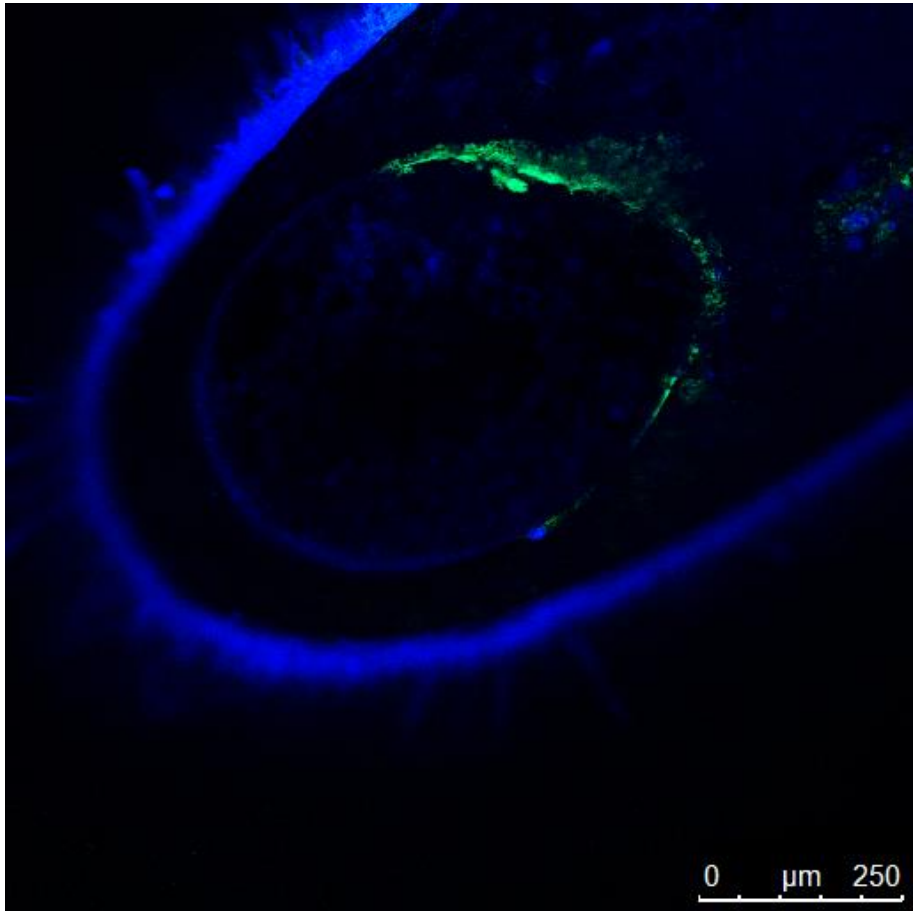


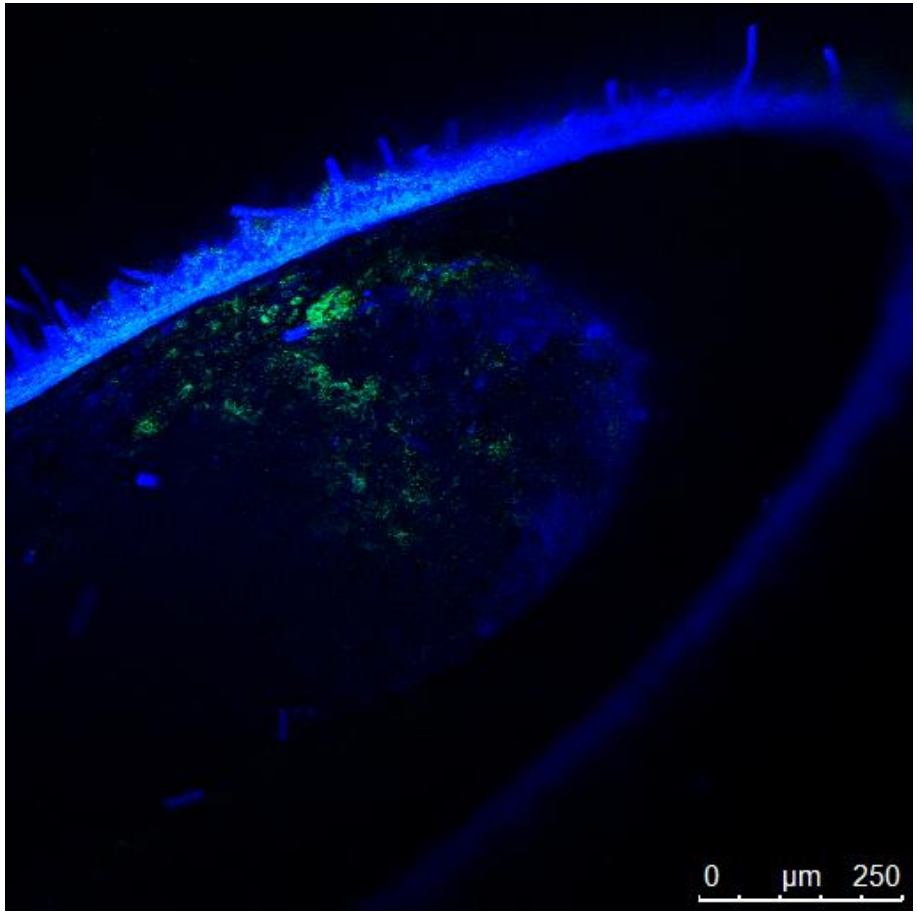
By plating 95-100% of these same seeds were contaminated at a level of ~10^8 cfu/ml of seed extract

Water-inoculated control



Highly infected seeds at 1 day after incubation on TBY[ab]





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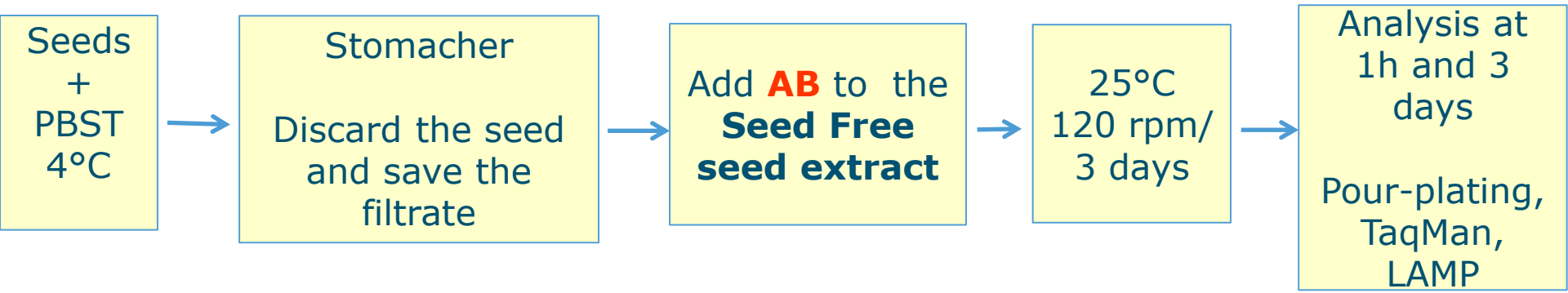
Conclusions

- Seeds were generated which were homogeneously and (very likely) internally infected with Cmm, both at a low and a high level of infection
- Infected seeds that were individually tested were frequently negative if directly tested by pour-plating in TBY^{ab}, but positive after incubation of seeds for 3 days on TBY^{ab}
- This result shows that dilution-plating of tomato seed extracts bears a risk for false-negative results
- Almost the total volume of the extracts of the individual seeds was plated; the negative results can therefore not be explained by the low infection levels

Development of enrichment procedure

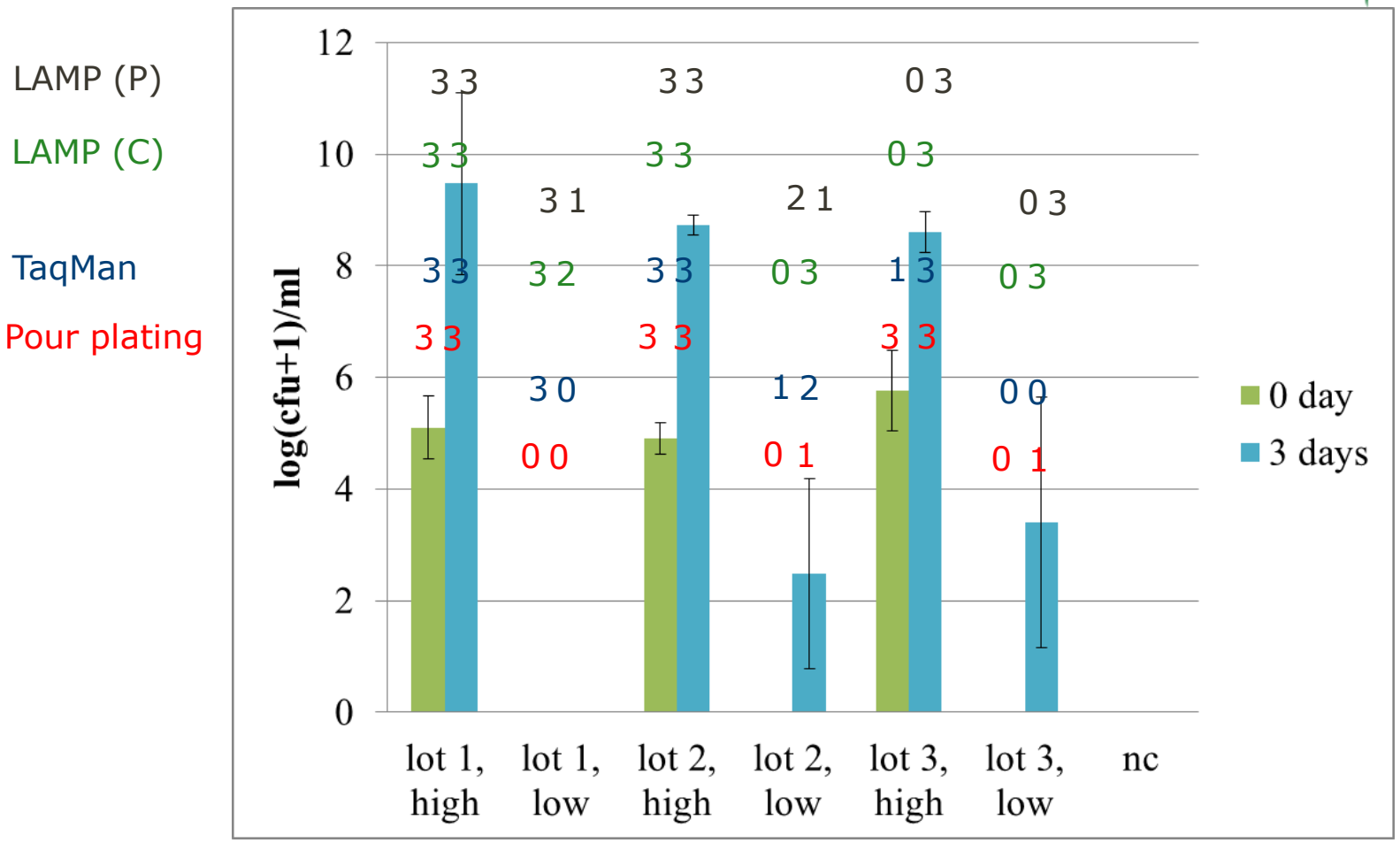
Development of the enrichment procedure using spiked seed lots

- 3 seed lots (De bolster N1, Bejo, Nunhems TOP136)
- 3 subsamples (1500 seeds/treatment)
- Spiked using 5 seeds with high and low levels of Cmm contamination



- TaqMan assay based on the RZ_Ptssk primers (Sen et al., 2013)
- Loop-mediated amplification (LAMP) (Yasuhara-Bell et al.,2013)
- The method used for extraction was EPICENTRE QuickExtract RNA Extraction.
- LAMP cox primers (Tomlinson et al., 2010b. Phytopathology100, 143–9)

Effect enrichment Cmm in tomato seed



Pour plating in TBY[ab];
LAMP (C): crude extract (Epicentre)
LAMP (P): pure DNA

Conclusions

- Enrichment for 3 days resulted in at least 1,000-fold increase of Cmm in seed lots spiked with highly (internally)-infected seeds.
- Cmm-infected seed samples negative in TaqMan became positive after enrichment

Evaluation of the enrichment procedure with naturally-infected seed lots

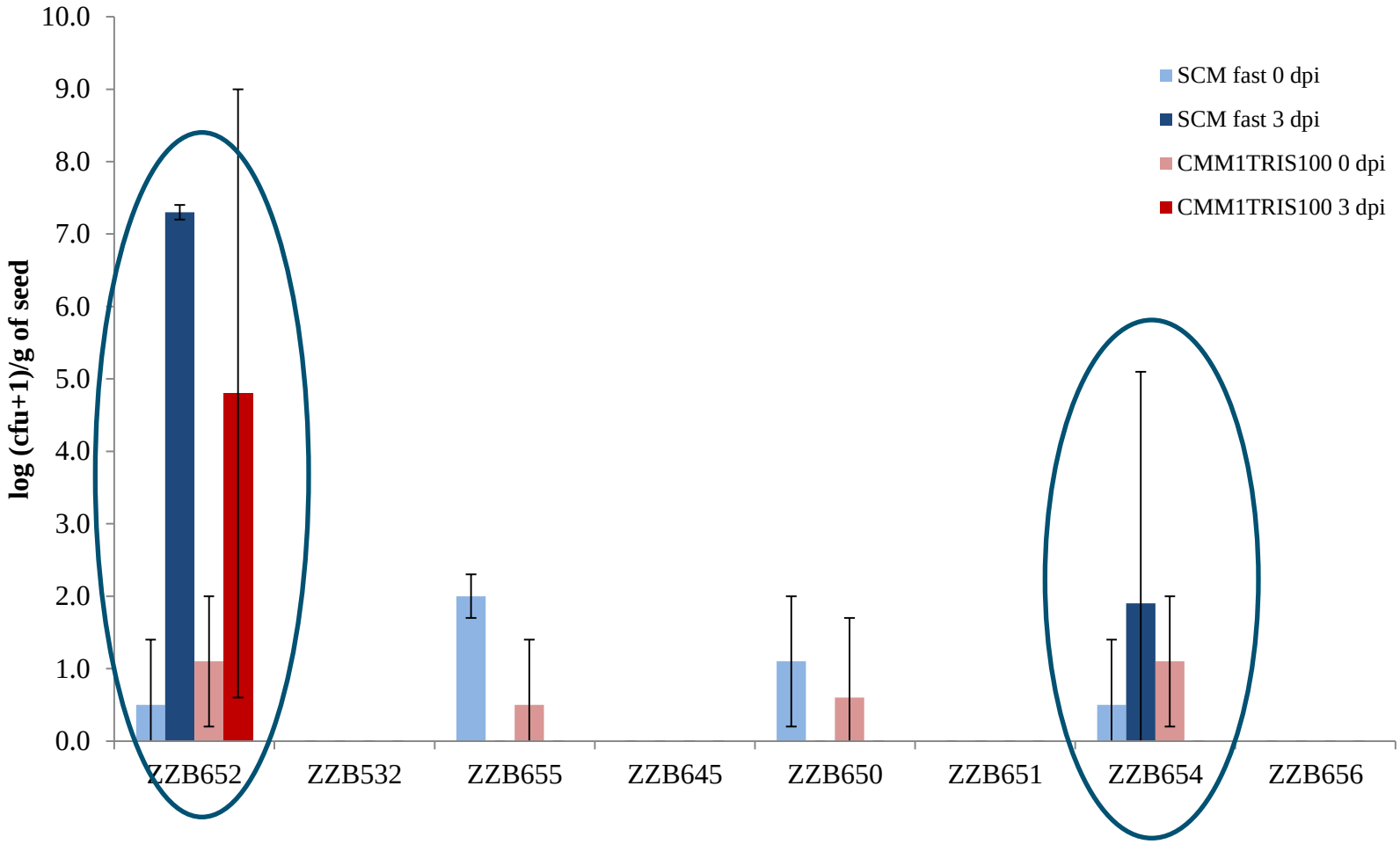
- The procedure was evaluated with 8 seed lots naturally infected with Cmm

Naturally infected seed lots					
Seed lots	**year**	**Treatment***	**background bacteria log(cfu+1/g of seed)**	**TaqMan 2015**	**Lamp 2015**
ZZB532			3,13	+	+
ZZB655	2012	HCL-TSP	3,50	+	+
ZZB645	2012	HCL-TSP	2,90	+	+
ZZB652	2012	HCL-TSP	2,52	+	+
ZZB650	2012	HCL-TSP	2,39	+	+
ZZB651	2012	HCL-TSP	2,95	+	+
ZZB654	2012	HCL-TSP	2,73	+	+
ZZB656	2012	HCL-TSP	2,88	+	+
* HCL- hydrochloric acid ; TSP- trisodium phosphate					

Evaluation of the enrichment procedure with naturally-infected seed lots

- ✓Three sub-samples of 9,000 seeds
- ✓Each seed lot were added to BIOREBA bag soaked in PBST and incubated as described before in the final protocol for enrichment
- ✓ The seed extract was analysed before and after enrichment by dilution plating on SCM fast and CMM1Tris100 and TaqMan assay

Pour-plating results



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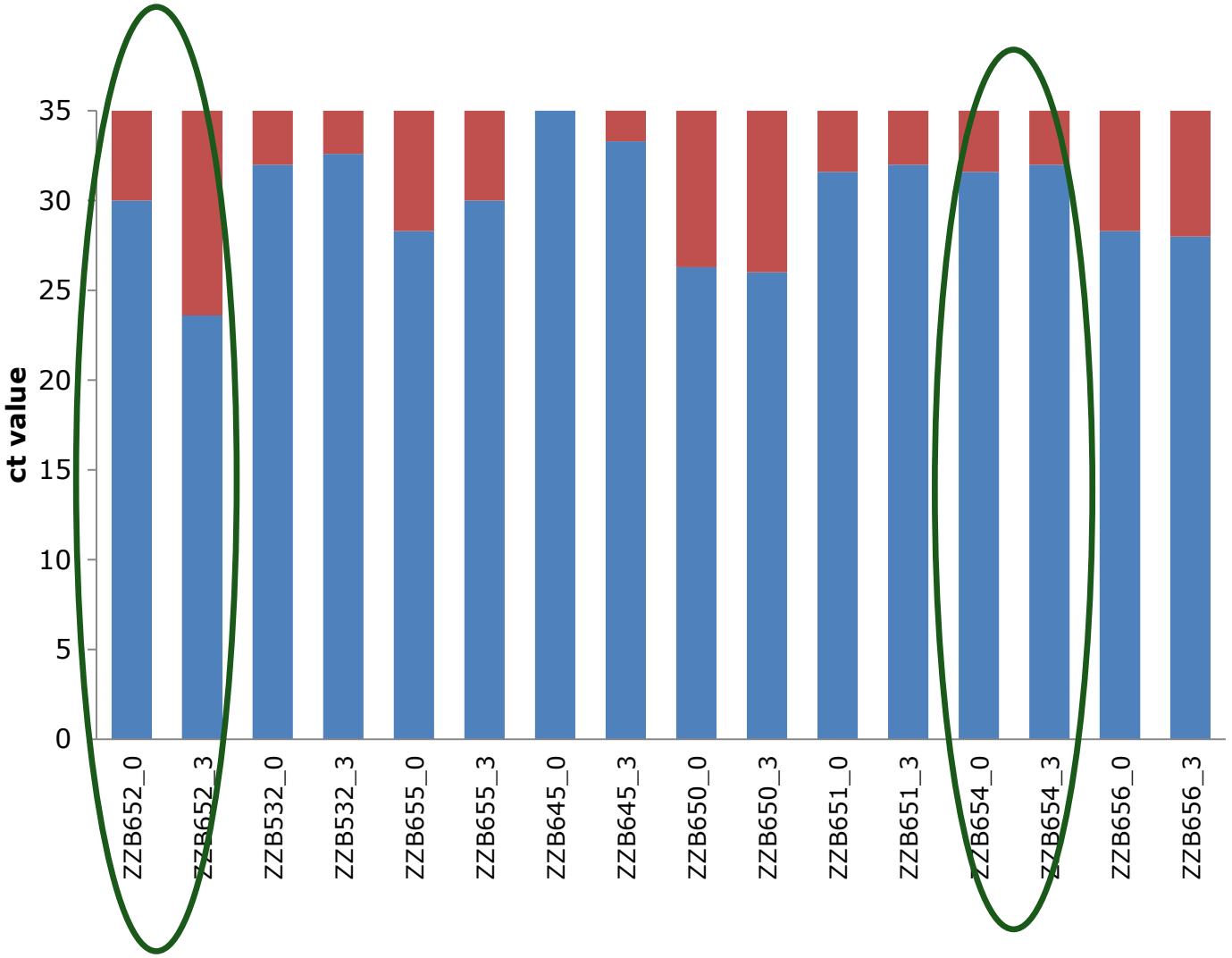
TaqMan Results



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Estimated incidence of Cmm positive seeds before and after enrichment in naturally contaminated tomato seed lots

Seed lots	Estimated incidence (%)			
	0 dpi		3 dpi	
	SCM fast	CMM1TRIS100	SCM fast	CMM1TRIS100
ZZB655	>0.04	0.01	0.00	0.00
ZZB652	0.01	0.04	> 0.04	0.04
ZZB650	0.04	0.04	0.00	0.00
ZZB654	0.01	0.04	0.01	0.00

Conclusions

- Enrichment can be easily integrated in the current protocol for detection of Cmm in tomato seeds (ISF, 2014).
- In the first part of the protocol the same steps are followed as in the protocol currently in use at the seed companies.
- Seed extracts can be analysed straight away by dilution plating, immunofluorescence or molecular techniques and additionally used for incubation under selective conditions.
- Enrichment may fail in case of damaged cells, cells in a VBNC state or the presence of high densities of micro-organisms that reduce the growth of Cmm in seed extracts

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