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Detection of pospiviroids on seeds of tomato and pepper

3rd meeting of the Panel on Virology and Phytoplasmaology
Thursday 16th 2014, Rome, Italy

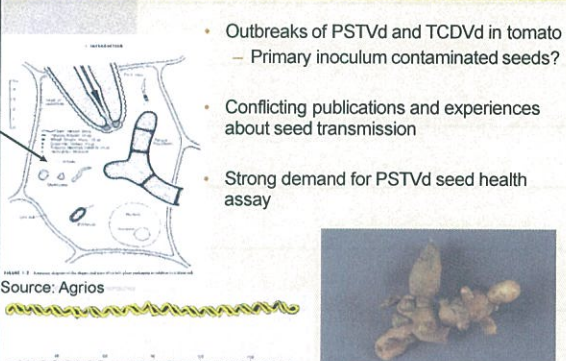
Harrie Koenraad, Ko Verhoeven (National Plant Protection Organization), André van Vliet, Agata Jodłowska, Michel Ebskamp and Maaïke Bruinsma (Naktuinbouw)

Overview presentation



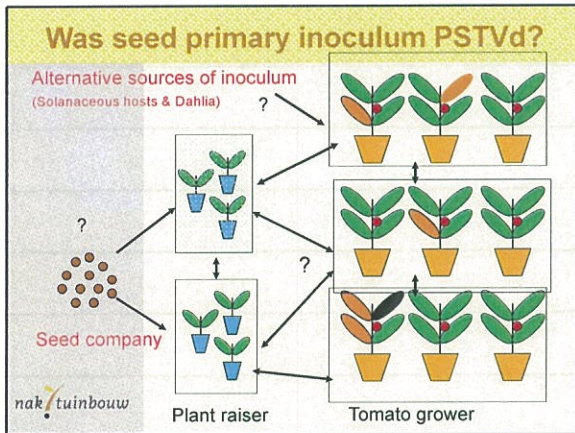
- Introduction pospiviroids
- Evolution of PSTVd assay for seeds of tomato
- Validation of PSTVd assay for seeds of tomato
- Development pospiviroids assays for tomato and pepper seeds
- Additional research at nVWA/ Naktuinbouw

Introduction



- Outbreaks of PSTVd and TCDVd in tomato
– Primary inoculum contaminated seeds?
- Conflicting publications and experiences about seed transmission
- Strong demand for PSTVd seed health assay

Source: Agrios



Evolution of PSTVd assay


- PSTVd version 1.0
 - Grow out of 300 plants and then rPAGE
- PSTVd version 2.0
 - Direct seed assay using 3,000 seeds
 - Manual RNA extraction (Epicentre kit)
 - TaqMan RT-PCR (Boonham *et al.*, 2004)
 - Internal amplification control (IAC) based on Cox amplicon (DNA & RNA)

Evolution of PSTVd assay

PSTVd version 3.0

- Adaptations in extraction
 - Lysis buffer PN1 (LCG)
 - Interscience bagmixer to extract "internally located" PSTVd/TCDVd/MPVd from seeds
- High throughput RNA extraction Sbeadex Maxi Plant Kit (LCG) – KingFisher
- New Internal amplification control (IAC): *nad5*, NADH dehydrogenase subunit 5 (only RNA)

Validation PSTVD version 3.0



EPPO & Dutch guideline for the validation

- Limit of Detection (LOD)
- Measuring range
- Analytical specificity
- Selectivity
- Reproducibility / Repeatability
- Robustness

• **Scope:**
 Detection of *Potato spindle tuber viroid* (PSTVd) and/or *Tomato chlorotic dwarf viroid* (TCDVd) in tomato seed with real-time RT-PCR based on TaqMan® technology

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Limit of Detection

- Lowest value, in a laboratory sample, of the target pathogen, which can still be determined with a certain degree of reliability
- Requirement: 1 "contaminated seed" detected/999 healthy seeds
- **Set-up**
- 4 contaminated seed lots:
 - 3 seed lots from PSTVd-inoculated tomato plants
 - PSTVd-PD01 / PD02 / PD03
 - 1 naturally infected TCDVd seed lot
 - TCDVd-Nakt01
- Ct value < 32 to allow sequence verification

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LOD results

Contaminated seed lot	Average Ct-value 1+/999-	
	Singleplex PSTVd/TCDVd	St. dev (n=8)
PSTVd-PD01	24.8	1.30
PSTVd-PD02	30.9	0.98
PSTVd-PD03	24.3	0.78
TCDVd-Nakt01	29.0	1.62

- All natural and artificial contaminated seed lots positive for PSTVd / TCDVd
- Variable viroid load

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Repeatability & Reproducibility

• 8 samples - 8 time points


sample	contaminated seed lot	ratio contaminated/not-contaminated seeds	not contaminated seed lot	Timepoint									
				1	2	3	4	5	6	7	8		
1 Repeatability	PSTVd-PD03	1/999	E	xx									
1 Reproducibility										x			
2 Repeatability	PSTVd-PD01	1/999	M							xx			
2 Reproducibility				x									
3 Repeatability	PSTVd-ZZB 498	100/900	AA		xx								
3 Reproducibility												x	
4 Repeatability	PSTVd-ZZB 498	100/900	N									xx	
4 Reproducibility					x								
5 Repeatability	PSTVd-PD03	5/995	G			xx							
5 Reproducibility													x
6 Repeatability	TCDVd-Nak01	5/995	L										xx
6 Reproducibility						x							
7 Repeatability	TCDVdNak01	10/990	Q					xx					
7 Reproducibility													x
8 Repeatability	PSTVd-PD01	5/995	O										xx
8 Reproducibility									x				

Repeatability & Reproducibility

	Sample 1: 1/999		Sample 2: 1/999		Sample 3: 100/900		Sample 4: 100/900					
	Repeat.	Repr.	Repeat.	Repr.	Repeat.	Repr.	Repeat.	Repr.				
PSTVd/TCDVd	24.3	23.6	23.4	24.5	24.6	23.4	30.1	29.3	30.3	26.7	28.0	28.4
PSTVd/TCDVd + nad5	24.5	24.0	24.2	24.5	24.7	23.7	29.8	29.2	30.1	26.4	27.4	28.2
detected	+	+	+	+	+	+	+	+	+	+	+	+

	Sample 5: 5/995			Sample 6: 5/995			Sample 7: 10/990		Sample 8: 5/995			
	Repeat.	Repr.		Repeat.	Repr.		Repeat.	Repr.	Repeat.	Repr.		
PSTVd/TCDVd	21.7	21.4	19.6	29.4	28.9	28.5	27.7	28.3	26.7	23.0	22.1	20.1
PSTVd/TCDVd + nad5	22.3	22.2	20.0	29.8	29.1	29.1	27.6	28.4	26.4	23.4	22.0	20.4
detected	+	+	+	+	+	+	+	+	+	+	+	+


• All positive samples detected: R&R 100%
 – No discrepancy between repeatability / reproducibility
 – No difference between singleplex or multiplex (+ nad5) PCR
 – All negative samples no PSTVd/TCDVd signal



Current practice PSTVd

- ISO17025 accreditation for tomato/PSTVd
- >2300 commercial tomato seed lots tested for PSTVd, TCDVd and MPVd
- 5 seed lots positive for PSTVd: all subs positive!
- All blind samples with PSTVd detected

Develop and validate new broad pospiroid assay for matrix tomato (and pepper) seeds



Pospiviroids tomato and peppers

viroid	tomato	pepper
CEVd	++	
CLVd	++	++
CSVd	+	
IrVd-1		
MPVd	+	
PCFVd	+	++
PSTVd	++	++
TASVd	++	++
TCDVd	++	
TPMVd	++	

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Yellow (artificially host)
Red (natural host)

Develop generic pospiviroid assay tomato (EU TESTA WP5)

Pilot experiments with PSTVd contaminated tomato seeds:

- Two tube generic pospiviroid "Botermans et al." assay
 - PSTVd not detected
- Use available Taqmans for other relevant pospiviroids (CEVd, CLVd, TASVd) (FERA)
- Design new Taqmans for PCFVd and TPMVd (Naktuinbouw)
- New viroid internal amplification control (Old Nad5 IAC highly variable in seeds (Ct 13-40)
 - DLVd (Dahliae latent viroid)
 - Wide spread non pospiviroid in dahlia
 - Spike at start of extraction each subsample
 - Ct DLVd \leq 32 for valid test result

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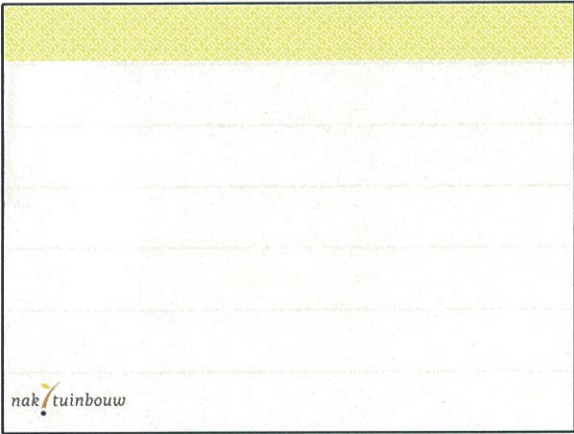
Genpospi for matrix tomato seeds

- Improved soaking of tomato seeds (delta ca. Ct 2-3)
 - GH+ buffer rather PN1 LGC
 - 30-60 min. at RT rather than overnight soak
 - Spike viroid (DLVd) rather than endogenous NAD5
- Extraction time of minimixer
 - 90 seconds OK

Multiplex* Taqman	Pospiviroid targets		Internal amplification control (IAC)
	FAM	VIC	TR
Tube A	PSTVd, TCDVd and MPVd*	PCFVd**	DLVd
Tube B	CEVd**, CLVd**		DLVd
Tube C	TPMVd**		Nad5 RNA
Tube D	TASVd*		No IAC

* FERA
** Naktuinbouw

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Genpospi version 1.0 pepper

- ZB13 pepper seeds positive in genpospi Taqmans (TASVd, CLVd or DLVd)
 - Sequence analysis NPPS (Ko Verhoeven)
 - TASVd (first report for pepper)
 - CEVd/CLVd (partial sequence)
 - Ca. 1 in 400 seeds (high load of TASVd)
- Biological relevance not known in pepper
- Workshop met LGC (Heiko Hauser) to investigate RNA extraction for pepper seeds



Optimization extraction buffers tomato and pepper seeds

Detection PSTVd and DLVd with 3 extraction with tomato seed ZB453 (N=8)

Extraction Buffer	Time	Cl target (approx.)
PSTVd/FAM6	15 min	25
	30 min	25
	30 min	25
DLVd/AC (Teesa Red)	15 min	28
	30 min	28
	30 min	28

Detection TASVd, CLVd and DLVd with 2 extraction buffers with pepper seed ZB13 (n=8)

Extraction Buffer	Time	Cl target (approx.)
CEN/CLW (FAM6)	30 min	25
	30 min	25
	30 min	25
DLVd (Teesa Red)	30 min	25
	30 min	25
	30 min	25




Additional research at nVWA/Naktuinbouw

Seed transmission experiments

- 100% TCDVd contaminated tomato seed: 4000 seedlings - no seed transmission (no transplant phase)
- 100% PSTVd contaminated tomato seeds: 1000 PSTVd transplanted tomato seedlings no seed transmission
- Ca. 1% TASVd/CLVd pepper seeds: 1000 seedlings underway

Seed transmission not very efficient for seeds of tomato!



Additional research at nVWA/Naktuinbouw

Taqman development


- "Botermans Taqman" not suitable for matrix seeds
- Marleen Botermans has designed new Taqmans

Identification direct Sequencing difficult in matrix seeds

- NGS for identity verification?



Spread PSTVd during tomato seed processing

- All "healthy" seeds covered with PSTVd



Conclusions

- Validated PSTVD assay available for matrix tomato seed with KF RNA extraction
- NPPS permission for broader pospiviroid specific Taqmans:
 - High throughput (KF-Sbeadex) for tomato seeds
 - Low throughput (Rneasy) for pepper seeds
- Much more PCR inhibition in matrix pepper seed but with improved washing & Sbeadex kit Kingfisher high throughput is now possible
- First report of TASVd on pepper seeds
- Extraction time in bagmixer and extraction buffer affect sensitivity of Taqman and especially classic detection strongly
- Very dependable new IAC mimics targets
- Ready to start with validation pospiviroid assay in framework of EU-TESTA (protocols available for EPPO)

<http://www.naktuinbouw.nl/sites/naktuinbouw.eu/files/20140801Tomatopopsiviroids.pdf>

<http://www.naktuinbouw.nl/sites/naktuinbouw.eu/files/20140801Pepperpospiviroids.pdf>

Thanks Marleen Botermans (nVWA), Ellis Meekes and Daniel Bakker (Naktuinbouw)



Thanks for your attention

Questions?

