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1 **Development and validation of a real-time RT-PCR test for screening pepper and tomato**
2 **seed lots for the presence of pospiviroids**

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15 Abstract

16 Potato spindle tuber viroid and other pospiviroids can cause serious diseases in potato and tomato
17 crops. Consequently, pospiviroids are regulated in several countries. Since seed transmission is
18 considered as a pathway for the introduction and spread of pospiviroids, some countries demand
19 for the testing of seed lots of solanaceous crops for the presence of pospiviroids. A real-time RT-
20 PCR test, named PospiSense, was developed for testing pepper (*Capsicum annuum*) and tomato
21 (*Solanum lycopersicum*) seeds for seven pospiviroid species known to occur naturally in these
22 crops. The test consists of two multiplex reactions running in parallel, PospiSense 1 and
23 PospiSense 2, that target Citrus exocortis viroid (CEVd), Columnea latent viroid (CLVd), pepper
24 chat fruit viroid (PCFVd), potato spindle tuber viroid (PSTVd), tomato apical stunt viroid (TASVd),
25 tomato chlorotic dwarf viroid (TCDVd) and tomato planta macho viroid (TPMVd, including the
26 former Mexican papita viroid). Dahlia latent viroid (DLVd) is used as an internal isolation control.
27 Validation of the test showed that for both pepper and tomato seeds the current requirements of a
28 routine screening test are fulfilled, i.e. the ability to detect one infested seed in a sample of c.1000

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29 seeds for each of these seven pospiviroids. Additionally, the Pospisense test performed well in an
30 inter-laboratory comparison, which included two routine seed-testing laboratories, and as such
31 provides a relatively easy alternative to the currently used tests.
32

33 Introduction

34 Pospiviroids are single-stranded circular RNA molecules consisting of around 360 nucleotides. The
35 genus *Pospiviroid* is in the family *Pospiviroidae,* with *Potato spindle tuber viroid* (PSTVd) being the
36 type species. Most pospiviroids can infect a wide range of plant species, including many
37 solanaceous ornamental and vegetable crops. Infected plants often remain symptomless, although
38 PSTVd and some other pospiviroids may cause serious diseases in potato and tomato crops (1, 2).
39 For this reason, many countries have implemented phytosanitary measures to prevent their
40 introduction and spread.
41 Pospiviroids may spread by vegetative propagation, mechanical transmission, and to a lesser
42 extent also by insects, pollen and seeds (3, 4).
43 The importance of seeds as a pathway for introduction and spread of pospiviroids in solanaceous
44 fruit crops is still a matter of debate. This is due to the fact that both successful- and failed
45 transmission from infested seeds to seedlings has been reported (5-8). Nevertheless, some
46 countries require mandatory testing of pepper (*Capsicum annuum*) and tomato (*Solanum*
47 *lycopersicum*) seed lots before import. Consequently, there is a need for reliable and cost-effective
48 tests for screening pepper and tomato seed lots for PSTVd and other pospiviroids identified in
49 these crops, i.e. Citrus exocortis viroid (CEVd), Columnea latent viroid (CLVd), pepper chat fruit
50 viroid (PCFVd), tomato apical stunt viroid (TASVd), tomato chlorotic dwarf viroid (TCDVd) and
51 tomato planta macho viroid (TPMVd, including the former Mexican papita viroid).
52 For detection of pospiviroids, several molecular tests are already available but they have their
53 limitations regarding analytical specificity and sensitivity. The generic tests described by
54 Botermans et al. (9), van Brunschot et al. (10), and Monger et al. (11) van were designed and
55 validated for generic pospiviroid detection in leaf material, but are not sensitive enough for testing
56 seed lots in which pospiviroid concentrations are generally lower. Other tests, such as the test

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57 described by Boonham et al. (12), are sensitive enough, but can only detect a limited number of
58 species. Naktuinbouw (12-15) designed and validated a generic seed test, which is currently
59 recommended by the International Seed Federation (16). This test consists of four parallel
60 reactions that allow detection of one infested seed in a sample of c.1000 seeds for each of the
61 seven pospiviroid species. A new test, therefore, should perform equally well and preferably
62 reduces the number of reactions.
63 This paper describes the development and validation of a real-time RT-PCR test (PospiSense) for
64 routine detection of the seven pospiviroid species in seeds of pepper and tomato. The test consists
65 of two multiplex reactions running in parallel with a single internal isolation control, and provides an
66 alternative to the currently used tests.
67

68 Materials and methods

69 Isolates used and confirmation of identity

70 Pospiviroid isolates and other pathogens used for test development and validation are presented in
71 Table 1. The identity of the majority of pospiviroid species was confirmed by sequence analysis of
72 the amplicons obtained by conventional RT-PCRs using different primer sets: Pospi1-FW/Pospi1-
73 RE and VidRE/FW (17), Pospi2-FW/ Pospi2-RE (18), the primers described by Shamloul et al. (19)
74 and AP-FW1/RE2 (20). Because of the lower analytical sensitivity of primers VidRE/FW (17) and
75 the primers described by Spieker (21) no amplicon or sequence data were obtained for CLVd
76 isolates from samples 6184939, PPS013 and PPS055. Therefore its presence and identity was
77 verified by a CLVd-specific real-time RT-PCR published by Monger et al. (11). Amplicons were bi-
78 directionally sequenced as described by Van de Vossenberg and Van der Straten (22). The identity
79 of the virus isolates was confirmed by ELISA or sequencing. The identity of *Clavibacter*
80 *michiganensis* subsp. *michiganensis* was confirmed by real-time PCR and a pathogenicity test.
81

82 Test development

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83 Complete genome sequences of the target species (CEVd, CLVd, PCFVd, PSTVd, TASVd,
84 TCDVd and TPMVd) were retrieved from NCBI GenBank and the sequence database of the
85 National Plant Protection Organization of the Netherlands. For all seven pospiviroids sequences of
86 over 130 isolates covering the intra-species variation were selected. Sequences were aligned with
87 the MAFFT alignment tool (23) in Geneious R8 (Biomatters) and manually adjusted. To minimise
88 the number of reactions, primer and probe design focused on the conserved regions shared by
89 different combinations of the seven species. Potentially suitable sites for primers and probes were
90 visually identified and the oligonucleotide design further optimised using PrimerExpress 3 (Thermo
91 Fischer Scientific). Primers and probes were tested in different combinations together with
92 published primers and probes for CLVd (11), resulting in the selection of primers and probes listed
93 in Table 2. Since the selected primers and probes could not be combined in one single reaction
94 without losing (analytical) sensitivity, the final design of the PospiSense test consisted of two
95 reactions run in parallel, named PospiSense 1 and PospiSense 2.
96 In both reactions, dahlia latent viroid (DLVd; genus *Hostuviroid*) was included as an (exogenous)
97 internal isolation control, which was detected by using the primers and probe (Table 2).
98

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Table 1. Overview of isolates (targets and non-targets) and control material used in this study

	Reference code / GenBank acc. no.	Original host	Tested material[a]	Test development	Analytical sensitivity	Analytical specificity	Selectivity	Repeatability Reproducibility Robustness	Inter-laboratory comparison	Diagnostic sensitivity diagnostic specificity relative accuracy
targets										
Pospiviroids										
Citrus exocortis viroid (CEVd)	4719338	*Hibiscus*	1	x		x				x
	4719389	*Hibiscus*	1	x		x				x
	3823889/ EU094208	*Solanum jasminoides*	1	x		x				x
	3823889/ EU094208	*Solanum jasminoides*	3		x		x	x	x	
	89002594/ AY372391	*Solanum lycopersicum*	1	x		x				x
	89002600/ AY372393	*Solanum lycopersicum*	1	x		x				x
	3123575/ EF192396	*Verbena*	1	x		x				x
Columnea latent viroid (CLVd)	6184939	*Capsicum annuum*	2							x
	6184939	*Capsicum annuum*	3		x	x	x	x	x	
	PPS013	*Capsicum annuum*	3		x	x	x	x	x	x
	PPS055	*Capsicum annuum*	3			x	x	x	x	x
	4812065	*Nemanthantus* sp.	1	x	x	x	x			x
	93007481/ AY372392	*Solanum lycopersicum*	1	x						x
	93007481/ AY372392	*Solanum lycopersicum*	2[b]		x	x				
	20904730	*Solanum lycopersicum*	2			x				
Pepper chat fruit viroid (PCFVd)	6184939	*Capsicum annuum*	2							x
	6184939	*Capsicum annuum*	3		x	x	x	x	x	
	3259237/ FJ409044	*Capsicum annuum*	1	x	x	x	x			x
	PPS013	*Capsicum annuum*	3		x	x	x	x	x	x
	20904730	*Solanum lycopersicum*	2			x				
Potato spindle tuber viroid (PSTVd)	5557027	*Capsicum annuum*	3	x		x				x
	5557051	*Capsicum annuum*	3			x				
	5558839	*Capsicum annuum*	3	x		x				x
	5558927	*Capsicum annuum*	3			x				
	5785531	*Capsicum annuum*	3	x		x				x
	6744916	*Capsicum annuum*	4	x		x				x
	PPS020	*Capsicum annuum*	3		x	x	x	x	x	x
	5895974/ AY372400	*Solanum commersonii*	1	x		x				x
	5458889	*Solanum lycopersicum*	1	x		x				x
	5558898	*Solanum lycopersicum*	2			x				
	5558900	*Solanum lycopersicum*	2			x				
	5785652	*Solanum lycopersicum*	2			x				
	5785664	*Solanum lycopersicum*	2			x				
	5785695	*Solanum lycopersicum*	2			x				
	6586364	*Solanum lycopersicum*	2			x				
	6586372	*Solanum lycopersicum*	2			x				
	M16826	*Solanum lycopersicum*	1	x		x				x
	N3/ X17268	*Solanum lycopersicum*	1	x		x				x
	3497501	*Streptosolen jamesonii*	1	x		x				x
Tomato apical stunt viroid (TASVd)	5348604	*Brugmansia*	5	x		x				
	5458774/ KX579067	*Capsicum annuum*	4	x		x				
	PPS055	*Capsicum annuum*	3		x	x	x	x	x	x
	3153272	*Cestrum*	1	x		x				x
	2010990	*Solanum lycopersicum*	1	x		x				x
	4127051	*Solanum lycopersicum*	1	x						
	4127051	*Solanum lycopersicum*	2[b]		x	x	x	x	x	x
	5962508	*Solanum lycopersicum*	1	x		x				x
Tomato chlorotic dwarf viroid (TCDVd)	3816013/ EF626530	*Brugmansia sanguinea*	1	x		x				x
	5345261	*Petunia*	2[b]		x	x	x	x	x	x
	5783657	*Petunia*	5	x						
	5783710	*Petunia*	5	x						
	3992641	*Solanum lycopersicum*	1	x		x				x
	4888596	*Solanum lycopersicum*	1	x		x				x
	22006456/ AY372399	*Solanum lycopersicum*	1	x		x				x
	A4	unknown	1			x				x

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Table 1. (Continued)

	Reference code / GenBank acc. no.	Original host	Tested material[a]	Test development	Analytical sensitivity	Analytical specificity	Selectivity	Repeatability Reproducibility Robustness	Inter-laboratory comparison	Diagnostic sensitivity diagnostic specificity relative accuracy
targets										
Tomato planta macho viroid (TPMVd)	OG1/ L78454	*Solanum cardiophyllum*	1	x		x				x
	3601768	*Solanum lycopersicum*	1	x		x				x
	3289954/ K00817	*Solanum lycopersicum*	1							x
	3289954/ K00817	*Solanum lycopersicum*	2[b]	x	x	x	x	x	x	x
non-targets										
Pospiviroidae										
Chrysanthemum stunt viroid (CSVd)	9501859/ U82445	*Petunia*	1			x				
	4308774	*Pericallis*	5			x				
Hop stunt viroid (HpSVd)	YP9352	*unknown*	5			x				
Iresine viroid 1 (IrVd-1)	4416011/ GU911350	*Celosia plumosa*	5			x				
	Naktuinbouw	*Verbena*	5			x				
Avsunviroidae										
Eggplant latent viroid (ELVd)	5421357	*Solanum melongena*				x				
Pepper and tomato infecting viruses										
Alfalfa mosaic virus (AMV)	Q0300019	*Solanum kurtzianum*	4			x				
Cucumber mosaic virus (CMV)	5473587	*Solanum lycopersicum*	5			x				
Pepino mosaic virus (PepMV)	3829631	*Solanum lycopersicum*	4			x				
Pepper mild mottle virus (PMMoV)	21005888	*Capsicum annuum*	5			x				
Potato virus Y (PVY)	4225768	*Solanum tuberosum*	4			x				
Tobacco mosaic virus (TMV)	6183848	*unknown*	4			x				
Tomato chlorosis virus (ToCV)	4343668	*Solanum lycopersicum*	5			x				
Tomato infectious chlorosis virus (TICV)	99913778	*Solanum lycopersicum*	5			x				
	22005209	*Solanum lycopersicum*	5			x				
Tomato mosaic virus (ToMV)	6184840	*unknown*	4			x				
Tomato spotted wilt virus (TSWV)	21007721	*Ligularia spp.*	4			x				
Tomato yellowleaf curl virus (TYLCV)	3181291	*Solanum lycopersicum*	5			x				
Clavibacter michiganensis subsp. michiganensis	ZZB 655	*Solanum lycopersicum*	2			x				
negative controls (seed)										
	PPS045	*Capsicum annuum*	3				x			x
	ZZB 379	*Capsicum annuum*	3				x			x
	6184891	*Solanum lycopersicum*	2				x			x
	ZZB 649	*Solanum lycopersicum*	2				x			x
Total # isolates				**33**	**13**	**70**	**17**	**11**	**11[c]**	**43**

[a] Tested material: 1 *Solanum lycopersicum:* leaf material; 2 *S. lycopersicum:* seed ; 3 *Capsicum annuum:* seed; 4 Solanaceaous test plant species other than *S. lycopersicum* ; 5 Original host species

[b] Sample consisting of 999 non-infested seeds and one seed infested with either TASVd, TCDVd or TPMVd, originating from an experimentally-infected tomato plant

[c] Eleven isolates in eight samples

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Table 2. Primers and probes sequences of PospiSense test

Primers & Probes	Sequence (5'-3')	Ref.
PospiSense 1		
PospiFW1	TGCGCTGTCGCTTCG	this paper
PospiFW5a	CCTTCCTTTCTTCGGGTTTC	this paper
PospiRV1	AGAAAAAGCGGCGCTTG	this paper
PospiRV2	TAGAGAAAAAGCGGTTCTCGG	this paper
PospiRV5a	GAAAAAGCACCTCTGTCAGTTGTA	this paper
CLVd-F	GGTTCACACCTGACCCTGCAG	(11)
CLVd-F2	AAACTCGTGGTTCCTGTGGTT	(11)
CLVd-R	CGCTCGGTCTGAGTTGCC	(11)
PospiP1a	*FAM*-CGGTGGAAACAACTG-*MGB*	this paper
PospiP3a	*FAM*-CGGCCTTCTCGCGCA-*MGB*	this paper
CLVd-P	*FAM*-AGCGGTCTCAGGAGCCCCGG-*BHQ1*	(11)
PospiSense 2		
PospiFW6a	GGATCTTTCTTGAGGTTCCTGT	this paper
PospiFW6b	GGAACTTTCTTGAGGTTCCTGT	this paper
PospiFW6c	TCTTTCCTTGTGGTTCCTGTG	this paper
PospiRV6a	CGACTTCCTCCAGGTTTCC	this paper
PospiP5	*FAM*-CTGCAGGGTCAGGTG-*MGB*	this paper
Internal Control		
DaVd1-FT	GCTCCGCTCCTTGTAGCTTT	this paper
DaVd1-RT	AGGAGGTGGAGACCTCTTGG	this paper
DaVd1-P	*Texas Red*-CTGACTCGAGGACGCGACCG-*BHQ2*	this paper

102

103

104 # Sample preparation and RNA extraction

105 ## Seeds

106 Samples of tomato seeds consisted of c. 3000 seeds, which were divided in three subsamples of
107 c.1000 seeds for testing, according to standard procedures used by seed testing laboratories in
108 Europe (16).
109 Seeds were processed by either using a Geno/Grinder (SPex SamplePrep P) or a BagMixer 100
110 (Interscience), depending on the laboratory's preference. When using the Geno/Grinder (dry
111 processing) 3x 1000 tomato seeds were transferred to a 50 ml tube containing a 14 mm steel ball.
112 Tubes were put upside down and seeds ground at 1500 rpm for 4 min (at least 95% seeds
113 crushed). After grinding, 20 ml GH+ extraction buffer ((9); modified by (24)) which included the
114 DLVd spike, was added to each tube. The DLVd-spike stock of 1 g DLVd-infected leaf material
115 homogenised in 10 ml GH+ buffer was used at a dilution of approximately 10^{-4} to achieve a Cq
116 value of about 28. To obtain homogenous solutions the tubes were shaken manually. When using

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117 the BagMixer (wet processing) subsamples of 1000 tomato seeds were transferred to a grinding
118 bag (BagPage 100ml (Interscience) and soaked in 20 ml GH+ buffer spiked with DLVd at room
119 temperature for 30-60 min and subsequently blended for 1.5 min at position 4.
120 For pepper seeds the same procedure was followed except that the subsamples of c.1000 seeds
121 were subdivided into 2x500 seeds before grinding with a Geno/Grinder for 7 min. After grinding 10
122 ml DLVd-spiked GH+ extraction buffer was added to each of the six tubes, followed by combining
123 and mixing of the contents of the two tubes of a subsample before further processing.
124 For RNA extraction, 1 ml of the seed homogenate was transferred into a 1.5 ml tube and 30 µl of
125 5M Dithiothreitol was added, followed by incubation in a thermoshaker at 850 rpm, 65°C for 15
126 min. The tubes were centrifuged at 16,000 g for 10 min. For manual RNA extraction using the
127 RNeasy plant mini kit (Qiagen), 750 µl of the supernatant was used following the manufacturer's
128 instructions. For large-scale RNA extraction on a Kingfisher KF96 system (Thermo Fisher
129 Scientific), using the Sbeadex Maxi Plant kit (LGC), 250 µl of supernatant was transferred to a
130 binding plate containing 600 µl of binding buffer and 50 µl Sbeadex particle suspension, following
131 the manufacturer's instructions.
132

133 Leaves

134 Sample preparation and RNA extraction from leaf material (Table 1) was performed according to
135 Botermans et al. (9).
136

137 PospiSense real-time RT-PCR

138 Table 2 lists the primer and probe sequences used for the PospiSense test. The PospiSense 1
139 reaction contained: 1x UltraPlex 1-Step ToughMix (Quanta Biosciences), 0.3 µM of each
140 PospiSense 1 and internal control primer, 0.1 µM of TaqMan probe PospiP1a, PospiP3a, CLVd-P,
141 0.2 µM of internal control TaqMan probe DaVd1-P, 2 µl RNA template and molecular grade water
142 to a final volume of 20 µl. The PospiSense 2 reaction included: 1x UltraPlex 1-Step ToughMix
143 (Quanta Biosciences), 0.3 µM of each PospiSense 2 and internal control primer, 0.1 µM of TaqMan
144 probe PospiP5, 0.2 µM of internal control TaqMan probe DaVd1-P, 2 µl RNA template and

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145 molecular grade water to a final volume of 20 µl. Both reactions used real-time RT-PCR: 10 min
146 50°C, 3 min 95°C, followed by 40 cycles 10 s 95°C and 1 min 60°C. Real-time RT-PCRs were
147 carried out in 96-well plates on a Bio-Rad CFX96™ Real-Time PCR system (Bio-Rad
148 Laboratories,) or a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific). After
149 verification of controls, a test result was considered positive if an exponential amplification curve
150 was produced for either the PospiSense 1 and/or the PospiSense 2 reaction.
151

152 Results

153 Test development and validation

154 Table 2 shows the primers and probes that were selected for further validation, based on the
155 results of the initial tests. To determine whether the PospiSense test is suitable for routine testing
156 of seed lots, the following performance characteristics were determined: analytical sensitivity,
157 analytical specificity, selectivity, repeatability and reproducibility, according to the EPPO standard
158 PM7/98 version 4 (25). In addition, the PospiSense test was compared with the currently most-
159 commonly used test (14, 15) for diagnostic sensitivity, diagnostic specificity and relative accuracy
160 (25). Table 1 indicates the isolates used to determine each of the performance characteristics.
161

162 Analytical sensitivity

163 To determine the analytical sensitivity, RNA-extracts of pepper or tomato seeds naturally infested
164 by CLVd, PSTVd or TASVd (one isolate each) were diluted in duplicate in RNA-extracts of non-
165 infested seeds. Testing of RNA extracts of decimal dilutions revealed that these three pospiviroids
166 showed 100% detection up to 1000, 10.000 and 100 times dilution respectively. Furthermore,
167 testing samples consisting of one tomato seed infested by either CLVd, TASVd, TCDVd or TPMVd,
168 and 999 non-infested seeds, produced consistent positive results. In comparison to the
169 Naktuinbouw test, the PospiSense test appeared less sensitive for detection of CEVd and TASVd,
170 (difference for CEVd ΔCq= 6.1 SD=3.2 n=6 and TASVd ΔCq= 4.0 SD=1.4 n=5, based on average

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171 values of both leaf and seed samples in a range of concentrations). Nevertheless, the PospiSense
172 test meets the requirements of detecting one infested seed in a sample of c.1000 seeds.
173

174 Analytical specificity

175 The analytical specificity was determined by testing infected leaf and infested seed samples by
176 target and non-target species (see Table 1). The PospiSense test gave positive results for all 51
177 tested isolates of the seven target pospiviroids, i.e. CEVd (6), CLVd (6), PCFVd (4), PSTVd (19),
178 TASVd (7), TCDVd (6) and TPMVd (3), thus showing coverage of the intra-species variability
179 (inclusivity). For 12 non-targets (exclusivity), no cross-reactions were observed, i.e. for hop stunt
180 viroid (hostuviroid), and most common pepper- and tomato-infecting viruses, i.e. alfalfa mosaic
181 virus, cucumber mosaic virus, pepper mild mottle virus, pepino mosaic virus, potato virus Y,
182 tobacco mosaic virus, tomato chlorosis virus, tomato mosaic virus, tomato spotted wilt virus and
183 tomato yellow leaf curl virus. In addition, no cross-reactions were found for the bacterium
184 *Clavibacter michiganensis* subsp. *michiganensis.* For four non-targets, i.e. Chrysanthemum stunt
185 viroid (CSVd), eggplant latent viroid (genus *Elaviroid*) and Iresine viroid 1 (IrVd-1), cross-reactions
186 (Cq= 27-37) were observed when present in high concentrations. Of these viroid species, however,
187 no natural infections in pepper and tomato have been reported. In addition, one out of two isolates
188 of tomato infectious chlorosis virus showed some cross-reactivity when present in high
189 concentration, which is not likely for seeds. Moreover during confirmatory testing, false positives
190 will be revealed and abolished. Therefore, the observed cross-reactions will not hamper the
191 application of the PospiSense test for screening seed lots.
192

193 Selectivity

194 To determine the effect of the matrix, RNA extracts of seeds containing RNA of each of the target
195 species were diluted in either RNA-extracts of non-infested seeds or water (Table 1). Test results
196 of serial dilutions of the RNA extracts were compared. For both pepper and tomato seeds only
197 minor differences were observed, i.e. average Δ Cq water: pepper seed= 0.4 and average Δ Cq

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198 water: tomato seed= 0.6. Regarding selectivity, therefore, it was concluded that no apparent matrix
199 effects occurred in both pepper and tomato seeds.
200

201 Repeatability and Reproducibility

202 Repeatability and reproducibility were determined by analysing sub-samples of pepper and tomato
203 seeds under the same experimental conditions (technical replicates) and under different
204 experimental conditions (date, operator, apparatus, *etc.*), including an inter-laboratory comparison.
205 Eight samples of pepper and tomato seeds infested by the seven relevant pospiviroids (11
206 isolates) were selected. The RNA extracts of these samples with medium to low relative infestation
207 rates (Cq values of targets varying between 20 and 32) were sub-sampled and tested by the three
208 participating laboratories, including two routine seed-testing laboratories. Qualitative interpretation
209 of the resulting data showed concordance for all (sub-) samples, both within and between
210 laboratories, and irrespective of variation in experimental conditions (Table 3a,b). Repeatability and
211 reproducibility were 100%, further demonstrating the robustness of the PospiSense test.
212

213 Diagnostic sensitivity, diagnostic specificity and relative accuracy

214 To determine the relative accuracy of the PospiSense test, test results were compared with the
215 results obtained with the with the most-commonly used pospiviroid seed test of Naktuinbouw
216 (2017a,b,c). In total, 43 samples including both infested and non-infested seed samples were
217 tested with both tests. Positive and negative results were compared qualitatively. The PospiSense
218 and the Naktuinbouw test both diagnosed the same number of positive (n=39; Naktuinbouw test
219 Cq 12-31, PospiSense test Cq 10-34) and negative (n=4) results. Consequently, diagnostic
220 sensitivity, diagnostic specificity and relative accuracy were all 100% in comparison with the
221 Naktuinbouw test.
222

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Table 3a. Results (Cq values) of the repeatability and reproducibility experiments in intra- and inter-laboratory setting (PospiSense 1)

Isolate	Matrix (seed)	PospiSense 1, Test Moment 1-6[a]					
		NPPO Lab				Lab 1	Lab 2
		1	2	3	4	5	6
PSTVd PPS020	Pepper	22/22	22			23	
TASVd + CLVd PPS055	Pepper	25/25	25			26	
PCFVd + CLVd PPS013	Pepper	24	25/24			28(VIC)[d]	
TCDVd 5345261[b]	Tomato	24	24/24				23
TPMVd 3289954[b]	Tomato			29/30	30		28
TASVd 4127051[b]	Tomato			ND/ND	ND		ND
PCFVd + CLVd 6184939	Pepper			23	22/22		22
CEVd 3823889[c]	Pepper			ND	ND/ND		ND

Table 3b. Results (Cq values) of the repeatability and reproducibility experiments in intra- and inter-laboratory setting (PospiSense 2)

Isolate	Matrix (seed)	PospiSense 2, Test Moment 1-6[a]					
		NPPO Lab				Lab 1	Lab 2
		1	2	3	4	5	6
PSTVd PPS020	Pepper	ND/ND	ND			ND	
TASVd + CLVd PPS055	Pepper	25/25	25			25	
PCFVd + CLVd PPS013	Pepper	37	37/37			38	
TCDVd 5345261[b]	Tomato	ND	ND/ND				ND
TPMVd 3289954[b]	Tomato			ND/ND	ND		ND
TASVd 4127051[b]	Tomato			32/32	32		31
PCFVd + CLVd 6184939	Pepper			37	37/37		37
CEVd 3823889[c]	Pepper			20	20/20		19

[a] 1-6 = Moment at which test is perfomed by a different operator

[b] RNA extract from sample consisting of 999 non-infested seeds and one seed infested with TASVd, TCDVd or TPMVd, originating from an artificially infected tomato plant.

[c] RNA extract from seed spiked with CEVd

[d] PCFVd specific probe was accidentally labeled with VIC instead of FAM fluorophore, explaining slightly different cq values

ND = not detected (negative test result)

223 Test results of internal control were positive (data not shown)

224

225 # Discussion

226 The newly developed PospiSense test has been shown to fulfil the requirements for routine testing
227 of pepper and tomato seed samples for the seven pospiviroid species known to occur naturally in
228 these crops. The validation data showed that for CLVd, TASVd, TCDVd and TPMVd the test allows
229 detection of at least one infested seed in a sample of 1000 seeds. For PSTVd similar results were
230 obtained for single seeds from a naturally infested seed lot. For CEVd no infested seed samples
231 were available, and for PCFVd only seed samples co-infested with CLVd. Therefore, the analytical

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232 sensitivity could not be experimentally determined. However, the results are expected to be similar,
233 because these pospiviroids are likely to share both physical and biological characteristics with
234 other members in the genus. Moreover, regarding test performance, the analytical sensitivity for
235 CEVd and PCFVd for leaf material is within the same range as the other pospiviroids. In addition,
236 the results of the wide range of targets and non-targets tested, as well as the absence of matrix
237 effects, showed its suitability for screening both pepper and tomato seeds. A 100% repeatability
238 and reproducibility were obtained during validation and inter-laboratory comparison, both further
239 demonstrating the robustness of the PospiSense test.
240 For routine screening of seed lots, the PospiSense test offers some improvements in comparison
241 with the currently used real-time RT-PCR pospiviroid tests. Firstly, the test is more sensitive than
242 the other (semi-) generic pospiviroid tests as described by Monger et al. (11) and Botermans et al.
243 (9), which both lack the sensitivity needed for reliable seed testing. Secondly, the PospiSense test
244 is less complex than the pospiviroid seed test of Naktuinbouw (14, 15) and its performance
245 characteristics generally comparable, although the analytical sensitivity of the PospiSense is
246 slightly lower for CEVd and TASVd. In addition, the comparison of both tests showed a 100%
247 agreement. However, in comparison to the Naktuinbouw test, the PospiSense test consists of two
248 instead of four parallel reactions and uses only one internal control (DLVd) and one fluorophore. In
249 both reactions, DLVd is spiked as internal isolation control. This control appeared a more
250 consistent control for seed testing than the host-derived nad5, which often produces variable Cq
251 values due to differences in cell physiology. The characteristics of the DLVd control are similar to
252 the targets and its secondary structure is likely to prevent it from degradation by RNases. Another
253 factor contributing to the lesser complexity of the PospiSense test is the choice of using the same
254 fluorophore for all target species, as it makes the interpretation of test results easier. There is little
255 chance of confusing results caused by cross-reactions between different primers and probes
256 and/or the presence of more than one pospiviroid species in a sample. Nevertheless, it is possible
257 to include additional fluorophores if discrimination among species at the screening stage is
258 desirable.
259 The PospiSense test has been developed for efficient testing of seeds by combining the detection
260 of seven pospiviroid species. This implies that in the case of a positive result, at least one

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261 pospiviroid species could be present and additional tests are needed for the identification of the
262 species. Specific real-time RT-PCR tests have been developed to detect CEVd, CLVd, TASVd
263 (26), and PCFVd (14, 15). For the closely related species PSTVd, TCDVd and TPMVd, the real-
264 time RT-PCR test described by Boonham et al. (2004) can be used for confirmation, but by
265 detecting all these three pospiviroids (except for one TPMVd isolate), the test is not able to
266 distinguish between these species. Consequently, these three species can only be distinguished
267 and identified by sequencing the amplicons obtained by conventional RT-PCR. Furthermore, it
268 should be noted that for confirmation, a different test, preferably targeting a different region of the
269 genome, should be used. However, the identification of pospiviroids in seed lots is not always
270 easy, since viroid concentrations are generally low. Identification has even become more
271 challenging because of the increased sensitivity of the recently developed real-time RT-PCR tests,
272 including the PospiSense test described in this paper. According to the International Committee on
273 Taxonomy of Viruses (27), the identification of viroids should be based on the analysis of their
274 complete genome. Complete sequences, however, are still difficult or impossible to obtain from
275 seed samples with low viroid levels, because conventional RT-PCR tests lack the required
276 sensitivity to produce full-length amplicons. In addition, in comparison to real-time RT-PCR tests,
277 conventional RT-PCRs are generally more prone to inhibition by matrix components. This means
278 that seed treatments might have more impact on the analytical sensitivity of the conventional RT-
279 PCR tests. For the identification of pospiviroid species in seed samples, the primer set Pospi1-
280 FW/Pospi1-RE described by Verhoeven et al. (17) appeared most suitable due to its relatively high
281 analytical sensitivity (28) When combining this test with the primer set Pospi2-FW/ Pospi2-RE (18),
282 complete genome sequences of all known pospiviroids (except for CLVd) can be obtained, since
283 these two primer pairs anneal at the same loci but in opposite polarity. However, often tailor-made
284 solutions are needed for the confirmation and identification of pospiviroids in seed samples, e.g.
285 concentration methods (29) nested-RT-PCR, or pooling of PCR-products for further testing.
286 In conclusion, the performance of the PospiSense test, combined with the need of only two parallel
287 reactions and a limited number of probes, shows its perspectives as an alternative test for
288 screening seed lots of solanaceous species.
289

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294

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