

1 **In-situ detection of Tomato spotted wilt orthospovirus from crude plant extracts using**
2 **Reverse Transcriptase- Recombinase Polymerase Amplification (RT-RPA) in endpoint and**
3 **real-time.**

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7 **Abstract**

8 Virus detection in early stages of infection could prove useful for identification and isolation of foci
9 *of inoculum before its spread to the rest of susceptible individuals via vectoring insects. However,*
10 *the low number of viruses present at the beginning of infection renders their detection and*
11 *identification difficult and requires the use of highly sensitive laboratory techniques that are often*
12 *incompatible with a field application.*

13 *To obviate this challenge, we designed a Recombinase Polymerase Amplification, a molecular*
14 *technique that makes millions of copies of a predefined region in the genome, in this case of*
15 *Tomato spotted wilt orthospovirus. The reaction occurs at 39 °C and can be used directly from*
16 *crude plant extracts without nucleic acid extraction. Notably, a positive result can be seen with*
17 *the naked eye as a flocculus made of newly synthesized DNA and metallic beads.*

18 *The objective of the procedure is to create a portable and affordable system that can isolate and*
19 *identify viruses in the field, from infected plants and suspected insect vectors, and can be used*
20 *by scientists and extension managers for making informed decisions for viral management.*
21 *Results can be obtained in situ without the need of sending the samples to a specialized lab.*

22 **Introduction**

23 *Tomato spotted wilt orthospovirus (TSWV) causes extensive losses worldwide to various crops*
24 *and ornamentals (Morsello & Kennedy, 2009) that can account to as much as 1 billion dollar*
25 *(Ohnishi et al., 2006). These losses are in part due to the generalist nature of the virus that can*
26 *infect up to 1000 species of plants, including monocots and dicots (Pappu, 2008) and has one of*
27 *the largest host range on record for plant viruses (Hanssen, Lapidot, & Thomma, 2010).*
28 *Furthermore, TSWV is transmitted in a propagative circulative manner by a dozen polyphagous*
29 *and hard to control thrips species (Webb, Tsai, & Mitchell, 1998).The virus has an ambisense*
30 *ssRNA genome, comprised of 3 segments named by size small (S), medium (M) and large (L),*
31 *surrounded by coat proteins and enclosed by a lipidic envelope (Adkins, 2000; Pappu, 2008). Like*
32 *for other plant viruses, virus and vector exclusion and eradication are the most effective control*
33 *measures. The use of ultraviolet-reflective mulches, for instance, has been proven to be useful in*
34 *avoiding primary spread (Funderburk et al., 2011).*

35 Early detection of TSWV and other plant pathogens is critical for adequate selection and
36 deployment of counter measures (Ohnishi et al., 2006). Common techniques for detection of
37 TSWV include RT-PCR (Roberts, Dietzgen, Heelan, & Maclean, 2000), immunostrips (Crosslin,
38 Mallik, & Gudmestad, 2009), ELISA (Gonsalves & Trujillo, 1986) and Next Generation
39 Sequencing (Hagen et al., 2011). While molecular assays necessitate expensive lab equipment,
40 can be time consuming and require trained personnel (Babu et al., 2017), antibodies based
41 immunostrips that can be used directly in the field do not offer a high degree of sensitivity, leading
42 to the possibility of false negative and missed virus detection. Losses caused by a lack of

43 detection can be significant, especially for an economically important virus, such as
44 TSWV. Providing a field-based, inexpensive, sensitive and easy-to-use detection assay would
45 allow to test infected material, even before the onset of symptoms, and would result in better
46 disease management.

47 Recombinase polymerase amplification (Piepenburg, Williams, Stemple, & Armes, 2006) is a
48 nucleic acid sequence based amplification (NASBA) (Compton, 1991) that allows amplification,
49 at low constant temperature (Lutz et al., 2010). Unlike Loop-mediated Isothermal amplification
50 (LAMP), another isothermal amplification technique which requires a temperature of 65 °C, RPA
51 can perform well at temperatures close to 37 °C, and even ambient temperature, although with
52 lower efficiency (Z. A. Crannell, Rohrman, & Richards-Kortum, 2014). Because of this, RPA
53 forgoes the need of a thermal cycler and bulky equipment and can be used in the field (Cabada
54 et al., 2017) (Robertson & Nicholson, 2005). Even more conveniently, all reaction components
55 can be lyophilized, allowing room temperature storage and transport under field conditions, and
56 the assay can be performed by untrained personnel (Lutz et al., 2010). Mechanistically, RPA
57 exploits a recombination and repair system found in phages (Bianco, Tracy, & Kowalczykowski,
58 1998); one of its reagents consist of a nucleoprotein complex (recombinase + primer) that
59 exchange non-template strand and primer, followed by extension by DNA pol, whilst proteins
60 stabilize nascent ssDNA. The end-point product is usually detected using a commercially
61 available amplification detection chamber or lateral flow devices (Lillis et al., 2016) which contain
62 proprietary mixes of antibodies that detect FAM and biotin from the amplified products (Rohrman
63 & Richards-Kortum, 2012).

64 In cases in which there is the need to follow RPA in real time, the design of the probe can be
65 modified to move the FAM molecule closer to the recombination site and quenched in the intact
66 probe. In this way, as the reaction happens, the FAM is cleaved and its concentration can be
67 detected with a fluorometer, as directly proportional to the amount of amplicon being produced
68 (TwistDx, 2009). Advantages of RPA are its readiness and affordability, specificity, sensitivity,
69 speed and easiness of use (Lobato & O'Sullivan, 2018) as well as the possibility of multiplexing
70 (Z. Crannell et al., 2016). Notably, the sensitivity and specificity of this reaction are comparable
71 and sometimes even superior to that of PCR (Cabada et al., 2017). Because of these reasons,
72 RPA is becoming a favorite technique for performing lab-on-a-chip microfluidics analyses that
73 require minimum incubation and can give real-time measurements (Lutz et al., 2010).

74 Binding-flocculation detection of amplicons is a cheaper and equally sensitive approach for
75 detection of RPA products. This techniques exploits the property of aggregation of newly-
76 synthesized nucleic acid and magnetic beads, which form stable floccules under acidic conditions,
77 indicating a positive result (Ng, Wee, West, & Trau, 2015). Interestingly, this same principle can
78 be used for a variety of applications, including the detection of methylation (Wee, Ngo, & Trau,
79 2015).

80 In this work, we present the creation of an RT-RPA assay specific for TSWV detection that can
81 be interpreted with amplification detection chambers, flocculation essays or by real time PCR. We
82 envision that such a test will become a useful addition to the toolkit already in use for TSWV
83 diagnosis.

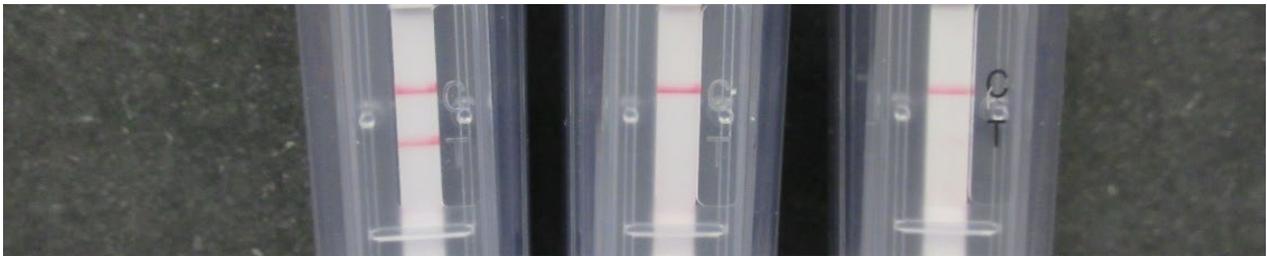
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86 Results

87 End-point RPA can be visualized in amplification detection chambers

88 Crude extract from a *Nicotiana benthamiana* (*N. benthamiana*) leaf infected with TSWV was used
89 for RT-RPA reaction and the results of the amplification were visualized in an Amplification
90 detection chamber (Agdia, Elkhart, IN). As seen in Figure 1, both the control (top) and the test
91 line (bottom line) were visible in the chamber where the extract from the infected leaf was loaded
92 (left chamber). This result, indicates a positive amplification, confirming the presence of the dual
93 labelled RT-RPA products and thus of TSWV in the mixture. A negative control, made with the
94 crude extract of a non-infected plant, and one where water was used instead of leaf extract were
95 also subjected to the same procedure. Results in Figure 1 (center and right chambers
96 respectively) show that for both controls only the control line (top line) was visible, indicating a
97 valid test but negative result.

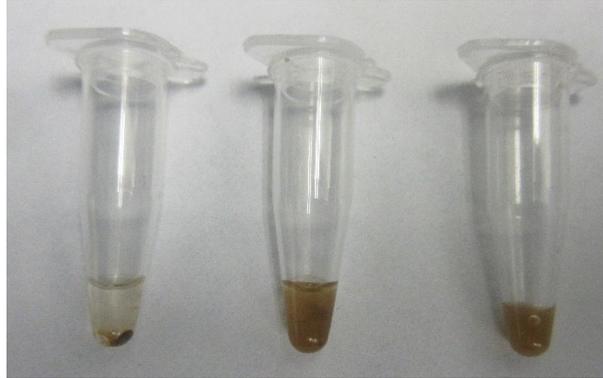


98

99 Figure 1: Result of end-point Reverse transcriptase RPA (RT-RPA) in amplification detection
100 chambers (Agdia, Elkhart, IN). Left: positive result for crude extract of a TSWV infected *N.*
101 *benthamiana* leaf, where both the control (top) and the test line (bottom) are visible. Center and
102 Right: negative results for crude extract of an uninfected leaf and water respectively, where only
103 the control line (top) is visible. NTC1: healthy *N. benthamiana*, NTC2: water.

104 End-point RT-RPA can be visualized by flocculation assay

105 After performing the RT-RPA amplification, the tubes were removed from the thermal block, SPRI
106 (Solid Phase Reversible Immobilization) magnetic beads were added to the amplified fraction,
107 washed with ethanol, followed by the addition of the acetate-based crowding agent. The tube that
108 contained the crude extract of the TSWV infected *N. benthamiana* showed a stable flocculus on
109 the bottom of the tube that did not break, despite flicking the tube repeatedly (Figure 2, left tube).
110 Since the flocculus collects all the beads in the mixture in one small mass, the solution in the tube
111 becomes clear. On the other hand, the vessel containing the non-target controls, did not form a
112 stable flocculus and, due to the even distribution of beads in the mixture, the mixture remained
113 opaque (Figure 2, center and left tube).

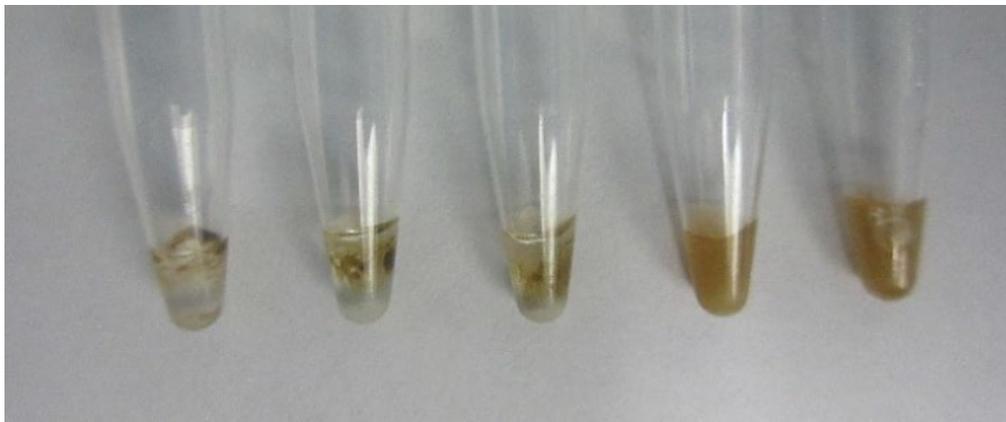


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115 Figure 2: Result of end-point RT-RPA with SPRI beads. Left: positive result, after rehydrating the
116 pellet and the amplification reaction, added SPRI beads react with the amplicon and form a stable
117 flocculus in presence of an acetate-based crowding agent. Center and right: Negative results
118 NTC1: healthy *N. benthamiana*, NTC2: water, the beads are spread throughout the solution
119 making it opaque.

120 **TSWV RT-RPA assay can detect multiple TSWV isolates in multiple plant species**

121 To ensure that our RT-RPA assay would detect different TSWV isolates and in different plant
122 species, we repeated the RT-RPA reaction for two more TSWV isolates: TSWV-Chrys5, isolated
123 from Chrysanthemum and maintained in *Nicotiana benthamiana*, TSWV-Tom2, isolated from
124 *Solanum lycopersicum* and maintained in *N. benthamiana*, and TSWV-PA01, isolated from
125 *Capsicum annuum* and maintained in *Emilia sonchifolia* (Margaria and Rosa 2016). All isolates
126 were originally detected via immunostrips (Agdia, Elkhart, IN) and isolated to single lesions to
127 ensure the presence of a single viral species. Our RT-RPA assay was able to detect all the three
128 isolates in all 3 plant species, even if with different sensitivity. Nevertheless, the reaction for
129 TSWV-PA01 was rather weak, when visualized in the amplification detection chambers, but
130 equally positive, when using the flocculation assay.



131

132 Figure 3: RT-RPA results for three isolates of TSWV revealed with a flocculation assay. TSWV-
133 Chrys5, TSWV-Tom2, TSWV-PA01, NTC1: healthy *N. benthamiana*, NTC2: water.

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139 Figure 4: Right: RT-RPA positive result for three isolates of TSWV revealed with an amplification
140 detection chambers. TSWV-Chrys5, TSWV-Tom2, TSWV-PA01 (faint test band), NTC1: healthy
141 *N. benthamiana*, NTC2: water.

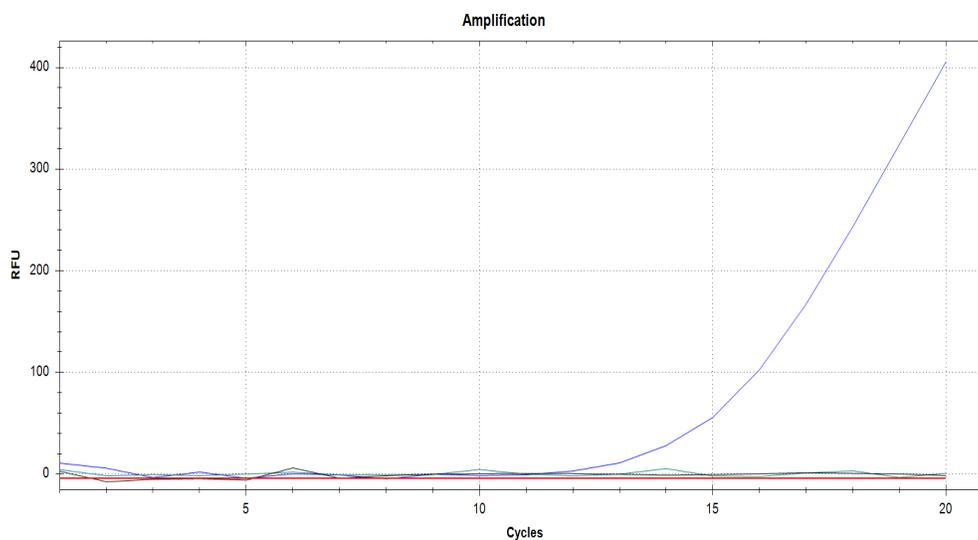
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144 **TSWV specific RT-RPA can be visualized by fluorescence in real time**

145 The RT-RPA amplification was followed in real time using the SYBR/FAM channel of a Bio-Rad
146 CFX 96 thermal cycler (Bio-Rad, Hercules, CA). As expected, a positive RT-RPA reaction showed
147 an amplification curve similar to curves typically generated by real-time PCR for the crude extract
148 of a TSWV *N. benthamiana* leaf. The two negative controls, containing both the crude extract of
149 an uninfected leaf and water (non-target controls) did not show an amplification curve.

150



151

152 Figure 5: Result of real-time RT-RPA visualized in the SYBR/FAM channel of a Bio-Rad CFX96
153 real time PCR thermal cycler kept isothermally at 39 °C. Like real time PCR, an amplification curve

154 is visible (blue). The non-target controls (black and green) did not produce an amplification curves
155 in the 20 minutes the reaction took place. Black lines NTC1: healthy *N. benthamiana*, NTC2:
156 water.

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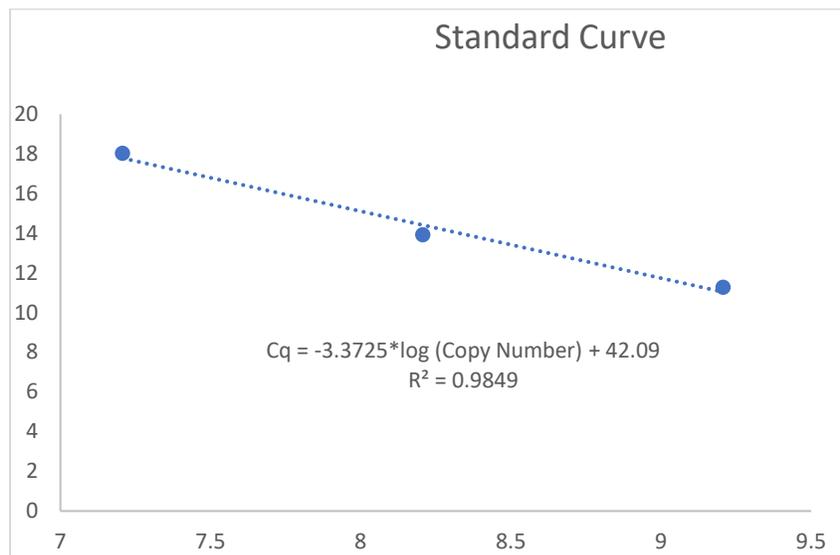
158 **Standard curve**

159 For standard curve analyses, a qRT-PCR product was purified from a completed PCR reaction;
160 then its concentration was measured and used to make serial dilutions. The number of copies of
161 template was calculated using the equation (1)

162 The standard curve, calculate by making a linear regression of the obtained Cq in the y-axis vs.
163 the \log_{10} of the calculated number of copies in the x-axis, is shown in Figure 6.

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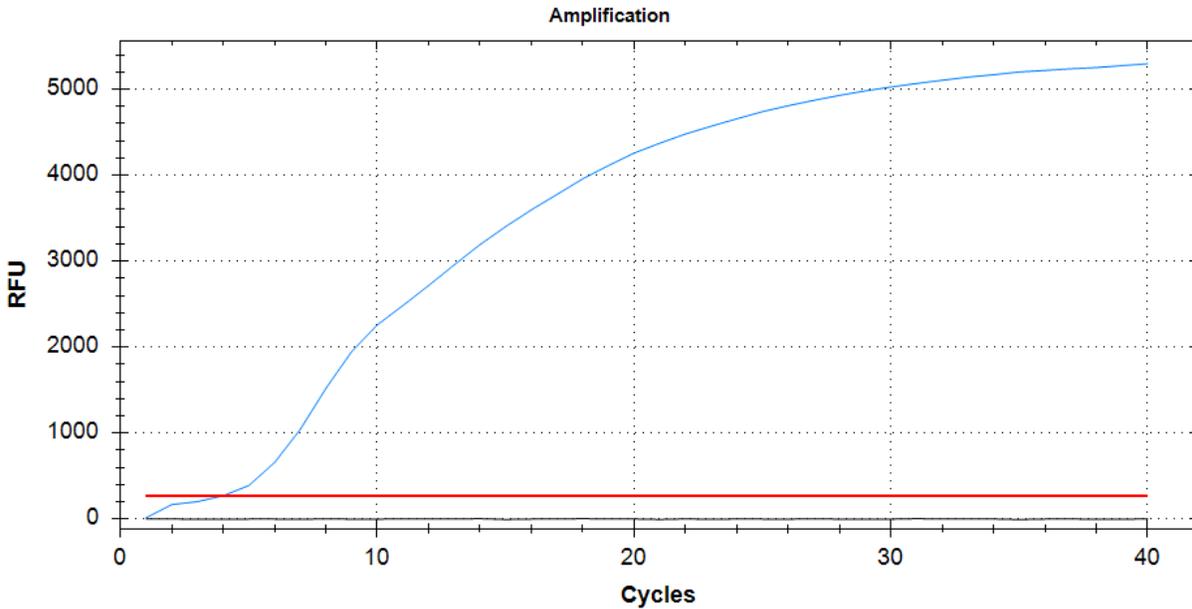


166

167 Figure 6: Standard curve computed for absolute quantification. The curve was built by purifying
168 the RT-qPCR amplicon and making 10-fold serial dilutions of it. The obtained Cq for the
169 dilutions was used for a linear regression with the estimated concentrations (measured by
170 nanodrop).

171 **Quantification of starting material**

172 Fifty microliters of the TSWV infected *N. benthamiana* extract were subjected to RNA extraction,
173 followed by a one-step real time PCR (Figure 7). The calculated Cq for TSWV in 50 microliters of
174 lysate was close to 1.61, which interpolated in the standard curve regression equation (1), shown
175 in Figure 6, gave an approximate of 6.02333E+12 copies of the amplified portion of the TSWV L
176 genomic segment). Since only 2 microliters of this solution were used for the crude extracts and
177 all its dilutions, this gives an estimated initial concentration of 2.40933E+11 genomic segments.



178

179 Figure 7: qRT-PCR Amplification curve. RNA was extracted from 50 μ l of plant lysate and
180 amplified via one-step qRT-PCR for 40 cycles. The amplification curve is visible with a Cq of
181 1.61 cycles.

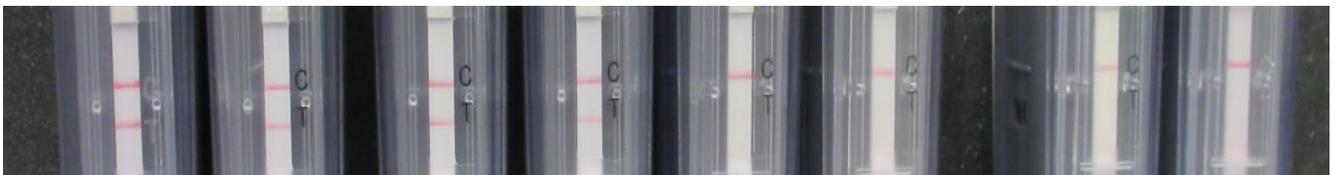
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183 Sensitivity assay

184 Serial dilutions of the crude extracts used for the above experiments were subjected to RT-RPA
185 and visualized in a detection chamber (Agdia, Elkhart, IN). After 20 minutes of incubation, all the
186 dilutions of the crude extract from the TSWV infected plant, from undiluted to 10^{-12} (~1 copy of
187 TSWV genomic segment) showed two bands, indicating a positive result and establishing the
188 theoretical sensitivity of the assay at 1 viral copy. The negative controls showed only the control
189 line (Figure 8).

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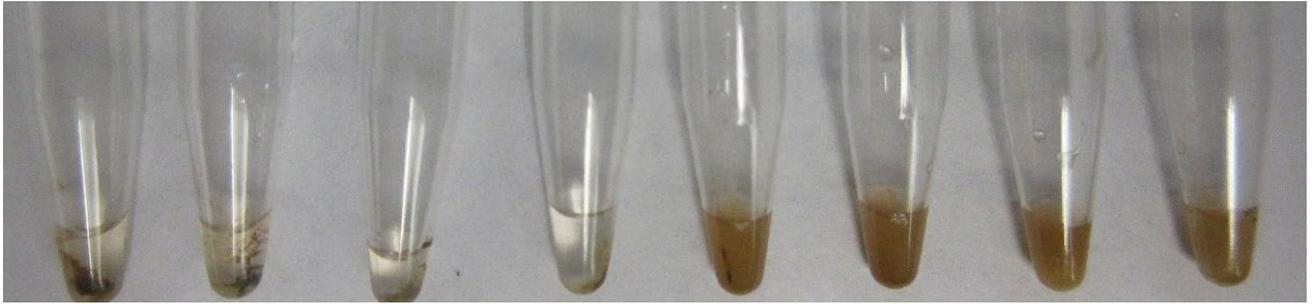


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193 Figure 8: Serial dilutions of a crude extract visualized in amplification detection chambers. From
194 left to right, undiluted sample, 10^{-4} (2^7 copies), 10^{-8} (2^4 copies), 10^{-12} (~1 copy) 10^{-16} (out of range),
195 10^{-20} (out of range) all showing both control and test lines indicating a positive result. To the
196 rightmost side, the two non-target controls NTC1: healthy *N. benthamiana*, NTC2: water were
197 tested, which only showed the control line.

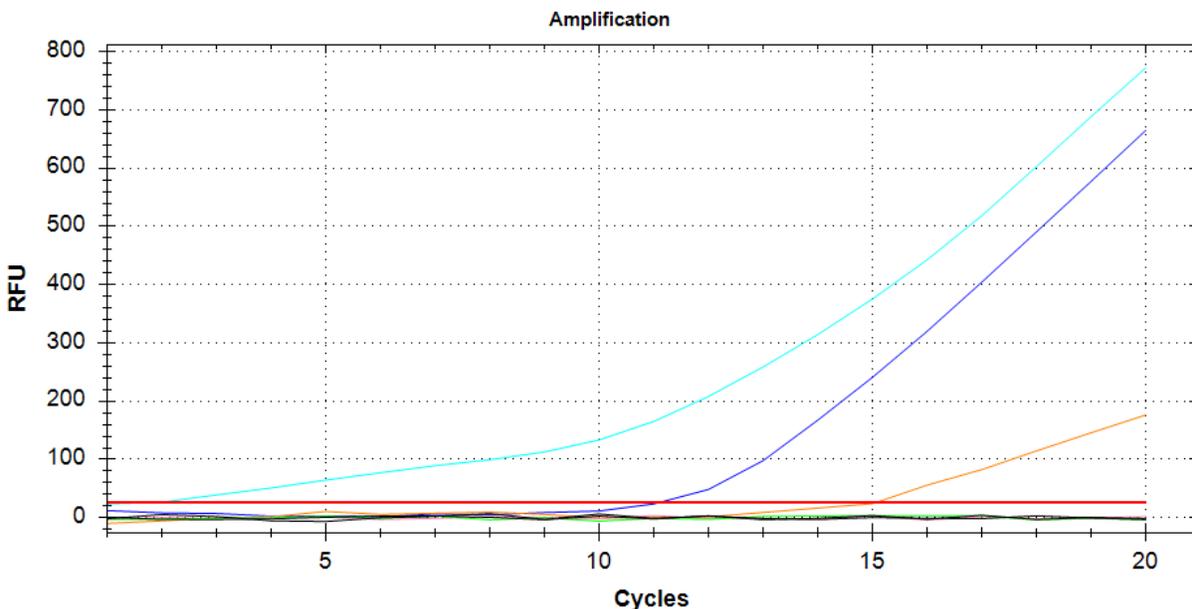
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199 Replicas of the 1:10 dilutions were interpreted with the flocculation assay, showing similar
200 results, all the dilutions, up to 10^{-12} showed a dark precipitate that remained stable even after
201 flicking the tubes repeatedly (Figure 9).



202
203 Figure 9: Serial dilutions of a crude extract visualized with SPRI beads. From left to right, undiluted
204 sample, 10^{-4} (2^7 copies), 10^{-8} (2^4 copies), 10^{-12} (~1 copy) 10^{-16} (out of range), 10^{-20} (out of range),
205 all showing a flocculus indicating a positive result due to the presence of the amplicon; 10^{-16} and
206 10^{-20} show no amplification. Last two tubes: NTC1: healthy *N. benthamiana*, NTC2: water.

207 Finally, the results for real time RT-RPA for some of the above dilutions from undiluted to 10^{-20}
208 in 10^{-4} increments showed differential amplification curves (Figure 10)



209
210 Figure 10: Serial dilutions of a crude extract visualized in the SYBR/FAM channel of a Bio-Rad
211 CFX96 real time PCR thermal cycler kept isothermally at 39 °C From left to right, teal: Crude
212 extract, blue: 10^{-4} (2^7 copies), orange: 10^{-8} (2^4 copies), pink: 10^{-12} (~1 copy), green: 10^{-16} (out of
213 range), yellow: 10^{-20} (out of range), Black: NTC1: healthy *N. benthamiana*, NTC2: water.

214 Discussion and Conclusions

215 Plant viruses are a threat to food security, because of the huge amount of losses (sometimes as
216 high as 100%, for the case of Tomato spotted wilt virus (TSWV) they cause on crops. As viruses
217 are cellular parasites, no curative measures are available for treating plants, other than

218 prophylactic interventions aimed at controlling their vector and avoid transmission to new hosts
219 (Nicaise, 2014)

220 RT-RPA has been already used for detection of other plant viruses, such as Rose rosette virus
221 (Babu et al., 2017), Plum pox virus (Zhang et al., 2014), Little cherry virus-2 (Mekuria, Zhang, &
222 Eastwell, 2014), Yam mosaic virus (Silva, Bömer, Nkere, Kumar, & Seal, 2015) as well as
223 detection at species/subspecies level of Potato virus Y and Wheat dwarf virus (Glais & Jacquot,
224 2015).

225 The results of the RT-RPA molecular detection technique developed in this study prove that crude
226 extracts of TSWV infected plants can be used directly for sensitive and reliable detection of the
227 virus with a portable but robust technique. In fact, we were able to tailor TSWV specific primers
228 and probes that work with Agdia's RT-RPA detection chambers and ABM flocculation beads. Our
229 results were confirmed by real time PCR amplification. In the case of real time PCR, it is
230 noteworthy that the appearance of amplification curves obtained was atypical and not linear,
231 suggesting that this assay is not the ideal one to quantify an RT-RPA product, but it should be
232 used more as a qualitative tool for assessing the presence or absence of target. This result is
233 expected, since the design of this assay is suitable for use with a fluorometer and was not
234 intended to be used in real time PCR thermal cyclers. The produced RT-RPA amplicons could
235 also be visualized and characterized using agarose electrophoresis, nonetheless, as other
236 researchers report, the assay can have a carryover of protein that can impose a limitation in the
237 proper migration of the amplified nucleic acid during the electrophoresis if a purification is not
238 performed (Babu et al., 2017).

239 As seen by Zhang et al. (2014) the level of sensitivity of RT-RPA is very high, and allowed us to
240 reach the theoretical detection of one copy of TSWV RNA segment. This unsurpassed sensitivity
241 is intrinsic to RT-RPA, that combines nucleic acid amplification with serological detection.

242 In conclusion, we were able to design a molecular technique for the rapid and sensitive detection
243 of *Tomato spotted wilt orthotospovirus* that is portable and can be interpreted using three different
244 tools: amplification detection chambers, SPRI beads + crowding agent and fluorometry, using a
245 modified probe. All these techniques can be used interchangeably for a qualitative detection of
246 TSWV and can be scaled up for lab-on-a-chip applications or point-of-care detection of virus, to
247 make quicker informed decisions in resource-limited environments, and without the need of
248 trained personnel.

249 **Materials and Methods**

250 **Plant rearing and infection**

251 *Nicotiana benthamiana* seeds were germinated in Sunshine #4 aggregate plus soil mix (Sungro,
252 Agawam, MA) for 1 week and the resulting seedlings were transplanted in 4" square pots and
253 reared for 2 weeks. After this, 18 plants were inoculated using around 1g of TSWV-Chrys 5
254 infected *N. benthamiana* tissue homogenized with mortar and pestle into chilled Paul's buffer (5
255 mM diethyldithiocarbamate –DIECA-; 1 mM Disodic Ethylenediaminetetraacetate –EDTA-; 5 mM
256 sodium thioglycolate in 0.05 M phosphate buffer pH 7) (Mautino, Sacco, Ciuffo, Turina, & Tavella,
257 2012; Van Leeuwen, Arrieta, Guiderdone, Turina, & Ciuffo, 2017) amended with a dash of
258 carborundum and activated charcoal. Other 18 *N. benthamiana* plants were inoculated using
259 TSWV-Tom2. All plants were kept in a growth chamber at 60% relative humidity and a photoperiod
260 of 16/8 hrs day/night for at least 7 days.

261 Similarly, seeds of *Emilia sonchifolia* were germinated in petri dishes for 1 week and transferred
262 to pots of Sunshine #4 aggregate plus soil mix (Sungro, Agawam, MA) and reared for 2 weeks in
263 a 16/8 hrs day/night photoperiod, relative humidity was not controlled for this case. With the
264 described process, TSWV-PA01 was inoculated and harvested 7 days after.

265 **Sample homogenization**

266 Leaf tissue was collected 7 days post infection, transferred to an extraction bag (Bioreba,
267 Switzerland) and mixed with 5 ml of phosphate buffered saline (VWR, West Chester, PA), hand
268 homogenized using a hand-held tissue homogenizer (Agdia, Elkhart, IN) and kept at room
269 temperature for the remainder of the experiment. Serial dilutions of this extract were made, when
270 necessary, by mixing 20 µl of extract with 180 µl of nuclease-free water and repeating this process
271 in series for up to 20 times.

272 **Amplification**

273 Endpoint: AmplifyRP Acceler8 (Agdia, Inc., Elkhart, IN) reaction pellets were rehydrated
274 accordingly to the manufacturer's instructions. Additionally, iScript Reverse Transcriptase (Bio-
275 Rad, Hercules, CA), was provided externally for converting RNA into cDNA, alongside modified
276 primers and a the Acceler8 probe, both provided by Integrated DNA Technologies (IDT, Coralville,
277 IA).

278 The probe and primers were designed according to the Assay Design Help Book from Agdia
279 (<https://d163axztq8am2h.cloudfront.net/static/doc/b0/4f/4e8d445a51f202f9f57f24a74c8d.pdf>).

280 The special considerations to design the probe are here summarized: Less than 50 nt long (42),
281 idSp is the abasic spacer (also known as THF) site that replaced a T, there are 30 spaces between
282 the Fluorophore (FAM) and the abasic site and at least 15 from this site and the 3' spacer. Our
283 TSWV specific Acceler8 Probe: 5'-/56-FAM/AT TGT ACA AAG GTT TGT TTC GGA ATA AAT
284 CTA GG /idSp/AT TCG CAA CCT AAT CT/3SpC3/-3' was designed starting at position 3070 to
285 3163 on the TSWV sequence with accession NC_002050.1 (L segment)

286 The primers were designed using Primer Blast (Ye et al., 2012) automatic parameters namely,
287 the target was set to TSWV with accession NC_002050.1 with the option of searching for other
288 isolates from the Refseq Representative Genome Database by limiting the organism field to
289 *Tomato spotted wilt orthotospovirus* (taxid:1933298), the maximum, optimum and minimum
290 primer sizes were set to 30, 33 and 36 nt respectively, the size of the amplicon was limited from
291 100 to 230 nt. Finally, a biotin was added to the 5' end of the reverse primer. The final primer
292 sequences are listed as follows Reverse: 5'-/5BiosG/ATATTGTTATAGAAGGTCCTAATGATT-3',
293 Forward: 5'- GAATCTATTATACCATTCTCAATCTCTTAGC -3'.

294 An abasic site on the middle of a probe is needed for a nuclease to cleave and release the blocking
295 group, allowing the polymerase contained as well in the mixture to extend the probe (Lobato &
296 O'Sullivan, 2018). The reverse primer contains a 5' biotin label and it forms with the probe a dual
297 labeled FAM-biotin amplicon that can be detected with antibodies (Daher, Stewart, Boissinot, &
298 Bergeron, 2016).

299 For performing the reaction, according to the manufacturer's procedure, dry reaction pellets in 0.2
300 ml tubes from the AmplifyRP® Discovery Kits (Agdia, Elkhart, IN) were hydrated with: 5.75 µl of
301 rehydration buffer; 0.5 µl of each, primers at 10 µM; 0.25 µl of 10 µM probe, 0.5 µl of iScript
302 Reverse Transcriptase (Bio-Rad, Hercules, CA), were mixed with a 2 microliters of crude plant

303 extract and with a reaction pellet. Following manufacturer's suggestions, the last 0.5 µl of 280 mM
304 Magnesium acetate (MgOAc) were added to the lid of the microfuge tube and spun down to
305 complete the 10 µl reaction.

306 Upon closing the vessel, the reaction was maintained at 39 °C for 20 min in a portable thermal
307 block (Agdia, Elkhart, IN), and the results interpreted afterward. For non-target controls, water
308 was used instead of the plant extract, as well as the extract from a healthy plant.

309 **Revealing of results: Amplification detection chambers**

310 The 0.2 ml tubes were collected from the portable thermal block and inserted in the designated
311 slot in the Amplification detection chambers (Agdia, Elkhart, IN). After this, the tube was smashed
312 into the amplification detection chamber, freeing both the RT-RPA product and the proprietary
313 solution and this device was left undisturbed while the liquids moved upwards through the lateral
314 flow immunostrip for 20 mins.

315 **Revealing of results: Flocculation essay**

316 For cheaper and in-field visualization methods, we also tried the method of (Koo, Wee,
317 Mainwaring, & Trau, 2016) for which, two volumes of SPRI (Solid Phase Reversible
318 Immobilization) magnetic beads (Applied Biological Materials, Richmond, BC, Canada) were
319 added to the amplified RT-RPA products, incubated for 5 mins and then vortexed thoroughly for
320 10 seconds.

321 The tubes containing the mixture were placed on a magnetic rack and the supernatant was
322 discarded and replaced by 50 µl of 80% ethanol, the mixture was then incubated for 5 min and
323 vortexed 10 seconds again. Finally, the tubes were placed into the magnetic rack again and the
324 ethanol was discarded, the tubes were open to air dry for 5 minutes and 50 µl of crowding solution
325 comprised of 3M sodium acetate and 20% Tween were added.

326 **Real time**

327 Like in the End point assay, pellets of AmplifyRP Acceler8 (Agdia, Inc., Elkhart, IN) were
328 rehydrated using rehydration buffer, MgOAc, primers, XRT probe and reverse transcriptase. The
329 XRT probe was designed according to the instructions from the Assay design book from Agdia,
330 and synthesized by Integrated DNA Technologies (IDT, Coralville, IA).; the same primers as the
331 end-point essay were used.

332 The XRT probe has the same sequence as the designed Acceler8 probe but contains a few
333 modifications for being used in real-time, according to the Assay design book. Namely: the FAM
334 was moved from the 5' end to one side of the abasic site and attached to a T, a black hole
335 quencher was added to the other side of the abasic site and attached to a T nucleotide as well.
336 Probe:5'-ATTGTACAAAGGTTTGTTCGGAATAAATC/iFluorT/AGG/idSp/AT/iBHQ-
337 1dT/CGCAACCTAATCT/3SpC3/-3'

338 For this case, to every dried amplification pellet, 5.75 µl of rehydration buffer were added; 0.5 µl
339 of each, primers at 10 µM; 0.25 µl of 10 µl XRT probe, 0.5 µl of iScript Reverse Transcriptase
340 (Bio-Rad, Hercules, CA), were mixed with a 2 microliters of crude plant extract and with a reaction
341 pellet. Following manufacturer's suggestions, the last 0.5 µl of 280 mM Magnesium acetate
342 (MgOAc) were added to the lid of the microfuge tube and spun down to complete the 10 µl
343 reaction.

344 For measuring the production of FAM, the vessels were inserted in a Bio-Rad CFX-96 system for
345 real time PCR with a custom-made program that consisted of 20 cycles at 39 °C, with a plate read
346 step every minute. The channel for SYBR/FAM that measures emission at 520 nm was used for
347 collecting fluorescence.

348 **Standard curve**

349 For standard curve analyses, a qRT-PCR product was purified from a complete PCR reaction
350 using the Zymo DNA Clean & Concentrator™-5 kit (Zymo Research, Irvine, CA) and eluted in 10
351 µl of nuclease-free water; then its concentration was measured by a Nanodrop 2000
352 spectrophotometer (Thermo Scientific, Wilmington, DE). Serial dilutions were prepared by adding
353 10 µl of the original amplicon to 90 µl of nuclease free water for each case. Then, the number of
354 copies of template was calculated using the following formula:

$$355 \text{ Number of copies} = \frac{(\text{amount in ng} * 6.022 * 10^{23})}{(\text{size in bp} * 1 * 10^9 * 650)} \quad (1)$$

356 Equation 1 . Where $6.022 * 10^{23}$ corresponds to the Avogadro's number for number of molecules
357 per mol, 10^9 nanograms of exist in one gram and 650 is the average molecular weight in
358 Daltons or g/mol of a pair of bases of DNA (double stranded). The standard curve was made by
359 making a linear regression of the obtained C_q in the y-axis vs. the log₁₀ of the calculated
360 number of copies in the x-axis.

361 **Quantification of starting material**

362 Fifty microliters of *N. benthamiana* lysate were transferred to a 1.5 ml microfuge tube and
363 amended with three volumes of TRIzol LS (Life technologies) and incubated for 3 minutes. Then,
364 a volume of 100% ethanol was added and vortexed for 10 seconds. Finally, Directzol (Zymo
365 Research) kit was used to extract the total RNA, following the manufacturer instructions.

366 The total RNA was checked in a Nanodrop (ThermoFisher Scientific) spectrophotometer and
367 diluted to 250 ng/µl to be used in real time PCR. The RT-qPCR reaction was performed using a
368 the iTaq™ Universal SYBR® Green One-Step Kit , containing 10 µl of 2X (Bio Rad, Hercules,
369 CA), 0.25 µl of iTaq™ Universal SYBR® Green (Bio-Rad, Hercules, CA), 1X iScript Reverse
370 Transcriptase (Bio-Rad, Hercules, CA), 10 mM of the primers L_TSTW_Fw_Short 5'-
371 GCTCTCCTRTCCACCATCTAC-3'; L_TSTW_Rv_Short 5'-TTGCTGTCAAGGCACACATTTT-3
372 (Iturralde-Martinez, 2017) designed to amplify a 136 bp amplicon in the TSWV L genomic
373 fragment.

374 The cycling conditions for the qRT-PCR included a reverse transcription at 50 °C for 10
375 min, an initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95
376 °C for 10 s, and combined annealing/extending at 60 °C for 30 s, according to the
377 manufacturer's instructions.

378 **Sensitivity assay**

379 Serial dilutions of crude extract were made by mixing 10 µl of lysate with 90 µl of nuclease-free
380 water, and then 10 µl of this solution were mixed with 90 µl more and so on (1:10). From each
381 dilution, 2 µl were taken directly and mixed with the master mix for each RT-RPA reaction.

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