EVALUATION OF PCR AND NON-RADIOACTIVE MOLECULAR HYBRIDIZATION TECHNIQUES FOR THE ROUTINE DIAGNOSIS OF *TOMATO LEAF CURL NEW DELHI VIRUS*, *TOMATO YELLOW LEAF CURL VIRUS AND TOMATO YELLOW LEAF CURL SARDINIA VIRUS*

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Running title: Molecular detection of ToLCNDV, TYLCSV and TYLCV

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SUMMARY.

The begomovirus *Tomato leaf curl New Delhi virus* (ToLCNDV) has been reported as a causal agent of leaf curl disease in tomato and other solanaceous crops and, more recently, affecting different cucurbitaceous crops. ToLCNDV was first detected in Asia and recently in Europe, in 2013. In the present analysis, we have evaluated the PCR and the non-radioactive nucleic acids spot hybridization (NASH) techniques together with two nucleic acids extraction protocols, for the routine diagnosis of ToLCNDV and its discrimination from the closely related *Tomato yellow leaf curl Sardinia virus* (TYLCSV) *and Tomato yellow leaf curl virus* (TYLCV). A protocol, designed to extract only the DNA, gave the best results with the PCR technique meanwhile the use of silica, which favors total nucleic acids extraction, was the best extraction protocol for the NASH. All allowed the specific detection of ToLCNDV but only some of the general begomovirus primers allowed the detection of all three viruses. The two ToLCNDV riboprobes analyzed by NASH, targeting the replicase and the coat protein genes, respectively, detected the virus with no cross-reaction with the TYLCV and TYLCSV infected extracts, obtaining a better detection limit with the replicase riboprobe. Direct comparison between the PCR and NASH techniques by the analysis of 42 field samples, revealed a good correlation between the two techniques. In addition, some samples were detected only by NASH due the presence of PCR inhibitors. The use of the PCR and NASH for the routine diagnosis of ToLCNDV is discussed.

Key words: Molecular detection methods; dig-RNA probe; Begomovirus; Routine diagnosis;

INTRODUCTION

Tomato leaf curl New Delhi virus (ToLCNDV) is a member of the genus *Begomovirus* belonging to the *Geminiviridae* family, characterized by icosahedral virions (18x30nm) joined by one of their faces, giving them geminate appearance. Specifically, ToLCNDV is the first begomovirus with bipartite genome detected in Europe (Juarez et al., 2014). The genome of this virus, like many members of this genus, is composed of two circular single-stranded DNAs named DNA-A and DNA-B (Padidam et al. 1995). DNA-A (2734 bp) encodes a protein associated with replication (Rep), the replication enhancer protein (Ren) interacting with the Rep, the transactivator protein (TRAP) that is involved in the activation of transcription controlled by the promoter of the CP and the suppression of RNAi, and capsid protein (CP), responsible for the encapsidation of viral RNAs and involved in intracellular virus movement, recognition of vector functions and systemic invasion of the genome through vascular tissue. DNA-B (2696 bp) encodes the nuclear shuttle protein (NSP) and movement protein (MP) and is responsible for the viral core movement, systemic movement and symptom expression. Both genomic components share a common region (CR) containing motifs necessary for the control of gene expression and replication initiation (Harrison et al. 2002; Pratap et al. 2011). At the moment, there have been identified different Spanish variants of ToLCNDV sharing a 97% nucleotide identity in both components of its genome with the Indian cucurbit strains (http://cienciacebas.wordpress.com).

ToLCNDV was first detected in India affecting tomato. Subsequently, it has been detected in various crops and species such as pepper (Khan et al. 2006), cucurbits (squash, cucumber, pumpkin, melon, watermelon, *Luffa cylindrica*, *Lagenaria leucantha* and *Momordica charantia*)(Sohrab et al. 2003; Tahir and Haider, 2005; Ito et

al. 2008; Juárez et al. 2014), potato (Garg et al. 2001; Usharani et al. 2004), papaya (Raj et al. 2008), cotton, eggplant and *Solanum nigrum* (Pratap et al. 2011). Besides several Asian countries such as India, Pakistan, Thailand, Indonesia and Bangladesh (Mizutani et al. 2011; Pratap et al. 2011), ToLCNDV has only been detected in zucchini and tomato in Spain (Juárez et al. 2014; Ruiz et al., 2015). ToLCNDV is transmitted by the whitefly *Bemisia tabaci*, in a persistent and circulatory way and although its transmission by contact or seed has not been demonstrated, it is possible to transmit it by artificial mechanical inoculation (Chang et al. 2010; Sohrab et al. 2013; López et al. 2015).

Although different sources of tolerance/resistance have been described (e.g. Saha et al., 2013; Lopez et al., 2015) the availability of quick, sensitive, economic and reliable diagnostic methods are still indispensable tools to control the virus incidence and reduce the significant economic losses caused by this devastating pathogen. Serological and molecular approaches like enzyme linked immunosorbent assay (ELISA), nucleic acid spot hybridization (NASH) and polymerase chain reaction (PCR) are generally used for the detection of geminiviruses (Navot et al. 1992). Though ELISA is more suitable for screening of a large number of samples due to high throughput and economy, NASH has been proved to be an alternative to the serological methods due to its comparable detection limit (Sánchez-Navarro et al. 1996; 1998), economy and the capacity to perform large-scale screening (Pallás et al. 1998; Sánchez-Navarro et al. 1999).

In the present work, we have compared two molecular detection techniques (PCR and NASH) and two different total nucleic acid extraction protocols for the detection of ToLCNDV and the closely related viruses, also present in Spain, *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Tomato yellow leaf curl virus* (TYLCV). For NASH analysis, direct application of leaf tissue extracts in citrate buffer, was also evaluated.

PCR analyses were performed either using ToLCNDV-specific (Gawande et al. 2007) or degenerate TYLCV/TYLCSV (Accotto et al. 2000) and begomovirus primers (Wyatt and Brown, 1996; Font, 2003). Meanwhile, the NASH was carried out using digoxigenin-labelled riboprobes designed to target the replicase (ToLCNDV) or the coat protein (ToLCNDV, TYLCSV and TYLCV) genes. The results revealed that both techniques permit the specific detection of ToLCNDV with a comparable detection limit.

MATERIAL AND METHODS

Virus sources and acid nucleic extraction methods

Virus isolates of the three different begomovirus species compared in the assays were collected in 2013 in three different provinces of the South of Spain: ToLCNDV from zucchini (Table 1, sample no. 1; Murcia), TYLCSV from tomato (Table 1, sample no. 2; Granada) and TYLCV from tomato (Table 1, sample no. 3; Almería). Twenty and eighteen samples of tomato and different cucurbits species (cucumber, melon, squash watermelon and zucchini), respectively, with typical symptoms of begomovirus infection were collected from the major production areas of Spain (Table 1).

Different extraction protocols were analyzed. Total nucleic acid extraction was performed using 0.1 g of leaf tissue and applying two different protocols: (i) The Silica capture extraction protocol (MacKenzie et al. 1997) that purifies total nucleic acids and (ii), the E.Z.N.A Plant DNA Miniprep Kit (OMEGA Biotech, Doraville, USA) (hereafter EZNA) designed to extract only the DNA. In both protocols, the extracted nucleic acids were resuspended in 200 µl of water (Silica) or elution buffer (EZNA) and stored at -80 ºC until use. Tissue homogenized directly with citrate buffer was also

123 assayed for NASH analyses in which the sample extraction and the membrane
124 application were performed as described previously (Sánchez-Navarro et al. 1998,
125 1999). Briefly, healthy and infected tissue were homogenized with 5 volumes of cold
126 extraction buffer (50mM sodium citrate, 5mM EDTA, pH 8.5) and directly applied
127 (1µl) onto nylon membranes.

128

129 **PCR and restriction digestion**

130 For PCR detection, four different primer pairs were used and their characteristics are
131 detailed in Table 2. Two sets were degenerate and designed to amply a fragment of 578
132 bp of different species of the genus *Begomovirus* (AV1048/AC494 and PGI/PGII);
133 another primer pair was specific for ToLCNDV (ToLCNDV up/down; 914 bp) and the
134 last pair (TY1/TY2) amplified 580 bp of the genome of two viral species TYLCV and
135 TYLCSV. PCR analyses were performed using DNA polymerase (Biotools DNA
136 Polymerase, B&M Labs S.A. Madrid, Spain) with each pair of primers and 2 µl of the
137 total nucleic acids (undiluted or from the corresponding dilutions) in a 20 µl reaction.
138 The PCR program consisted in initial denaturation for 2 minutes at 94ºC and 35 cycles
139 of 94ºC for 1 minute, annealing temperature appropriate for specific primers (Table 2)
140 for 1 minute and 72 ºC for 1 minute, plus a final incubation of 10 minutes at 72ºC to
141 finish the incomplete PCR fragments, except that for ToLCNDV annealing and
142 extension temperature were maintained for 2 and 3 minutes, respectively. Ten
143 microliters of the amplified PCR products were analyzed in 1.2% agarose gels in 1x
144 TAE buffer, stained with ethidium bromide and visualized under UV light. The
145 remaining ten microliters of the PCR reaction obtained with degenerate primers for
146 begomovirus species detection (AV494/AC1048 and PGI/PGII) and primers which

147 amplified TYLCV and/or TYLCSV (TY1/TY2) were directly digested with *Eco*47I
148 (*Ava*II) enzyme in a total volume of 20 µl, following the manufacturer´s instruction. All
149 the digestion reactions were analyzed in a 3% agarose gel in 1x TAE buffer stained with
150 ethidium bromide and visualized in a UV transilluminator.
151

152 **Synthesis of specific cDNA clones**

153 PCR reactions were carried out as described above using specific primers (Table 2),
154 containing the *Xho*I and *Sal*I restriction sites at the 5' and 3' ends, respectively. For the
155 detection of ToLCNDV two regions, corresponding to part of the replicase (296 nt;
156 primers 2558s and 2557As) and the coat protein (CP) (737 nt; primers 2565s and
157 2566As) genes, were selected. In the case of TYLCSV and TYLCV, the target region
158 corresponded to the CP gene, covering a region of 518 nt (TYLCSV; primers 2530s and
159 2531As) or 751 nt (TYLCV; primers 2381 and 2382). The amplicons were digested
160 with the *Xho*I and *Sal*I restriction enzymes and the resultant fragments were inserted in
161 the plasmid pBluescript SK+, previously digested with the *Xho*I enzyme and
162 dephosphorylated. In the case of the amplified ToLCNDV CP fragment, the *Xho*I and
163 *Sal*I digestion generated two fragments of 457 and 278 nt, due to the presence of an
164 internal *Xho*I restriction site. In this case, the fragment of 457 nt was selected for the
165 subsequent processes. The incorporation of the purified PCR fragment in the plasmid
166 pBluescript SK+ in the right orientation allows the inactivation of the original *Xho*I site
167 by the compatible *Sal*I site. This permits the use of the new 5' proximal *Xho*I for the
168 synthesis of the riboprobe or the incorporation of a new PCR fragment, if desired (Peiró
169 et al. 2012).
170

171 **Synthesis of the digoxigenin-labeled riboprobes and hybridization procedure**

For the synthesis of the riboprobes, 1 µg of the corresponding plasmid was linearized with *Xho*I restriction enzyme, purified by phenol–chloroform extraction and precipitated with ethanol. The linearized plasmid was used to synthesize the riboprobe as described previously (Mas et al. 1993; Pallás et al. 1998). One µl of total nucleic acids (undiluted or from the corresponding dilutions) extracted using the Silica or the EZNA protocols, were directly applied onto positively charged nylon membrane (Roche Diagnostics GmbH, Manheim, Germany), air dried and cross-linked by UV crosslinker (700 x 100 $\mu J/cm^2$). Prehybridizations and hybridizations with the riboprobes were conducted as described previously, with the only difference of the 60ºC selected temperature (Pallás et al. 1998; Sánchez-Navarro et al. 1999). All riboprobes were used at the same concentration in the hybridization solution (20 ng/ml). Chemiluminiscent detection using CSPD reagent as substrate was performed as recommended by the manufacturer (Roche Diagnostics GmbH, Manheim, Germany). Films were exposed for 30 minutes.

RESULTS

Detection of ToLCNDV, TYLCSV and TYLCV by PCR

Initially, we analyzed the capacity of the begomovirus AV494/AC1048 primers (Wyatt and Brown 1996) to detect the three viruses. No amplification was observed in the TYLCSV infected extract (Fig. 1, TYLCSV), indicating the inability of the AV494/AC1048 primers to detect this virus. The expected amplicon of 578 nt was observed in the ToLCNDV infected extract until dilutions 10^{-2} (Silica) and 10^{-4} (EZNA) meanwhile for TYLCV infected extracts it was observed until dilutions 10^{-1} (Silica) and 10^{-3} (EZNA) (Fig. 1, ToLCNDV and TYLCV). Both amplicons rendered the expected fragments corresponding to ToLCNDV (578 nt) or TYLCV (300 nt + 278 nt) after the

197 *Eco*47I (*Ava*II) digestion. During the analysis, it was observed that the undiluted
198 samples of the EZNA extraction protocol rendered negative results (TYLCV) or
199 reduced levels (ToLCNDV) of the expected amplicons, indicating the presence of
200 putative inhibitors. When the same samples were analyzed using the general
201 begomovirus primers PGI/PGII (Wyatt and Brown 1996), we observed the expected
202 amplicon of 578 nt in the three infected extracts using both Silica and EZNA extraction
203 protocols, indicating the capacity of such primers to detect the three viruses (Fig. 2).
204 The subsequent *Eco*47I (*Ava*II) digestion of the amplified products confirmed the
205 identity of each virus by generating a fragment of 578 nt (ToLCNDV), two fragments of
206 300 nt and 278 nt (TYLCV) or three fragments of 360 nt, 150 nt and 68 nt (TYLCSV).
207 The detection limit of the ToLCNDV and TYLCSV infected extracts corresponded to
208 the dilutions 10^{-3} and 10^{-2}, respectively, independently of the extraction protocol used
209 meanwhile for the TYLCV extract, the expected amplicon was observed up to the 10^{-2}
210 and 10^{-3} dilutions of the Silica and EZNA protocols, respectively. As observed with the
211 AV494/AC1048 primers, the majority of undiluted samples from both Silica and EZNA
212 protocols, rendered negative results. Finally, we detected the three viruses using specific
213 primers for ToLCNDV (ToLCNDV up/down; Gawande et al. 2007) or for both
214 TYLCSV and TYLCV viruses (TY1/TY2; Accotto et al. 2000)(Fig. 3). The use of the
215 ToLCNDV up/down primers allowed the specific detection of ToLCNDV up to the
216 dilution 10^{-3} of the infected extract, independently of the extraction protocols used (Fig.
217 3A). In the case of the TY1/TY2 primers we observed a better detection limit using the
218 EZNA protocol. Thus, the TYLCSV infected extract was detected until dilutions 10^{-4}
219 and 10^{-5} of the Silica and EZNA protocols, respectively. Similar situation was observed
220 using the TYLCV infected extract in which the expected amplicon was observed until
221 dilutions 10^{-3} and 10^{-4} of the Silica and EZNA protocols, respectively. In all cases, the

identity of the target virus was confirmed after the *Eco*47I (*Ava*II) digestion (Fig. 3B). Summarized, the detection limit obtained using the EZNA extraction protocols was 10 times higher when compared to the Silica protocols for both TYLCSV and TYLCV infected extracts. As observed for the other primers combinations, the expected amplicons could not be observed from undiluted extracts or their amplification was significantly reduced, indicating that such effect was likely due to the presence of inhibitors rather than to the primers used.

Detection of ToLCNDV, TYLCSV and TYLCV by nucleic acid spot hybridization (NASH)

In the next step, we analyzed the three infected samples by NASH using different digoxigenin-labelled riboprobes that recognize the coat protein gene of TYLCV, TYLCSV and ToLCNDV but also the replicase gene of ToLCNDV. Previously, we observed that the detection limit of the four riboprobes was similar, allowing the detection of picograms per microliter of quantified complementary transcripts (data not shown). The four probes were then evaluated in terms of sensitivity and specificity to discriminate between the three closely related viruses. To do this and to compare with the PCR detection technique, the same samples analyzed previously were applied onto four identical nylon membranes that were hybridized separately with the corresponding riboprobe (Fig. 4). In addition to the 1:10 serial dilutions analyzed previously, we also included a different set of 1:5 serial dilutions of all samples to determine more precisely the detection limit.

Considering the two extraction protocols used (Silica and EZNA), we observed equal (ToLCNDV replicase probe) or better results (all coat protein -CP- probes) using the

246 Silica extraction protocol, allowing the detection of samples 5 to 10 times more diluted.
247 Regarding the detection of ToLCNDV-infected extracts, we observed better results
248 using the replicase probe, with a detection limit of 5 to 10 times higher (5^{-5} and 10^{-4})
249 when compared to the CP riboprobe (5^{-4} and 10^{-3}). Both riboprobes were very specific,
250 showing no hybridization signal in any of the other TYLCV and TYLCSV infected
251 extracts. Similar results were observed using the TYLCSV CP probe, which allowed the
252 specific detection of the TYLCSV infected extract until 5^{-5} and 10^{-4} dilutions, with no
253 hybridization signals in the non-related infected extracts, except for the undiluted
254 TYLCV Silica extract in which we observed a very weak hybridization signal. In the
255 case of the TYLCV CP probe, the end point detection limit corresponded to the 5^{-3} and
256 10^{-2} dilutions, although we observed cross-hybridization signal only with the TYLCSV
257 infected extract.

258

259 **Analysis of field samples by PCR and NASH**

260 Finally, we performed a direct comparison between the PCR and the NASH detection
261 methods for the three target viruses by analyzing 42 field samples including cucumber,
262 melon, squash, watermelon, zucchini and tomato using all set of PCR primers analyzed
263 herein and the replicase (ToLCNDV) and CP (TYLCV and TYLCSV) riboprobes
264 (Table 1). The samples were extracted using the EZNA protocol but in the case of the
265 NASH, we also analyzed the samples by a fast protocol in which the tissue is
266 homogenized with citrate buffer and directly applied onto the membrane (Sánchez-
267 Navarro et al. 1998; 1999). Regarding the PCR results, no divergences were observed
268 between the different primers used except for the AV494/AC1048 combination, which
269 rendered negative results for all TYLCSV-positive samples. Interestingly, the PGI/PGII
270 primers allowed the detection of all positive samples obtained with the other primer

271 combinations, indicating their utility for the routine detection of the three viruses. When
272 the same extracts were analyzed with the NASH, we observed that the 89.6% of the
273 PCR positives (26 out 29) were correctly detected and identified, although the EZNA
274 extraction protocol was less sensitive for the NASH methodology. Samples 16, 22 and
275 42 were positive for the ToLCNDV, TYLCV and TYLCSV, respectively but negative
276 using the NASH, suggesting a virus concentration below the NASH detection limit,
277 although we can not discard the presence of a related virus since the PCR amplicons
278 were not sequenced. When the plants were analyzed using the fast citrate buffer
279 protocol, we observed that the 82.7% of PCR positives (24 out 29) were correctly
280 detected and identified. In this analysis, the PCR positives samples 29 (TYLCV) and 37
281 (TYLCV) were also negative by NASH. Finally, we observed three samples that were
282 positive by NASH and negative by PCR, independently of the primers used. Thus,
283 samples 6 (melon), 7 (squash) and 40 (tomato) were positive for ToLCNDV (6 and 7)
284 and TYLCV (40), using both the EZNA and the citrate protocols. These results could
285 indicate the presence of putative inhibitors in the PCR reaction, which was confirmed
286 by successful amplifications from diluted nucleic acid preparations (data not shown).

287

288 **DISCUSSION**

289 The availability of a reliable detection method for the routine diagnosis of a specific
290 pathogen is a necessary tool that, in coordination with other actions, would help to
291 control and potentially eradicate the disease. In some cases, serological detection
292 methods are limited mainly due to the lack or the low reactivity of the corresponding
293 antibody. In this sense, only very recently a commercial antibody against the
294 ToLCNDV has been available (DSMZ, German Collection of Micoorganisms and Cell

295 Cultures), thereby limiting its routine diagnosis, especially in large-scale surveys, being
296 an urgent problem in Europe after the first detection of ToLCNDV in 2013 (Font and
297 Alfaro-Fernández 2014; Juárez et al. 2014). In the present study, we have evaluated two
298 molecular detection methods, the PCR and NASH, and two extraction protocols, for the
299 routine analysis of ToLCNDV and its discrimination from the closely related viruses
300 named TYLCV and TYLCSV. Comparing the two extraction protocols, we observed
301 better results using the EZNA procedure for the PCR technique meanwhile the Silica
302 protocol gave the best results for the NASH. This discrepancy probably reflects the
303 type of nucleic acids that are purified with the corresponding extraction protocol. Thus,
304 while EZNA procedure only purifies total DNA, the Silica extraction method also
305 purifies total RNA (including the viral messenger RNAs) that are only detected by the
306 NASH.

307 Regarding the PCR technology, all primers used in the present work allowed the
308 specific detection of ToLCNDV in different hosts (zucchini, melon, cucumber and
309 squash) although the best sensitivity was observed with the degenerate AV494/AC1048
310 primers (Wyatt and Brown, 1996). However, such general begomovirus primers were
311 unable to detect the TYLCSV, as was already indicated by Accotto et al. (2000). In the
312 other hand, the begomovirus PGI/PGII primers (Font, 2003) allowed the generic
313 detection of the three viruses. In spite of the good detection limit, we also observed that
314 PCR gave some false negatives in the undiluted extracts, probably due to the presence
315 of inhibitors compounds. This effect together with the requirement of a commercial
316 extraction protocol and, in the case of some primers, an extra *Eco*47I (*Ava*II) digestion
317 step, could limit the use of the PCR described herein for the large-scale field screening.
318 Part of these limitations could be overcome by avoiding the DNA extraction step using

319 plant tissue printing on small pieces of nylon membranes (Atzmon et al. 1998) or 3MM
320 paper (Navas-Castillo et al. 1998).

321 An alternative to the PCR detection could be the NASH. Here we have developed for
322 the first time nonradioactive riboprobes for the detection of ToLCNDV. The two
323 ToLCNDV riboprobes analyzed permit the specific detection of the virus with no cross-
324 reaction with the related TYLCV and TYLCSV infected extracts. Similar specificity
325 was observed using the CP riboprobe of TYLCSV but not with the probe designed for
326 TYLCV, which cross-hybridized with the TYLCSV-infected extracts. Blast analysis
327 revealed that both ToLCNDV probes were up to 74% identical to the TYLCV or
328 TYLCSV isolates available in the database meanwhile the CP probes of TYLCV and
329 TYLCSV were up to 81% and 82% identical to the TYLCSV and TYLCV isolates,
330 respectively. In the case of the TYLCSV probe, we observed three TYLCV sequences
331 showing 99-100% identity (GenBank Accession numbers: Z86067, Z86068 and
332 EF423644) that correspond to a recombinant isolate (Garcia-Andres et al. 2007), or
333 putative TYLCSV isolates initially classified as TYLCV. Furthermore, cross-reactivity
334 between TYLCV and TYLCSV probes covering the full genome has been previously
335 reported (Accotto et al. 2000). In spite of the observed cross-reactivity between the two
336 TYLCV and TYLCSV probes observed on initial samples, such effect was not observed
337 during the analysis of 42 field samples, except for just one sample (sample #2). The
338 direct comparison between PCR and NASH revealed that the detection limit of the PCR
339 was similar or 100 times higher than the NASH using the Silica or the EZNA extraction
340 protocols, respectively. Differences of two orders of magnitude in the detection limit
341 between PCR and NASH are reported for other viruses (Sánchez-Navarro et al. 1998)
342 although in our case, such differences are compensated using the Silica extraction
343 protocol, probably due the inherent purification of the target viral RNA.

344 The two studied techniques showed good correlation: 89.6% of the samples identified in
345 PCR were also recognized in NASH experiments. Interestingly, some positive samples
346 were only detected by NASH, indicating the presence of PCR inhibitors in the plant
347 extracts. Finally, the observation that the 82.7 % of the PCR positive samples were
348 detected using a fast extraction protocol that does not require any special manipulation
349 (Sánchez-Navarro et al. 1996, 1999) make the NASH very suitable for large-scale
350 analysis. In addition, the possibility to detect different viruses and/or viroids by mixing
351 the probes (Saldarelli et al. 1996; Sánchez-Navarro et al. 1999) or by using a unique
352 polyprobe (Herranz et al. 2005; Cohen et al. 2006; Aparicio et al. 2009; Lin et al. 2011;
353 Peiró et al. 2012) makes the NASH a very interesting detection method for the routine
354 diagnosis of such plant pathogens.

355

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488

489 **Table 1.** Comparative analysis for the presence of ToLCNDV, TYLCV and TYLCSV in
490 field samples, performed by PCR-RFLPs using degenerate (begomovirus species
491 detection) and specific primers (TYLCV, TYLCSV and ToLCNDV detection) and by
492 nucleic acids spot hybridization (NASH) using specific riboprobes for ToLCNDV
493 (replicase), TYLCV and TYLCSV. All samples were extracted with the EZNA
494 protocol. In the case of NASH, the samples were also analyzed using the citrate buffer
495 protocol.

496

Sample code	Collection date	Area	Host	PCR-RFLPs (Begomovirus)		PCR-RFLP (TYLCV/ TYLCSV)	PCR ToLCNDV	NASH (EZNA/Citrate buffer)		
				AV494/AC1048	PGI/PGII			ToLCNDV	TYLCV	TYLCSV
1	Sep-2013	Murcia	Zucchini	+ ToLCNDV	+ ToLCNDV	na[a]	+	+ / +	- / -	- / -
2	Oct-2013	Granada	Tomato	-	+ TYLCSV	+TYLCSV	-	-/-	(+)/-	+/+
3	Sep-2013	Almería	Tomato	+ TYLCV	+ TYLCV	+ TYLCV	-	-/-	+/+	-/-
4	Sep-2013	Murcia	Zucchini	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	- / -	- / -
5	Sep-2013	Murcia	Zucchini	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	- / -	- / -
6	Sep-2013	Murcia	Melon	-	-	na	-	+ / +	- / -	- / -
7	Sep-2013	Murcia	Squash	-	-	na	-	+ / +	- / -	- / -
8	Sep-2013	Murcia	Zucchini	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	- / -	- / -
9	Sep-2013	Granada	Zucchini	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	- / -	- / -
10	Sep-2013	Granada	Zucchini	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	- / -	- / -
11	Oct-2013	Almería	Melon	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	- / -	- / -
12	Sep-2013	Almería	Cucumber	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	- / -	- / -
13	Sep-2013	Murcia	Squash	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	- / -	- / -
14	Oct-2013	Granada	Cucumber	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	-/ -	- / -
15	Oct-2013	Granada	Cucumber	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	- / -	- / -
16	Oct-2013	Granada	Cucumber	+ ToLCNDV	+ ToLCNDV	na	+	- / -	- / -	- / -
17	Oct-2013	Granada	Cucumber	-	-	na	-	- / -	- / -	- / -
18	Mar-2014	Granada	Cucumber	-	-	na	-	- / -	- / -	- / -
19	Apr-2014	Granada	Watermelon	-	-	na	-	- / -	- / -	- / -
20	May-2014	Granada	Cucumber	+ ToLCNDV	+ ToLCNDV	na	+	+ / +	- / -	- / -
21	May-2014	Valencia	Zucchini	-	-	-	-	- / -	- / -	- / -

22	Sep-2013	Murcia	Tomato	+ TYLCV	+ TYLCV	+ TYLCV	-	- / -	- / -	- / -
23	Sep-2013	Murcia	Tomato	-	-	-	-	- / -	- / -	- / -
24	Sep-2013	Granada	Tomato	+TYLCV	+TYLCV	+TYLCV	-	- / -	+ / +	- / -
25	Sep-2013	Granada	Tomato	+TYLCV	+TYLCV	+TYLCV	-	- / -	+ / +	- / -
27	Sep-2013	Granada	Tomato	+TYLCV	+TYLCV + TYLCSV	+TYLCV +TYLCSV	-	- / -	+ / +	+ / +
28	Sep-2013	Granada	Tomato	-	-	-	-	- / -	- / -	- / -
29	Sep-2013	Granada	Tomato	-	+ TYLCV	+TYLCV	-	- / -	+ / -	- / -
30	Oct-2013	Granada	Tomato	+TYLCV	+TYLCV	+TYLCV	-	- / -	+ / +	-
31	Oct-2013	Granada	Tomato	-	-	-	-	- / -	- / -	- / -
32	Oct-2013	Granada	Tomato	-	-	-	-	- / -	- / -	- / -
33	Oct-2013	Granada	Tomato	-	-	-	-	- / -	- / -	- / -
34	Oct-2013	Granada	Tomato	-	-	-	-	- / -	- / -	- / -
35	Oct-2013	Granada	Tomato	+TYLCV	+TYLCV	+TYLCV	-	- / -	+ / +	- / -
36	Oct-2013	Granada	Tomato	+TYLCV	+TYLCV	+TYLCV	-	- / -	+ / +	- / -
37	Dec-2013	Murcia	Tomato	+TYLCV	+TYLCV	+TYLCV	-	- / -	+ / -	- / -
38	Jan-2014	Granada	Tomato	-	+ TYLCSV	+TYLCSV	-	- / -	- / -	+ / +
39	Jan-2014	Granada	Tomato	-	+ TYLCSV	+TYLCSV	-	- / -	- / -	+ / +
40	May-2014	Valencia	Tomato	-	-	-	-	- / -	+ / +	- / -
41	May-2014	Valencia	Tomato	+TYLCV	+TYLCV	+TYLCV	-	- / -	+ / +	- / -
42	May-2014	Cantabria	Tomato	-	+ TYLCSV	+TYLCSV	-	- / -	- / -	- / -

[a]na: not analysed

499

500 **Table 2.** Primer pairs used in the PCR assays for the detection of begomovirus species and for

501 the amplification of the NASH probes.

502

Primer name	Nucleotide sequence (5´-3´) [a]	Target viral species	Expected Fragment (bp)	Acc. No. and location in the genome[c]	Annealing Temp. (ºC)	Reference
AV494 AC1048	GCCYATRTAYAGRAAGCCMAG GGRTTDGARGCATGHGTACATG	Begomovirus species	578 (CP[b])	NC_001439 494-1070	60	Wyatt and Brown, 1996
PGI PGII	GCCYATGWAYMGRAAGCC GTTWGAVGCATGHGTRCAWGCCAT	Begomovirus species	578 (CP)	X61153 447-1023	48	Font, 2003
ToLCNDV-up ToLCNDV-do	GAACTATGGTGAAGCGACCAGCAGA ACACAGGTCCTTAGGTACCTGG	ToLCNDV	914 (CP)	KF749223 275-1190	52	Gawande et al. 2007
TY-1 TY-2	GCCCATGTAYCGRAAGCC GGRTTAGARGCATGMGTAC	TYLCV, TYLCSV	578 (CP)	X61153 447-1024	55	Accotto et al. 2000
2565-s 2566-As	GACTCGAGCGACCAGCAGATATCATC TATGTCGACATCCGGATTTTCAAAGTAGC	ToLCNDV	737 (CP)	KF749223 286-1021		Herein
2558-s 2557-As	AAACTCGAGCACTGAAGCAGTGGAC TATGTCGACTGTGTTAGCCAGGGCCTG	ToLCNDV	296 (Repb)	KF749225 1341-1621		Herein
2530-s 2531-As	CTTCTCGAGCCCGTATACCAGCCGTGC TCAGTCGACACTGCTCCTTCATCCC	TYLCSV	518 (CP)	JN859137 376-893		Herein
2381-s 2382-As	AACTCGAGCGACCAGGCGATATAATC TATGTCGACATAGAAATAGATGCGTATTTTC	TYLCV	751 (CP)	AF071228 324-1074		Herein

503 [a] Restriction sites of the *Xho*I and *Sal*I are underlined.

504 [b] 'CP' and 'Rep' refer to the coat protein and replicase genes, respectively.

505 [c] Numbers refer to the corresponding nucleotide of the sequence available in the indicated
506 accession number of the GenBank database.

507

508

509 **Table 3.** Relationship of the detection limits obtained with the PCR and the NASH

510 detection techniques.

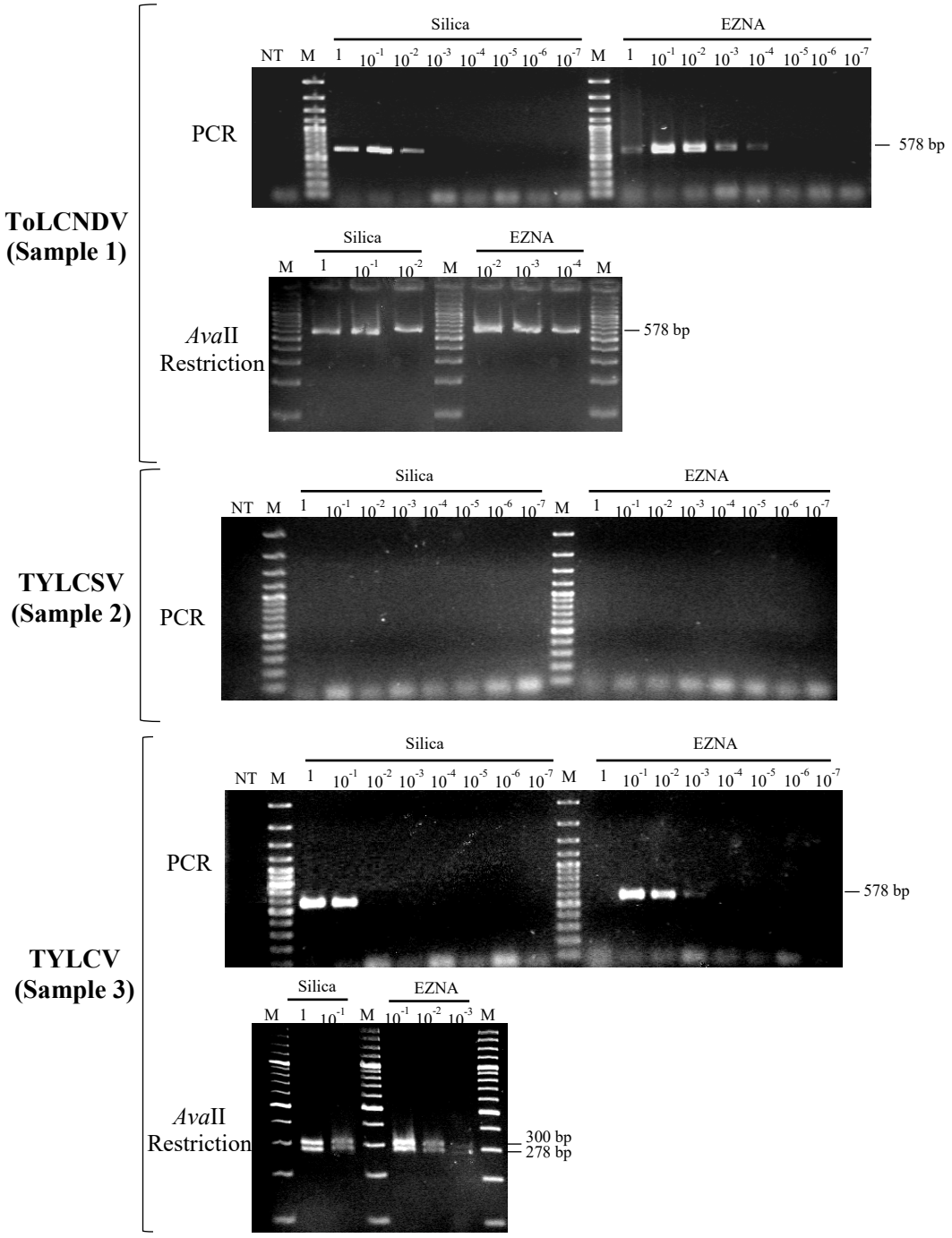
		Detection limit					
		ToLCNDV (sample 1)		TYLCSV (sample 2)		TYLCV (sample 3)	
		Silica	EZNA	Silica	EZNA	Silica	EZNA
PCR	AV494/AC1048	10^{-2}	10^{-4}	-	-	10^{-1}	10^{-3}
	PGI/PGII	10^{-3}	10^{-3}	10^{-2}	10^{-3}	10^{-1}	10^{-2}
	TY1/TY2	-	-	10^{-4}	10^{-5}	10^{-3}	10^{-4}
	ToLCNDV up/down	10^{-3}	10^{-3}	-	-	-	-
NASH							
	Replicase probe	$10^{-4}(5^{-5})$	$10^{-4}(5^{-5})$	-	-	-	-
	CP probe	$10^{-3}(5^{-4})$	$10^{-3}(5^{-4})$	$10^{-4}(5^{-5})$	$10^{-3}(5^{-4})$	$10^{-2}(5^{-3})$	$10^{-1}(5^{-1})$

511

512

513

Figure 1



514

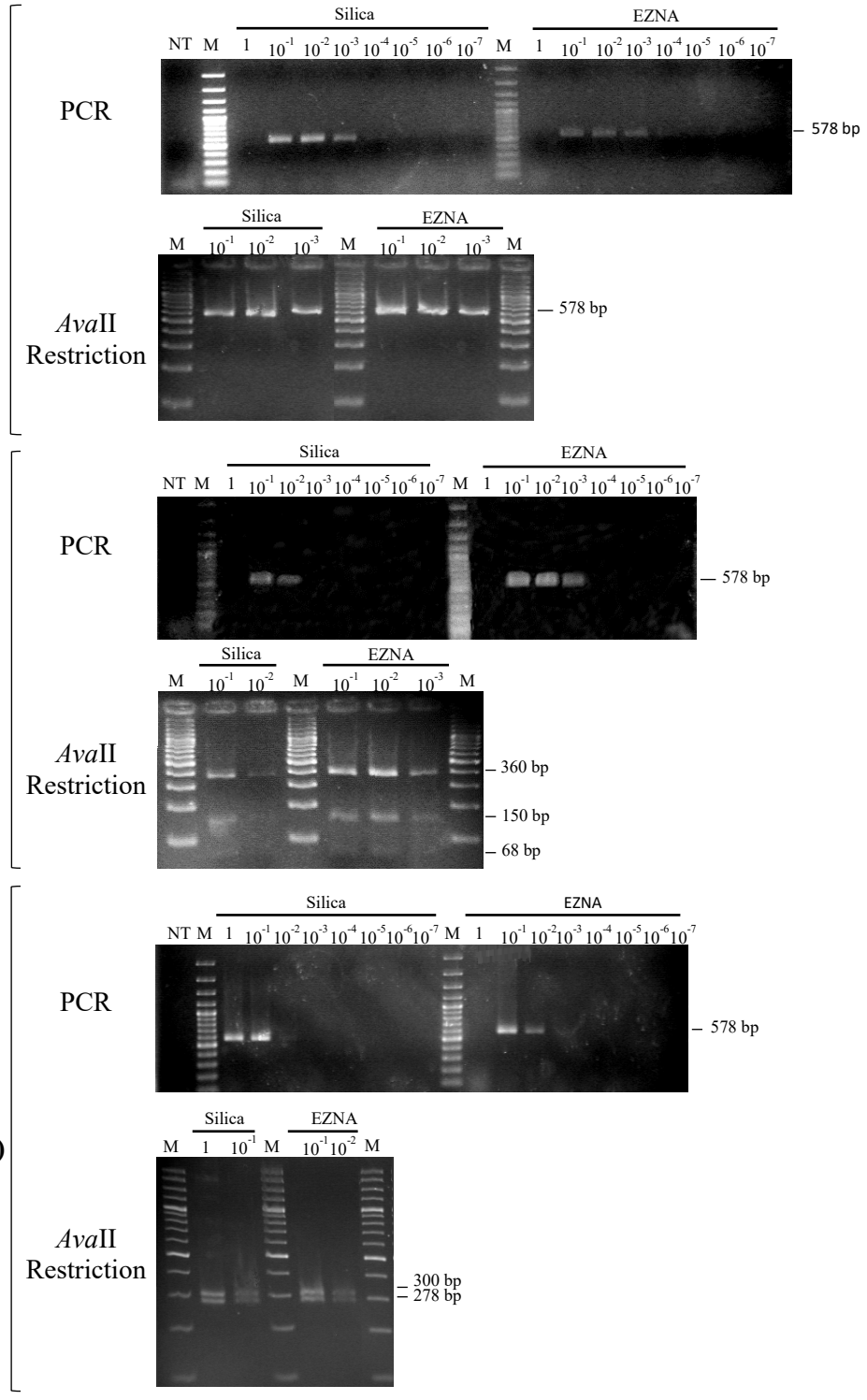
515 **Fig. 1** PCR detection of ToLCNDV (sample 1, Table 1), TYLCSV (sample 2, Table 1)
516 and TYLCV (sample 3, Table 3) using degenerate primers AV494/AC1048 for
517 begomovirus genus (Wyatt and Brown 1996) followed by restriction analyses of the
518 PCR products with endonuclease *Eco*47I (*Ava*II) to identify the viral species. Ten-fold

519 dilutions (10^{-1} to 10^{-7}) of the Silica and EZNA nucleic acid extraction protocols were
520 assayed. The amplicons of 578 bp, corresponding to begomovirus genus and the *Eco*47I
521 restriction patterns for ToLCNDV (578 bp), TYLCSV (360+150+68 bp) and TYLCV
522 (300 bp and 278 bp) on agarose gels are indicated. Lanes NT and M correspond to no
523 template control and to the 100 bp molecular weight marker, respectively.

524

525

Figure 2



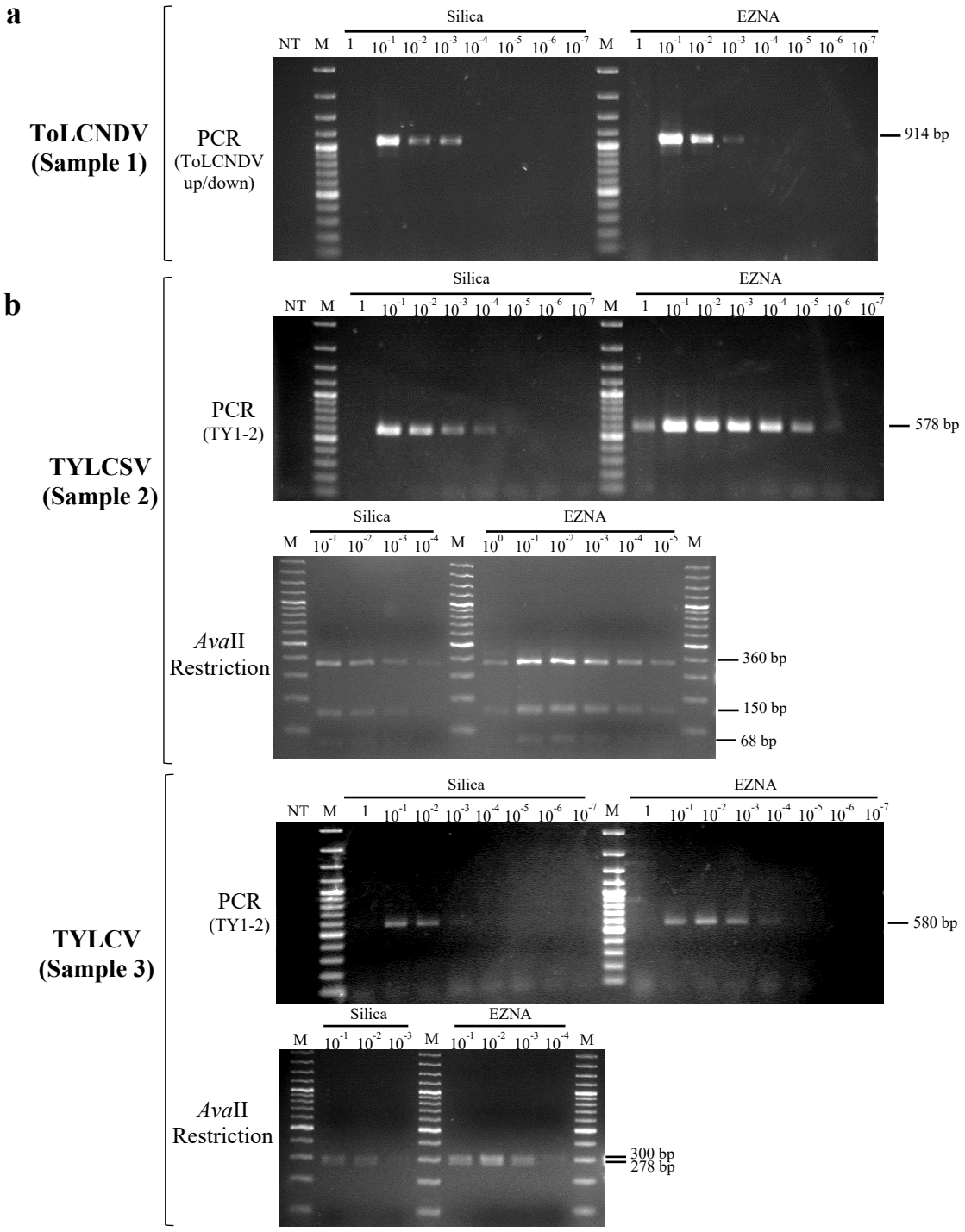
526

527 **Fig. 2** PCR detection of ToLCNDV (sample 1, Table1), TYLCSV (sample 2, Table 1)
528 and TYLCV (sample 3, Table 3) using degenerate primers PGI/PGII for begomovirus
529 genus (Font, 2003) followed by restriction analyses of the PCR products with

endonuclease *Eco*47I (*Ava*II) to identify the viral species. Ten-fold dilutions (10^{-1} to 10^{-7}) of the Silica and EZNA nucleic acid extraction protocols were assayed. The amplicons of 578 bp, corresponding to begomovirus genus and the *Eco*47I restriction patterns for ToLCNDV (578 bp), TYLCSV (360, 150 and 68 bp) and TYLCV (300 and 278 bp) on agarose gels are indicated. Lanes NT and M correspond to no template control and to the 100 bp molecular weight marker, respectively.

537

Figure 3



538

539 **Fig. 3** PCR detection of ToLCNDV (sample 1, Table 1), TYLCSV (sample 2, Table 1)

540 and TYLCV (sample 3, Table 1) using (a), specific primers for ToLCNDV (ToLCNDV

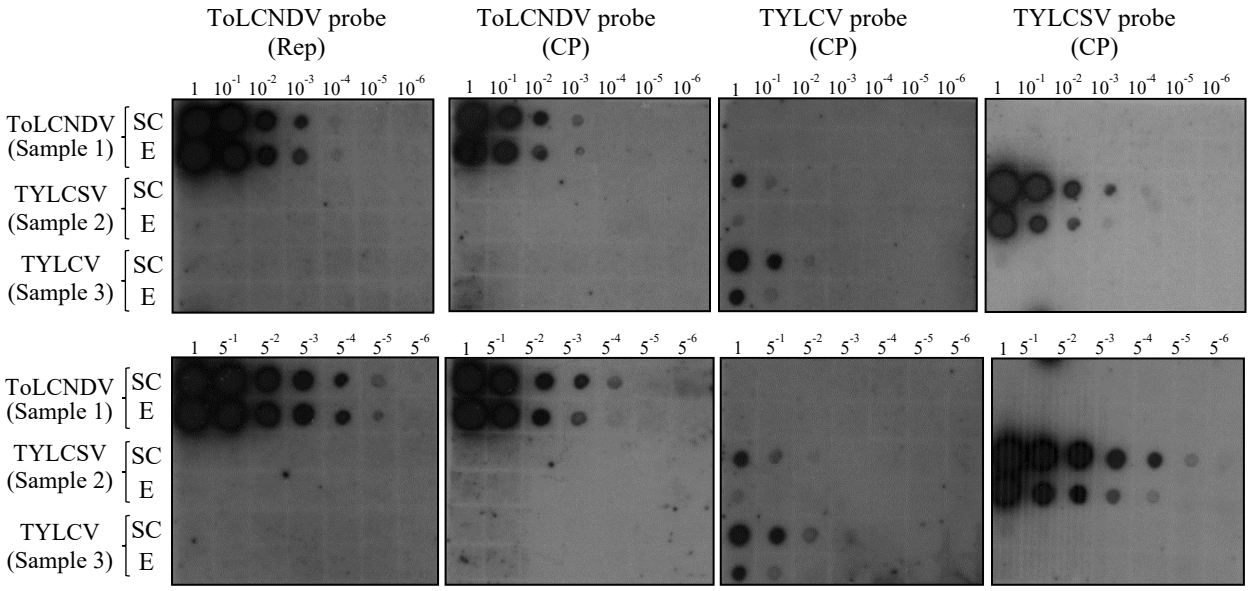
541 up/down; Gawande et al. 2007) and (b), degenerate primers TY1/TY2 (Accotto et al.

542 2000) specific for TYLCSV and TYLCV. Ten-fold dilutions (10^{-1} to 10^{-7}) of the Silica
543 and EZNA nucleic acid extraction protocols were assayed. The amplicons of 914 bp or
544 578 bp corresponding to ToLCNDV or the TYLCSV and TYLCV viruses, respectively,
545 are indicated as well as the *Eco*47I (*Ava*II) restriction patterns that permit the
546 identification of TYLCSV (360, 150 and 68 bp) and TYLCV (300 and 278 bp) virus
547 species. Lanes NT and M correspond to no template control and to the 100 bp molecular
548 weight marker, respectively.

549

550

Figure 4



551

552 **Fig. 4** Detection of ToLCNDV, TYLCV and TYLCSV in infected tissue by nucleic acid
553 spot hybridization (NASH). Infected zucchini (ToLCNDV; sample 1, Table 1) or
554 tomato (TYLCSV, sample 2; TYLCV, sample 3 in Table 1) tissues were extracted with
555 the EZNA (E) or Silica (SC) protocols and applied onto nylon membranes. Undiluted
556 samples and fivefold (5^{-1} to 5^{-6}) or tenfold (10^{-1} to 10^{-6}) dilutions with sterile water were
557 dotted onto nylon membranes and analyzed using the individual riboprobe (virus name-
558 probe) targeting the replicase (Rep) or the coat protein (CP) genes. Numbers at the top
559 of the figure represent the dilutions performed on the original undiluted samples.
560 Chemiluminescent detection was carried out after 30 min exposure.

561