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Application of FTA®Cards to Sample Microbial Plant Pathogens for PCR and RT-PCR

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Abstract

For the detection of microbial plant pathogens, like fungi, bacteria, viruses and viroids, methods based on nucleic acids have gained importance as the availability of sequence information increased. This requires well-established extraction procedures that are cheap, non-laborious, safe and reliable. The paper cards introduced by Flinders Technology Associates, acronym FTA®cards, offer a simple tool to sample and preserve nucleic acids from many kinds of biological specimen and have been already tested for their potential to sample and process several plant pathogens in PCR and RT-PCR. We have tested FTA cards for the sample preparation of a broader range of plant pathogens with different NA contents and subsequent amplification by PCR, RT-PCR as well as multiplex PCR.

Introduction

The first attempt of a tissue print from Plum pox virus-infected leaves on filter paper for RT-PCR detection of the virus was published by Olmos et al. (1996). The described method was further developed into FTA®-cards where the paper was impregnated with several chemicals to assist in lysing cells and protect the released nucleic acids from degradation. It is asserted that at least DNA survives storage on dry cards for several years at room temperature (Smith and Burgoyne 2004), whereas RNA needs storage at minus 20–80°C (Roy and Nassuth 2005). Besides the advantage of improved stability, another advantage is the loss of infectivity of the adsorbed material, making transportation of samples, still suitable for nucleic acid-based detection, easier and safer (Moscoso et al. 2005).

There are many reports where nucleic acids (NA) from blood, saliva and also human and animal pathogens have been sampled on FTA cards for detection by PCR or RT-PCR. Reports on the application of the FTA cards for plants or their pathogens, however, appeared rather rarely (Lin et al. 2000; Drescher and Graner 2002; Ndunguru et al. 2005; Roy and Nassuth 2005; Suzuki et al. 2006; Owor et al. 2007a).

Up to now the status for usage of FTA cards with plants and their pathogens can be described as follows. The proof of principle has been published by Ndunguru et al. (2005). They were able to recover viral DNA or RNA from several plant tissues pressed on FTA cards. The recovered NA provided efficient template to start up several molecular methods such as PCR, cloning and sequencing. The viruses used in their study include ssDNA geminiviruses, tobacco mosaic virus (TMV) and two potyviruses. FTA cards were employed as well in a relatively large-scale field sampling of ssDNA virus-infected plant tissues (Owor et al. 2007a). The authors showed that NA recovered from FTA cards were comparable to those obtained by other classical methods like CTAB. They also experimentally proved that the NA is stable up to 8–9 months storage on the FTA cards. Roy and Nassuth (2005) described sampling and application of FTA cards for RNA viruses, mRNA from plants as well as plant genes, and were the first to describe the possibility to work on DNA, RNA and RNA virus from the same sample successfully. Suzuki et al. (2006) have shown that NA recovered from FTA cards could be used in genotyping assays as well as for the detection of NA from higher fungi. They released the NA from the fungal tissue by treatment in a microwave oven before application to the FTA cards.

In this study, we have tested the suitability of FTA cards for the extraction of NA from a broader range of microbial plant pathogens and for the first time for a genomic dsRNA and for a viroid. Also, we report here the first use of NA recovered from FTA cards in a multiplex PCR.

Material and Methods

Pathogens

The pathogens used in this study together with their propagation hosts and methods are summarized in Table 1.

Table 1
Plant pathogens evaluated for FTA sampling: their nucleic acid types and ways of propagation before FTA-card sampling

Nucleic Acid-Type	Pathogen		Propagation hosts or Propagation method
	Acronym	*Name*	
circular ssDNA	WmCSV	*Watermelon chlorotic stunt virus*	*Citrullus lanatus*
dsRNA		Mycovirus *China 9*	*Fusarium graminearum*
(−)ssRNA	TSWV	*Tomato spottet wilt virus*	*Nicotiana rustica; N. benthamiana*
(+)ssRNA	TMV	*Tobacco mosaic virus*	*N. tabacum*, Samsun nn
	CMV	*Cucumber mosaic virus*	*N. glutinosa*
	LChV 1	*Little cherry virus 1*	*Prunus avium*
	LChV 2	*Little cherry virus 2*	
circular ssRNA	PSTVd	*Potato spindle tuber viroid*	*Solanum esculentum*
dsDNA	*Acidovorax valerianellae*		agarplates
dsDNA	*Agrobacterium tumefaciens*		agarplates
dsDNA	*Ralstonia solanacearum*		agarplates
dsDNA	*Fusarium graminearum*		agarplates
dsDNA	*Phytophthora ramorum*		agarplates/*Rhododendron spec*

TMV, tobacco mosaic virus; CMV, cucumber mosaic virus; TSWV, tomato spotted wilt virus.

Nucleic acid isolation

Total NA from virus-infected plants were extracted by silica as described by Rott and Jelkmann (2001). Bacterial DNA was released by one freeze-thaw-cycle followed by a heat treatment at 100°C for 2 min. Fungal total NA was extracted by grinding the mycelium in liquid nitrogen, followed by the phenol/chloroform method described by Verwoerd et al. (1991). Isolated nucleic acids were quantified and assayed for purity photometrically using a picodrop device (Picodrop Limited, Saffron Walden, UK).

FTA-sample preparation

In principle, sample preparation and application onto the FTA-cards was carried out as described in the Whatman website (http://www.whatman.com/Protocols.aspx). A kit containing the FTA Classic cards, a Harris 2-mm punch and the cutting mat was used (Fig. 1).

Viroids and plant viruses were either applied directly to the card by pressing symptomatic leaves onto the sample area (Fig. 1). Alternatively, the infected plant tissue was ground at a 1 : 10 (w/v) dilution in 10 mM Tris-EDTA pH 8.0 (TE-buffer) and, after homogenization, 60–100 μl was spotted onto the sample area.

Bacteria were usually taken as single colony resuspended in 100 μl TE-buffer and spotted to the card or 120 μl of an overnight culture was applied to the card.

From lower fungi, like *Oomycota*, a small amount of mycelium was suspended in TE-buffer and applied directly to the card without homogenization. Filamentous fungi, like *Fusarium spec*., were homogenized and then applied to the cards. Briefly, 50–100 mg of mycelium was put into a 2-ml reaction vessel together with 3 iron beads (3 mm diameter), frozen in liquid nitrogen and shaken in a Retsch MM 200 Bead Mill at a frequency setting of 24 for 2 min. The ground mycelium was resuspended in 500 μl TE-buffer and spotted gradually onto the FTA card. The cards were dried for at least 1 h at room temperature and then sealed in a plastic bag for storage.

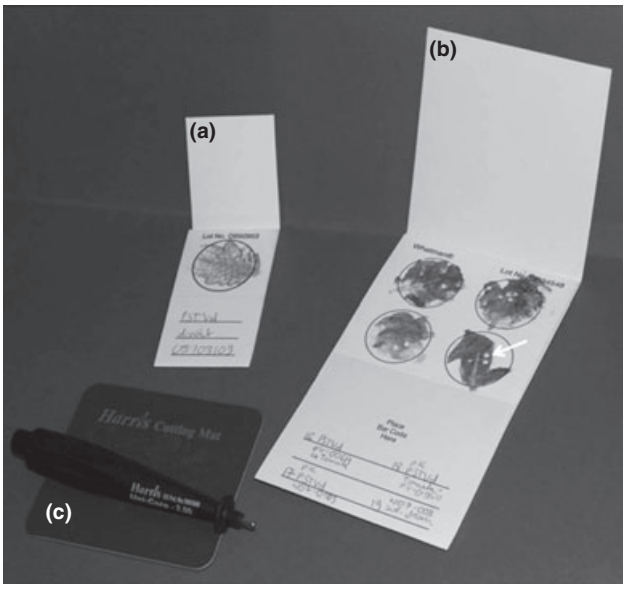

Fig. 1 FTA cards and equipment for punching plugs. a: Microcard with directly applied tomato leaf infected with PSTVd. b: Classic card with 4 application sites directly printed as in **a**. The arrow points to holes where plugs had been removed for testing. c: Cutting mat and 2-mm Harris Punch

Preparation of FTA-card samples for PCR

A Harris punch with a diameter of 2 mm was used to cut out a 2-mm plug from a card where the sample had been applied (arrow Fig. 1). The punch was cleaned as described in the protocols supplied by Whatman using 70% ethanol.

For each PCR, one plug was used. The plug was washed three times for 5 min each with 200 μl FTA purification solution (Whatman) directly in the PCR tube. In the next step, the plug was washed two times for 5 min each with 200 μl TE-buffer, finally dried in a Speedvac centrifuge (Savant Instr., Holbrook, NY, USA) and then used directly as template for a PCR of 25 μl volume. All preparation steps were performed at room temperature.

Preparation of FTA-card samples for RT-PCR

Pathogens with ssRNA genomes were processed with a modified procedure because ssRNA is not retained as strongly as DNA on the paper matrix (Ndunguru et al. 2005). Briefly, ssRNA was eluted from the plug with 400 μl sterile elution buffer (TE-buffer, 200 μg/ml glycogen and 2 mM DTT) by incubation on ice for 15 min with mixing every 5 min. After removal of the plug, the remaining RNA was precipitated by addition of 0.1 vol of 3 M sodium acetate pH 5.2 plus 1 vol 100% ice-cold isopropanol and incubated for 1 h at −20°C. The precipitate was sedimented at 12 000 g for 20 min, washed once with 75% ethanol, centrifuged again at 12 000 g for 10 min. The supernatant was discarded, and the pellet was dried in a Speedvac centrifuge. The RNA was then dissolved in 50 μl sterile double distilled water and used directly for the RT-reaction, otherwise the material was stored for future use at −20°C.

Nucleic acid amplification

The PCR conditions were performed principally as described by Heinze et al. (2006). One FTA plug was applied per each PCR. Approximately 5–6% of the cDNA reaction was used as a template for the subsequent PCR.

The primer sequences used for cDNA synthesis and PCR are summarized in Table 2. The PCR conditions for the detection of the different microorganisms and controls are summarized in Table 3. The PCR was performed in 0.2-ml PCR tubes using the cyclers T1-96 or Professional-96 from Biometra. The enzyme used for PCR amplification was FIREPol® DNA Polymerase

Table 2
Primer sequences, annealing temperatures and expected amplicon sizes for each given primer pair

Primer name	Base sequence	Amplicon size in bp	Annealing-Temp
Mycovirus China 9			
4Rev4	5′-CTT GGA CTC TGT GTT AAC ACC-3′	267	57°C
3prime_For	5′-GCG GCA ATC AGT GGT CAA A-3′		
Fusarium graminearum			
tri4 123bprev	5′-CCC TCT CAA GAA GTA CAT CAC-3′	150	58°C
tri4 123bpfw	5′-GAA ATG TTA GCA AGT GTT CTC C-3′		
Tri5 126bprev	5′-ATC GCT GCT GTC ATC CA-3′	150	58°C
Tri5 126bpfw	5′-ACT ACA GGC TTC CCT CCA-3′		
Tri6 123bprev	5′-CAA AGT CTT GGT TGA ATC CAC-3′	150	58°C
Tri6 123bpfw	5′-CTT TGT CCC AGA TCT AAA CGA-3′		
Agrobacterium tumefaciens			
vir C1	5′-ATC ATT TGT AGC GAC T-3′	730	50°C
vir C2	5′-AGC TCA AAC CTG CTT C-3′		
ipt f	5′-GAT CG(G/C) GTC CAA TG(C/T) TGT-3′	427	50°C
ipt r	5′-GAT ATC CAT CGA TC(T/C) CTT-3′		
A	5′-ATG CCC GAT CGA GCT CAA GT-3′	224	50°C
C	5′-TCG TCT GGC TGA CTT CTT TCG TCA TAA-3′		
Acidovorax valerianellae			
AVO-FOR	5′-TTT TGT ACG GAA CGA AAA AGC TGT-3′	948	58°C
AVO-REV	5′-AGA CCC GGG AAC GTA TTC ACC-3′		
Ralstonia solanacearum			
RS-1-F	5′-ACT AAC GAA GCA GAG ATG CAT TA-3′	718	55°C
RS-1-R	5′-CCC AGT CAC GGC AGA GAC T-3′		
TMV			
Bluni 1	5′-ATT GTG GAS GGA AAA (G/A)CA CT-3′	950	60°C
Bluni 2	5′-GT(G/A) GTT GAT GAG TCC (T/C)TG GA-3′		
CMV			
CMV ATG CP for	5′-ATG GAC AAA TCT G(AG)A TC(AT) (AC)CC-3′	772	59°C
CMV TAG CP rev	5′-CTG GAT GGA CAA CCC GTT-3′		
TSWV			
TSWV-N-ATG	5′-ATG TCT AAG CTT AAG CTC AC-3′	650	60°C
TSWV-N-Ende	5′-GCT GGA GCT GAG TAT AGC-3′		
TOSPO-Uni 1	5′-AGA GCA ATC GTG TCA-3′	450	56–62°C
TOSPO-Uni 2	5′-ATC AAG CCT TCT GAA GGT CAT-3′		
WmCSV			
A_313_CP_s	5′-ATG GCG AAG CGA ACA GGA GAT AT-3′	567	65.0°C
A_880_CP_c	5′-CGA CCA CGG TGG CAT GAA ACT TTC-3′		
B_531_BV1s	5′-ATG CGT CGC TAT GAT GGG ACA CC-3′	493	65.0°C
B_1024_BV1_as	5′-GAC GCT GAT TTT CCT TCA CTC GT-3′		
PSTVd			
69–88	5′-CCCTGAAGCGCTCCTCCGAG-3′	359	62°C
89–113	5′-ATCCCCGGGGAAACCTGGAGCGAAC-3′		
Aktin			
Aktin 1-675	5′-AGT TGC TGA CTA TAC CAT GC-3′	400	56°C
Aktin 2-676	5′-GAC AAT GGA ACT GGA ATG GT-3′		
NAD			
NAD5 antisense	5′-CTC CAG TCA CCA ACA TTG GCA TAA-3′	150	55°C
NAD5 sense	5′-GAT GCT TCT TGG GGC TTC TTG TT-3′		

TMV, tobacco mosaic virus; CMV, cucumber mosaic virus; TSWV, tomato spotted wilt virus.

Table 3
PCR cycling programs applied for each pathogen tested

T (°C)	Time	Cycles	*T* (°C)	Time	Cycles	*T* (°C)	Time	Cycles	*T* (°C)	Time	Cycles
	CMV			TMV			TSWV			WmCSV	
95	04:00	×29	94	05:00	×25	94	04:00	×29	95	03:00	×30
95	00:30|		94	01:00|		94	00:30|		95	01:00|	
59	00:45|		60	00:45|		60	01:00|		65	00:30|	
72	01:00|		72	01:00|		72	01:00|		72	00:45|	
						72	05:00		72	10:00	
72	05:00		72	05:00							
	LChV1			China 9			PSTVd			*F.graminearum*	
4	04:00	×35	94	04:00	×29	94	04:00	×30	94	04:00	×30
94	01:00|		94	00:20|		94	00:30|		94	00:20|	
53	01:00|		57	00:20|		62	01:00|		58	00:20|	
72	01:00|		72	00:30|		72	01:00|		72	00:45|	
72	10:00		72	05:00		72	05:00		72	05:00	
	Ph. ramorum			*A. tumefaciens*			*A. valerianella*			*R. solanacearum*	
94	04:00	×33	94	03:00	×30	95	05:00	×35	95	05:00	×30
94	00:35|		94	01:00|		94	01:00|		95	01:00|	
62	00.55		50	01:00|		58	01:00|		55	00:45|	
72	00:50		72	01:00|		72	01:00|		72	01:00|	
72	|10:00		72	10:00		72	05:00		72	10:00	
	General primers for control of:										
	RT-reaction on NAD			PCR-reaction on Actin							
94	04:00	×29	94	04:00	×25						
94	01:00|		94	00:30|							
55	01:00|		56	01:00|							
72	01:00|		72	01:00|							
72	05:00|		72	05:00								

TMV, tobacco mosaic virus; CMV, cucumber mosaic virus; TSWV, tomato spotted wilt virus.

(AS Solis Biodyne, Tartu, Estonia), and cDNA was synthesized with reverse transcriptase (M-MLV-RT; Promega, Mannheim, Germany) according to the manufacturer protocol.

Evaluation of PCR results

The PCR products were separated on 1.3% agarose gels in TAE-buffer, pH 8, containing 0.2 μg/ml ethidium bromide (Sambrook and Russell 2001). After separation, the gels were illuminated with 254 nm UV-light and documented by a digital camera.

Results

Pathogens with RNA genomes

As examples for plant pathogens with single-stranded RNA genomes, we have chosen potato spindle tuber viroid (PSTVd), tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), little cherry virus1 (LChV1) and tomato spotted wilt virus (TSWV) representing pathogens with high and low titres in their host plants, as well as encapsidated and unencapsidated RNA or even an enveloped virus. From all these pathogens, infected leaves were printed directly on the FTA cards or applied as homogenized leaf extract. For comparison, the RNA was extracted from the same infected plants using a silica-based method.

Figure 2 shows the results for three of the above-mentioned pathogens. In Fig. 2a, CMV is shown as an example for a (+) stranded ssRNA virus with a very high virus titre in plants. This explains the high performance of such a template in RT-PCR regardless of the extraction procedure (lane 2–6). Comparable results (data not shown) were obtained for the other high titre virus, TMV. Controls using water as template or empty plugs were negative (lane 7).

The results with the viroid PSTVd from infected tomato plants are shown in Fig. 2b. Here, we tested the reproducibility using plugs from two different cards, where leaf material had been pressed directly to the cards. It is evident from the very faint band in lane two that the amount of viroid RNA may not be evenly distributed on the sampling surface. Applying more amounts of cDNA to the PCR compensate for the weak signal and led to results comparable with PCR products of silica-extracted total NA (lanes 8 + 9).

Detectable PCR products from the enveloped ambisense RNA virus TSWV (Fig. 2c, lanes 2 and 3) were obtained only by a nested PCR to templates prepared either from the FTA card or from total NA extracted by the silica method.

The results with the LChV1 from infected cherry leaves were negative with all FTA sampled probes,

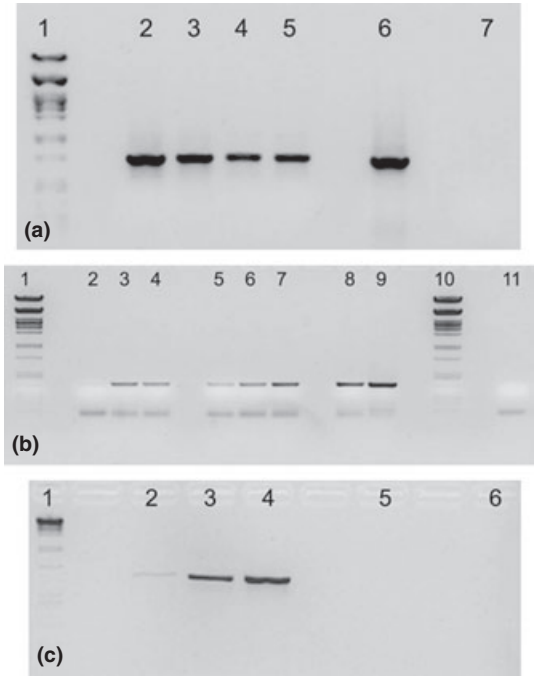

Fig. 2 RT-PCR results from two RNA viruses and one viroid. The RT-PCR products of the viruses were separated on a 1.3% agarose gel in TE-buffer for 30–60 min at 120 V and visualized by ethidiumbromide staining. a: Cucumber mosaic virus from *N. glutinosa* leaves. **1** λ-*Pst*I Marker, **2** and **3** FTA®card, directly pressed leaf tissue, 2-mm plug, 2 μl cDNA template; **4** and **5** FTA®card, leaf homogenate, 1 : 10 (w/v) in TE, and treated as for 2 and 3; **6** Silica-extracted total NA; **7** water control. The amplicon size is 772 base pairs (bp). b: Potato spindle tuber viroid from tomato leaves. **1** and **10** λ-*Pst*I Marker; **2** FTA®card 1, directly pressed leaf tissue, 1 μl cDNA template; **3** FTA®card 1, directly pressed leaf tissue, 2 μl cDNA template; **4** FTA®card 1, directly pressed leaf tissue, 3 μl cDNA template; **5** FTA®card 2, directly pressed leaf tissue, 1 μl cDNA template; **6** FTA®card 2, directly pressed leaf tissue, 2 μl cDNA template; **7** FTA®card 2, directly pressed leaf tissue, 3 μl cDNA template; **8** Silica-Extraktion No.1, 1 μl cDNA template; **9** Silica-Extraktion No. 2, 1 μl cDNA template; **11** water control. The amplicon size is 359 bp. c: Tomato spotted wilt virus from *N. rustica* leaves after nested RT-PCR. **1** λ-*Pst*I Marker; **2** FTA®card, directly pressed leaf tissue, 2-mm plug for RNA extraction, 2 μl cDNA; **3** Silica-extracted total NA, 2 μl cDNA; **4** positive control PCR from a plasmid clone; **5** water control 1st PCR; **6** water control 2nd nested PCR. The amplicon size is 450 bp

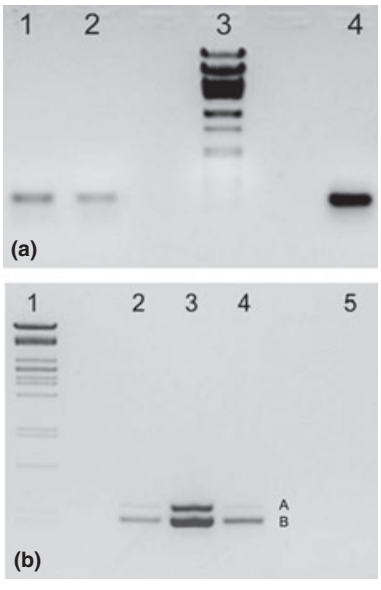

Fig. 3 RT-PCR results for a dsRNA and PCR results for a ssDNA virus. The PCR products of the viruses were separated and visualized as described in Fig. 2. a: Amplified products for NA extracts from *F. graminearum* infected with a dsRNA Mycovirus. **1** FTA®card, RNA released from 2-mm plug, RT-PCR; **2** FTA®card, 2× washed in TE, RT-PCR with 2-mm plug as template; **3** λ-*Pst*I Marker; **4** phenol-extracted total NA from infected mycelium. For each PCR, 2 μl cDNA was used for the PCR reactions. The amplicon size is 150 bp. b: PCR products for NA extracts from Watermelon Chlorotic Stunt Virus from *Citrullus lanatus* leaves. Sample lanes: **1** λ-*Pst*I Marker; **2** FTA®card, directly pressed leaf tissue, 2-mm plug as template; **3** Silica-extracted total nucleic acid (NA), 4 μl total NA as template; **4** Silica-extracted NA, 3 μl as template; **5** water control. The two bands in lanes 2–4 represent the duplex PCR products for the two genomic DNA segments of WmCSV: A (567 bp) and B (493 bp)

whereas the silica-extracted total NA produced positive results (data not shown). This was probably due to the very low virus content in the applied plant material because at least 300 mg of tissue was used for the NA extraction.

As a dsRNA pathogen, we have chosen a virus from *Fusarium graminearum*, called tentatively China-9. The primers for RT-PCR were directed against a short sequenced piece with a RNA-polymerase motive.

Because there is no experience with the retention of dsRNA to FTA cards, we have examined two different methods. After the mycelium was ground in liquid nitrogen, suspended in TE-buffer and applied to the FTA cards, plugs were treated in two different ways. In method 1, we have treated the plugs the same way as for DNA-containing plugs. In the second method, we have eluted and precipitated the dsRNA material like described for ssRNA. In Fig. 3a, lanes 2 and 1 represent the RT-PCR products for dsRNA templates prepared by method 1 and 2, respectively. The signals with both procedures were quite comparable and much weaker than that of phenol-extracted NA from the same mycelium (lane 4).

DNA pathogens

As an example for pathogens with an ssDNA genome we used the Begomovirus WmCSV, with the same direct application technique as described before for RNA viruses. Because it has already been described by others that ssDNA behaves like dsDNA, we have treated the plugs like described for dsDNA. As expected, the primers amplified both DNA species of the segmented genome from FTA cards and from silica-extracted total NA (Fig. 3b). The signal from the FTA plug (lane 2) was as strong as that obtained with 3 μl total NA extract (lane 4).

As typical dsDNA pathogens, we have used three plant pathogenic bacteria and two different plant pathogenic fungi. All three bacteria could be easily detected from FTA cards and in comparable intensities to PCR products from bacterial cells treated by heat shock before PCR. In case of *A. tumefaciens* multiplex PCR

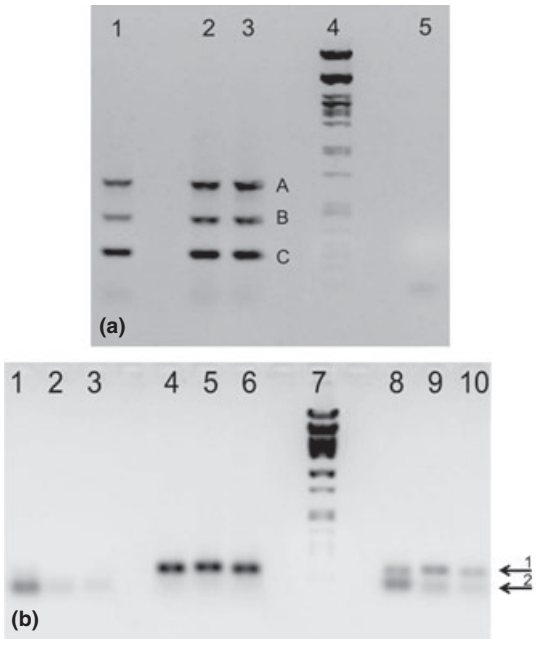

Fig. 4 PCR results for the bacterium *Agrobacterium tumefaciens* and the fungus *Fusarium graminearum*. The PCR products were separated and visualized as described in Fig. 2. a: Multiplex PCR results obtained for *Agrobacterium tumefaciens*. **1** FTA®card; **2** Colony-PCR, *A. tumefaciens* culture, untreated; **3** Colony-PCR, *A. tumefaciens* culture, heat shocked (99°C); **4** λ-*Pst*I Marker; **5** water control. A = *vir* C → primer: *vir* C1/*vir* C2 (730bp); B = Cytokinine → primer: *ipt* f/*ipt* r (427 bp); C = *vir* D → primer: A/C (224 bp). b: PCR products for NA extracts from *Fusarium graminearum* amplified with genomic primers. **1** control H_2O Tri4; **2** control H_2O Tri5; **3** control H_2O Tri 6; **4** Phenol-extracted NA Tri4; **5** Phenol-extracted NA Tri5; **6** Phenol-extracted NA Tri6; **7** λ-*Pst*I Marker; **8** FTA®card Tri4; **9** FTA®card Tri5; **10** FTA®card Tri6. Tri 4, Tri5 and Tri6 indicate the primer pairs of three genes in the trichothecen pathway used for amplification (see Table 2). The amplicon sizes are 150 bp each

with three different primer pairs was successfully employed as shown in Fig. 4a.

Moreover, both of the plant pathogenic fungi could be detected without problems from FTA cards. Whereas higher fungi needed a homogenization step before application to the FTA card, mycelium of the Oomycete *Phytophthora* was applied directly to the cards and was lysed by the embedded chemicals (results not shown). The results obtained with several primer pairs against trichothecen (a mycotoxin)-processing genes from *F. graminearum* are summarized in Fig. 4b. The amount of PCR products obtained from FTA plugs (lane 8–10) was low but nevertheless identical to those obtained with phenol-extracted total NA (lane 4–6, arrow 1) from the same mycelium. An unspecific PCR product was detected in the negative control as well as in the positive samples (Fig. 4b, arrow 2), which probably represent primer dimer.

Several trials to use the direct-press application of *P. ramorum*-infected Rhododendron leaves failed to produce positive result.

Discussion

Our efforts to examine the validity of FTA cards for the PCR or RT-PCR detection of different microbial plant pathogens have clearly shown that independent of the type of NA the card method is suitable. This can be said in general for DNA pathogens, where according to several published reports, the storage of sampled cards at room temperature and the survival of PCR suitable NA have been proven even for ssDNA viruses (De Swart et al. 2001; Smith and Burgoyne 2004; Rajendram et al. 2006; Owor et al. 2007a). The sensitivity as well as reproducibility of the FTA card method compared to other classical NA-extraction procedures was in our hands satisfactory although the intensity of bands obtained from PCR cards was in many cases slightly reduced. One possible reason may be the inability to ascertain the amount of DNA introduced with the plug into the PCR. The main constraint for the FTA-card method seems to be the low concentration of pathogen DNA in the sampled plant tissue leading to false negative results. This was probably the case for *P. ramorum*-infected Rhododendron leaves and may be solved by increasing the number of PCR cycles, but was not tested by us.

Using FTA cards, dsRNA and dsDNA were detected with relatively weak signals when they were from fungal origin. However, the detection of dsDNA from bacteria produced much stronger signals. This indicates that the detection threshold is independent of the ds-nature of the applied NA, but most likely on the amount of the NA introduced into the FTA card and hence into the PCR. Needless to say that according to the extraction procedure applied in this study, bacterial cells would release more NA than fungal cells on FTA cards. In addition, ssDNA seems not to behave different from dsDNA when sampling on FTA cards and using detection by PCR as shown by us with a single-stranded DNA plant virus, as well as on larger scale by others (Ndunguru et al. 2005; Owor et al., 2007b).

The detection of ssRNA viruses, independent of sense, was in our hands quite successful. The procedure we have applied differed from that described by Roy and Nassuth (2005), confirming the recommendations by Whatman, although we could omit the RNAse inhibitors in the elution buffer without problems. The successful detection of pathogen RNA, ranging from a few hundred to several thousand nucleotides, by RT-PCR indicates that the size of RNA does not influence the sampling success as already shown by the publications of other groups using tissue printing on untreated filter paper (Olmos et al. 1996; Weidemann and Buchta 1998). The different results we have obtained with the different viruses are obviously due to the different target concentrations that are introduced into the RT-PCR as suggested by the influence of the amount of cDNA used for PCR, the reaction of the RT-PCR to virus titres in the plant and the differences between direct printing of infected leaves versus application of homogenized leaf sap. We would recommend the use of FTA cards for the routine sampling of biological specimens. However, we would confer to the statement of Roy and Nassuth (2005) that one should determine beforehand the optimal conditions for the preamplification steps.

Table 4
Qualitative performance of NA extracted with different methods in PCR or RT-PCR

	Extraction method		
	FTA cards*		
Pathogen type and source	Direct press	With a pretreatment	Classical methods
Fungi (DNA)			
Higher fungi from agar plates	ND	+[a]	+++[1]
Lower fungi from agar plates	+[b]	ND	ND
Lower fungi from infected leaves	–[c]	ND	ND
Bacteria (DNA)			
From agar plates		+++[d]	+++[2]
From liquid culture		+++[e]	+++[2]
Viruses			
ssDNA: WmCSV from inf. leaves	+[f]	ND	+ to ++[3]
dsRNA: mycovirus from inf. mycelium	ND	+[g]	+++[1]
ss(–)RNA: TSWV enveloped	+[f]	ND	++[3]
ss(+)RNA: (high titre): TMV from plant	+++[f]	++[h]	+++[3]
CMV from plant			
ss(+)RNA (low titre): LChV1, LChV2	–[f]	ND	+[3]
Viroid			
Circular ssRNA: $PSTV_d$ from plant	+[f]	ND	+[3]

*For PCR use 2-mm plugs, wash in purification solution and TE-buffer, dry and add to the PCR master mix.
–,no product; +, faint band; ++, intense band; +++, highly intense band; ND, not determined; TMV, tobacco mosaic virus; CMV, cucumber mosaic virus; TSWV, tomato spotted wilt virus.
1: Homogenized in liquid nitrogen then extracted by Phenol-chloroform.
2: Heat denatured, diluted and applied to the PCR.
3: Extracted with a standard silica-based method.
[a]homogenized in liquid nitrogen, suspended in buffer, spotted on card.
[b]suspend mycelium in buffer, spot on card.
[c]squash symptomatic leaf on card.
[d]resuspend in buffer, heat shock and apply onto FTA card.
[e]heat shock and spot on card.
[f]squash symptomatic leaf on card, extract RNA from an 2-mm plug, redissolve in 50 μl buffer and use 2–3 μl for reverse transcription reaction. Use 2–3 μl cDNA for PCR.
[g]homogenize mycelium as in a and spot on card. Treat 2-mm plugs like described for PCR and perform RT-PCR either directly on the plug or elute the dsRNA from the plug by standard methods and use in RT-PCR.
[h]homogenize symptomatic leaf in buffer at 1 : 10 (w/v) ratio and apply onto the FTA card.

It was interesting to observe no inhibitory effects of plant material after direct printing of leaves on the FTA cards, probably due to a good denaturing capability by the impregnating chemicals. Also, the different forms of PCR detection methods that we used were not noticeably influenced by the use of FTA plugs. Multiplex, nested and semi-nested PCR were possible under conditions known from regular PCR detection procedures using NA isolated according to standard protocols.

In addition to the two application methods used by us in the described experiments, i.e. direct printing without homogenization and application of homogenized material onto the card surface, we have tested the microwave procedure described by Satoshi Suzuki et al. (2006) with fungi. They have placed mycelium together with buffer into wells of a microtiterplate and heated this in a microwave oven. After two or more successive heating, the content was applied onto a FTA card. Unfortunately, although described for *Aspergillus oryzae* to be efficient, we failed to achieve results using this method with *F. graminearum*.

In Table 4, the qualitative performance of NA recovered from the FTA cards in PCR or RT-PCR is compared to those obtained by other classical methods. The procedure by which each NA was applied onto the FTA cards is described in the legend for Table 4. It is obvious from Table 4 that viroids and viruses from easy to squash plant tissue can be detected from FTA cards in comparable sensitivity to standard procedures. The same is true for bacteria. In case of fungi, even after good homogenization, the release of nucleic acids on the FTA cards is not optimal, leading to weaker signals of PCR products.

In summary, the results obtained in this study together with the information collected from other reports, proof the suitability of using FTA cards for sampling, storage, transport and molecular detection of NA from a broad range of biological specimens. Compared to the classical extraction protocols, the method is safe, time saving and reproducible. However, more optimization is required to increase the sensitivity of the detection, especially for rare templates and their release from rigid tissues.

For educational purposes, like laboratory classes, the method has the advantage that noxious chemicals can be avoided, fume hoods are not necessary, and valuable time can be saved.

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