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Protocols

In-field capable loop-mediated isothermal amplification detection of *Turnip yellows virus* in plants and its principal aphid vector *Myzus persicae*

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ABSTRACT

Widespread *Turnip yellows virus* (TuYV) infection causes severe seed yield and quality losses in rapeseed (*Brassica napus*) crops grown in broadacre agricultural systems worldwide. Current TuYV detection protocols are expensive and time consuming, and can have poor specificity and sensitivity. Typically, they are used as a diagnostic tool to test already symptomatic plants, limiting their practical value to reactive disease management. To improve diagnostic services so that they provide earlier, cheaper, faster, more specific and sensitive TuYV detection, novel and innovative protocols that utilise new technology are required. A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed to detect TuYV in crude and total RNA extractions of leaf material and its principal aphid vector *Myzus persicae*. The assay was based on a set of six primers, highly sensitive and specific to TuYV, derived from a TuYV isolate originating from the south-west Australian grainbelt. TuYV was readily detected in 1 in 100 dilutions of (i) infected to uninfected leaf material, and (ii) viruliferous to non-viruliferous *M. persicae*. Furthermore, detection was successful in a majority of aphids stored for at least 8 weeks in various trapping and storage substances, including 30% ethylene glycol, sticky trap glue and 70% ethanol. This RT-LAMP assay protocol enables quicker and cheaper diagnosis for TuYV than currently adopted laboratory-based diagnostic techniques. Ultimately, it has the potential for earlier in-field TuYV detection in combination with aphid trapping surveillance programs.

1. Introduction

Turnip yellows virus (TuYV; Family *Luteoviridae*, Genus *Polerovirus*) is host phloem limited and persistently aphid-transmitted (circulative and non-propagative) (Duffus and Russell, 1972; Mayo and D'Arcy, 1999; Schliephake et al., 2000). TuYV infects a wide range of economically important crop hosts worldwide including cabbage (*Brassica oleracea*), chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), field pea (*Pisum sativum*), lentils (*Lens culinaris*), lettuce (*Lactuca sativa*) and rapeseed (*B. napus*) (Coutts et al., 2006; Coutts and Jones, 2000; Graichen and Rabenstein, 1996; Latham and Jones, 2001; Makkouk et al., 2001; McLean and Price, 1984; Moreno et al., 2004). Rapeseed, grown in broadacre cropping systems for its oilseed which is used for production of cooking oil, margarine, animal meal and biofuel (Economic Research Service, 2016; Ge et al., 2017; Raymer, 2002), is the second largest oil crop in the world and therefore one of the most economically valuable crops affected by TuYV. When rapeseed crops are infected by TuYV at high incidences they incur significant yield losses (> 40%), decreases in seed oil content, and undesirable increases in erucic acid and glucosinolate contents (Graichen, 1995, 1997; Graichen and Schliephake, 1999; Jay et al., 1999; Jones et al., 2007; Smith and Hinckes, 1985).

TuYV is not seed transmissible, therefore its entire infection reservoir to economically important crops is externally sourced from nearby weed, pasture and crop volunteer hosts *via* migrant aphid vectors (Coutts et al., 2006). *Myzus persicae* (green-peach aphid, *Hemiptera*: *Aphididae*) is the principal TuYV vector due to its high virus transmission efficiency (> 90%), generally early arrival into crops, and sparse colonisation habits which result in rapid plant-to-plant migration and extensive virus spread (Duffus and Russell, 1972; Schliephake et al., 2000; Wallis, 1967). *Brevicoryne brassicae* (cabbage aphid, *Hemiptera*: *Aphididae*) is considered of minor TuYV vector importance due to its low transmission efficiency (Schliephake et al., 2000), generally later arrival into crops, and dense vertical colony production on individual plants that are concentrated on crop edges thereby constraining virus spread (Severtson et al., 2015). Epidemiological modelling of the TuYV-rapeseed pathosystem in the south-west Australian grainbelt (Mediterranean-type environment) demonstrates that high levels of rainfall

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prior to sowing (late-Summer to early-Autumn) drive the establishment of a large infection reservoir and aphid vector population. This results in viruliferous aphids arriving in the crop at an early growth stage and high TuYV infection incidences in the crop. Conversely, when there is little to no rainfall in this period, the infection reservoir is smaller, resulting in viruliferous aphids arriving much later and lower incidences in the crop (Maling et al., 2010).

In broadacre farming systems, correct diagnosis of TuYV infection in rapeseed crops is critically important. Symptoms expressed by TuYV-infected rapeseed plants, *i.e.* lower leaf reddening, interveinal chlorosis and stunting (Coutts et al., 2010), are easily mistaken for other stresses such as nutrient deficiencies resulting in misdiagnosis and potentially wasteful application of fertilizers (Graichen and Schliephake, 1999). Conversely, nutrient deficiencies or other stress symptoms may be mistaken for symptoms of TuYV infection that may result in unnecessary application of expensive insecticides (*e.g.* sulfoxaflor) to eliminate aphids and minimise further spread. Laboratory diagnostic services for TuYV detection in commercial crops currently involve testing leaf samples using reverse transcription-polymerase chain reaction (RT-PCR) or serological methods such as enzyme linked immunosorbent assay (ELISA) and tissue blot immunosorbent assay (TBIA) (D'Arcy and Hewings, 1986). These techniques are time-consuming and expensive, requiring specialist laboratory facilities and skilled operators. Although ELISA and TBIA enable much higher sample throughput, they can have poor specificity and sensitivity, and are susceptible to cross-reaction with plant proteins (*e.g.* Boonham et al., 2014; Duffus and Russell, 1972; Hauser et al., 2000; Webster et al., 2004). These protocols provide diagnosis following an influx of viruliferous aphids, their proliferation through the crop and initiation of an epidemic, often upon observation of symptomatic plants at a time when control options (such as the application of expensive systemic insecticides) are reactive and potentially ineffective. New diagnostic protocols aimed at detecting TuYV in crops prior to plant symptom expression are required to improve TuYV diagnostic services, and delivery and efficiency of disease management strategies.

Loop-mediated isothermal amplification (LAMP) is a rapid and sensitive molecular diagnostic technique run under a single reaction temperature (Notomi et al., 2000) that can now be performed with user-friendly and portable machines featuring real-time result display (*e.g.* Optigenes Genie® II and III). Reverse transcription-LAMP (RT-LAMP) is used for detection of some RNA plant viruses in their host plant and insect vectors in total RNA extractions (*e.g.* Tien Le et al., 2010; Zhou et al., 2012). For example, using crude leaf extractions in which leaves were ground in NaOH using a shaker and neutralised immediately with dilution in Tris-HCl buffer, RT-LAMP has been used to detect *Cucumber green mottle mosaic virus* (CGMMV; Family *Tombusviridae*, Genus *Tobamovirus*) (Li et al., 2013). Furthermore, RT-LAMP is effective for detection of the semi-persistently transmitted *Cucurbit chlorotic yellows virus* (CCYV; Family *Closteroviridae*, Genus *Crinivirus*) in tobacco whitefly (*Bemisia tabaci*) taken from sticky insect traps for up to 14 days after being caught. For this, *B. tabaci* were homogenized with a pestle in RNase inactivation agent and heated at 60 °C for 5 min (Okuda et al., 2015). Using RT-PCR, *Potato leafroll virus* (PLRV; Family *Luteoviridae*, Genus *Polerovirus*) can be detected in total RNA extractions of aphid specimens that were caught in yellow pan traps and stored in 70% ethanol at room temperature for at least 7 years (Singh et al., 1995). This is also effective for detection of a single PLRV-viruliferous aphid in groups of up to 30 non-viruliferous aphids (Singh et al., 1997). Crude extraction and RT-LAMP has yet to be utilised to detect plant viruses in their aphid vectors taken from traps soon after being trapped or when stored for extended periods.

A RT-LAMP assay that could detect TuYV in crude extractions of viruliferous aphids caught on sticky traps or in pan traps, in the presence of multiple non-viruliferous aphids, would be in-field capable and highly advantageous for use in surveillance trapping programs and epidemiological studies. Such an assay could enable earlier TuYV detection by intercepting viruliferous aphids as they move from external host reservoirs to emerging crops, thereby providing a more useful diagnostic service to growers than is currently provided. Furthermore, portable LAMP-capable technology would enable in-field opportunistic real-time testing of suspected green bridge host reservoirs and symptomatic crop plants. This paper describes a series of experiments done to develop and validate a rapid, sensitive and specific in-field capable RT-LAMP assay for TuYV detection in (i) dilutions of infected in uninfected leaf material, and viruliferous in non-viruliferous aphids and (ii) viruliferous aphids stored for extended periods in yellow sticky trap glue, 30% ethylene glycol, 70% ethanol and orange oil.

2. Materials and methods

2.1. Plants, virus culture and aphid colonies

Plants of rapeseed cv. Cobbler were used to maintain aphid colonies, TuYV cultures and in experiments. They were grown in potting mix and maintained at 16 to 22 °C (daily min. to max.) in an insect-proofed air-conditioned glasshouse. A culture of TuYV isolate WA-1 (Coutts and Jones, 2000) was maintained in rapeseed plants infested with *M. persicae*. TuYV-infected and healthy *M. persicae* colonies used in experiments were kept inside separate aphid rearing cages (Bugdorm, Australia) located in different air-conditioned controlled environment rooms (held at 20 °C with a 16 h photo-period). The *M. persicae* colony was originally collected from a field site in the south-west Australian grainbelt. For use in experiments, aphids second instar and older taken from TuYV-infected plants were putatively classed as viruliferous and those reared on uninfected plants non-viruliferous. Leaf samples from plants infected with TuYV isolate WA-1 were used as positive controls in ELISA, RT-PCR and LAMP. Plants in the healthy colony were tested regularly by RT-PCR and ELISA to ensure their uninfected status was maintained.

2.2. ELISA

To perform preliminary confirmation of TuYV infection in culture plants used for experiments, leaf samples were extracted singly in pH 7.4 phosphate-buffered saline (PBST; 10 mM potassium phosphate, 150 mM sodium chloride, Tween 20 at 5 ml/liter, and polyvinyl pyrrolidone at 20 g/liter) using a mixer mill (Retsch, Germany). Sample extracts were tested for TuYV by double-antibody sandwich ELISA (Clark and Adams 1977) using BWYV polyclonal antiserum (Sediag, France, cat. no. BWY-SRA 5000). All samples were tested in duplicate wells in microtiter plates. Sap from TuYV-infected and healthy rapeseed leaf samples were always included in paired wells to provide positive and negative controls, respectively. The substrate was p-nitrophenyl phosphate at 1.0 mg/ml in diethanolamine, pH 9.8, at 100 ml/liter. Absorbance values (A405) were measured in a microplate reader (Bio-Rad Laboratories, USA). Positive absorbance values were always more than 3 times those of the healthy sap controls.

2.3. Total RNA and crude extractions

Total RNA extraction was conducted using a QIAGEN RNeasy plant mini-kit according to manufacturer instructions (QIAGEN, Australia). For crude aphid extraction, a polypropylene pellet pestle driven by a pellet pestle motor (Sigma-Aldrich, USA) was used to grind aphids in a 1.5 mL tube containing 30 µL PBST buffer. For crude leaf extraction, 5 mL plastic tubes containing 50 mm^2 leaf discs with three 5 mm ball bearings and 1 mL PBST buffer were shaken by hand for 30 s. For both plant and aphids, 10 µL of crude extract was diluted in another 1 mL of PBST buffer before being used as template for the LAMP reaction mix.

Table 1
Primers used for loop-mediated isothermal amplification of *Turnip yellows virus*.

Primer	Type	Position on genome[a]	Length (nt)	Sequence 5'–3'
F3	Forward outer	897-914	18	TGATGTCACCCTCCTCCG
B3	Backward outer	1084-1102	19	AGTGTCCTCCTTCCGTGTG
FIP	Forward inner	970-991 and 926-945	42	TGCATTTTGCTAGGTTGGCAGCATTGGGAAGGACTGTTAGGC
BIP	Backward inner	1019-1040 and 1064-1083	42	ATGGCTGGGTTAGCGGTTATGCGCTCAGGACCATAACATCGG
LF2	Loop forward outer	946-964	19	TGACGTTGGCCGCTTTACA
LB2	Loop backward outer	1041-1062	22	CGAGATTGTAGGCTCAGAAGGT

[a] Genome position according to the reference nucleotide sequence of TuYV isolate WA-1 (ERS2791624).

Table 2
Turnip yellows virus detection in dilutions of leaf and aphid extractions by reverse transcription polymerase chain reaction (RT-PCR) and reverse transcription loop mediated isothermal amplification (RT-LAMP).

	RT-LAMP				RT-PCR			
	Leaf		Aphid		Leaf		Aphid	
Dilution	Crude[a]	Total[b]	Crude	Total	Crude	Total	Crude	Total
1/1	3/3[c]	3/3	3/3	3/3	2/3	3/3	2/3	3/3
1/10	3/3	3/3	3/3	3/3	2/3	3/3	2/3	3/3
1/20	3/3	3/3	3/3	3/3	2/3	3/3	2/3	3/3
1/50	3/3	3/3	3/3	3/3	2/3	3/3	2/3	3/3
1/100	3/3	3/3	3/3	3/3	2/3	3/3	1/3	3/3
0/100	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

[a] For crude aphid extractions, *Myzus persicae* apterae ground up in 30 µL PBST buffer with a polypropylene pestle driven by a pellet pestle motor. For leaves, rapeseed leaf discs shaken in 5 mL tube containing 1 mL extraction buffer and three ball bearings.
[b] Total RNA extracted using a QIAGEN RNeasy mini kit according to manufacturer's instructions.
[c] Number of repeat experiments TuYV-detected/total number of repeat experiments.

2.4. RT-PCR

Two-step RT-PCR was performed to amplify the open reading frame (ORF) 3 RdRp gene nucleotide (nt) sequence of TuYV using primers TuYV1_3299 F (5′-CGTAAGTTGCAAGTAAGGGAAAC-3') and AS5 (5′-CCGGTTCYBCGTCTACCTATTTDG-3'). To obtain cDNA, reverse transcription was performed using an ImProm-II™ Reverse Transcription System with random primers (Promega, Australia). The cDNA was used to perform PCR amplification using goTaq® DNA polymerase (Promega, Australia) with the reaction consisting of an initial incubation at 95 °C for 1 min followed by 35 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 40 s, and a final extension at 72 °C for 3 min.

2.5. RT-LAMP primer design and cross reactivity

The LAMP specific primer set (consisting of F3, B3, FIP, BIP, LF2 and LB2 primers) used in this study was derived from the ORF1 gene nt sequence of TuYV isolate WA-1 (ERS2791624) using PrimerExplorer V5 software (available at http://primerexplorer.jp/lampv5e/index.html) with default settings (Table 1). Primer specificity was determined by testing the primer set against TuYV isolate WA1 and four other south-west Australian TuYV isolates (obtained from Coomalbidgup, Irish Town, Jerramungup and Wellstead in 2017), three other poleroviruses, *Phasey bean mild yellows virus* (PBMYV), PLRV and three luteoviruses *Barley yellow dwarf virus* (BYDV), *Cereal yellow dwarf virus* (CYDV) and *Soybean dwarf virus* (SbDV). All isolates were obtained from south-west Australian grainbelt field sites.

2.6. RT-LAMP assay

All RT-LAMP reactions were done using a dual-block (eight reaction wells per block) Genie® II instrument (Optigene, United Kingdom). In a total volume of 25 µL, the reaction mixture contained 1 µL total RNA extraction or 3 µL diluted crude extraction template, 15 µL ISO-004 master mix (Optigene, United Kingdom), 0.5 pmol each of F3 and B3, 2 pmol FIP and BIP and 1 pmol LF2 and LB2, 0.25 U of *Avian myeloblastosis virus* (AMV) reverse transcriptase, and 2 µL RNase free water (total RNA extraction template only). Each set of eight reactions always included a water (reaction negative) and total TuYV RNA positive control in wells seven and eight, respectively. The reaction mixture was incubated at 65 °C for 30 (dilution experiments) or 45 min (trap experiments) followed by an annealing step for 10 min. Results were analysed in real-time *via* amplification and annealing graphs. Based on assay runs performed during initial testing of the primer set and assessment of primer set cross-reactivity, a sample was considered positive if fluorescence exceeded 10,000 and peaked within the incubation time, and annealing temperatures were within 1 °C of those of the positive controls. These standards are specific to the assay protocol described in this study.

2.7. TuYV detection in dilutions of leaf and aphids

Two experiments were done to assess and compare the capability of RT-LAMP and RT-PCR to detect TuYV-infected material diluted in uninfected material. In the first experiment, a single TuYV-infected rapeseed leaf disc was tested individually or combined with 9, 19, 49 or 99 uninfected leaf discs by RT-LAMP and RT-PCR using crude or total RNA extractions. As a negative control, 100 uninfected leaf discs were included. In the second experiment, the same was done with a single viruliferous aphid individually or in 9, 19, 49 or 99 non-viruliferous aphids. As a negative control, 100 non-viruliferous aphids were included. In both experiments, each combination of assay, dilution and extraction method was repeated three times.

2.8. TuYV detection in aphids from trapping and storage substances

To investigate in-field capability of the TuYV RT-LAMP assay, viruliferous aphids were stored in four different substances used in aphid trapping and storage. These were (i) glue on yellow sticky traps (Bugs for Bugs, Australia), (ii) 30% ethylene glycol commonly used in pan traps (*e.g.* Thackray et al., 2000), (iii) 70% ethanol used for specimen storage, and (iv) orange oil (De-Solv It, Vardon Industries, Australia) used to dissolve sticky trap glue from aphid specimens. Aphids were tested on six occasions: after 2 h, and 1–4 and 8 weeks. For each substance at each storage time, four aphids were removed using a fine tipped paintbrush and underwent crude extraction individually. These were tested by RT-LAMP with live non-viruliferous and viruliferous aphids as negative and positive controls, respectively. Aphids from orange oil were tested up to 4 weeks, only. The remaining 27 µL of crude extract from each aphid underwent total RNA extraction and was tested by RT-PCR. To ensure these substances did not produce false positives, 3 µL of sample substrate (without aphids) was tested before

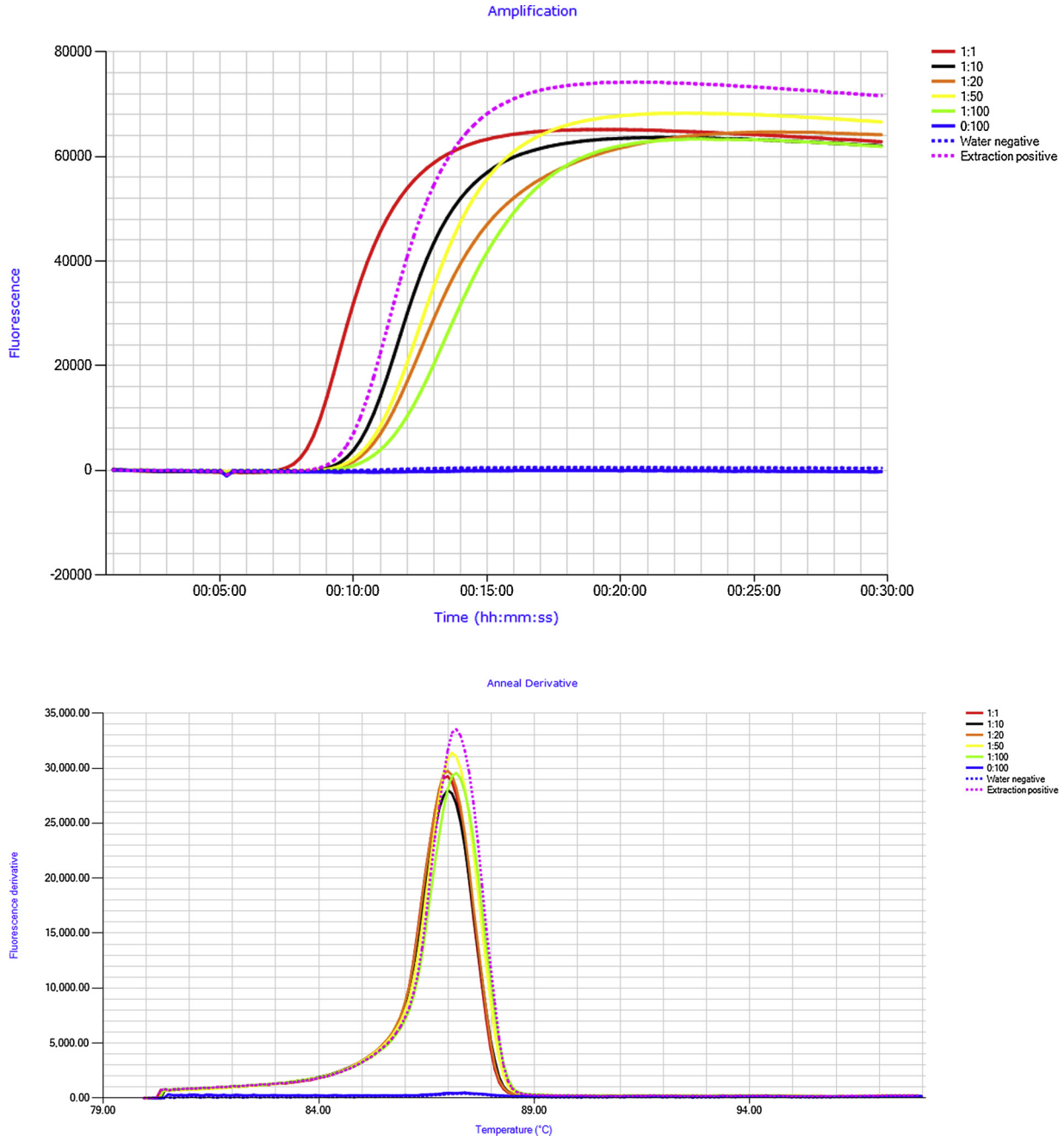

Fig. 1. Amplification and anneal derivative curves for reverse transcription loop-mediated isothermal amplification of *Turnip yellows virus* in crude extractions of dilutions of one viruliferous aphid in 9, 19, 49 and 99 non-viruliferous aphids[a].
[a]Controls included = water negative and total TuYV RNA extraction positive (extraction positive).

and after the experiment and confirmed negative by RT-LAMP and RT-PCR. This experiment was repeated once.

3. Results

3.1. Primer specificity

When the LAMP primer set was tested against five isolates of TuYV, three other poleroviruses and three luteoviruses, only TuYV isolates reacted positively, the other virus isolates tested did not cross-react.

3.2. Detection in dilutions of leaf and aphid material

TuYV was detected by RT-LAMP and RT-PCR in crude and total RNA extractions of both infected leaf material and viruliferous aphids. For both crude and total RNA leaf and aphid extractions tested by RT-LAMP, TuYV was detected in all three repeat experiments at all dilution steps (Table 2, Fig. 1). For crude leaf and aphid extractions tested by RT-PCR, TuYV was detected in two of three repeat experiments for all dilution steps, except for aphids when TuYV was detected in one of three repeat experiments at 1/100 dilution. For total RNA leaf and aphid extractions tested by RT-PCR, TuYV was detected in all three repeat experiments at all dilution steps. For both total RNA and crude extractions tested by RT-LAMP and RT-PCR, TuYV was never detected in the samples with non-viruliferous aphids or uninfected leaf discs (0/100).

Table 3
Reverse transcription loop-mediated isothermal amplification *Turnip yellows virus* detection rate and amplification time in aphids stored in various substances.

	Sticky trap glue		30% ethylene glycol		70% ethanol		Orange oil	
Time in substance	Positive detection[a]	Difference from positive (min)[b]	Positive detection	Difference from positive (min)	Positive detection	Difference from positive (min)	Positive detection	Difference from positive (min)
2 hours	7/8	1.9 ± *1.3*[c]	8/8	−0.7 ± *0.9*	8/8	3.8 ± *1.2*	8/8	−1.0 ± *1.4*
1 week	8/8	2.1 ± *1.8*	8/8	2.5 ± *1.6*	7/8	−2.5 ± *1.3*	8/8	4.3 ± *1.1*
2 weeks	8/8	1.2 ± *4.0*	8/8	1.3 ± *1.2*	8/8	−2.8 ± *1.0*	4/8	1.0 ± *2.4*
3 weeks	8/8	5.9 ± *1.8*	8/8	7.4 ± *2.7*	8/8	4.3 ± *1.8*	6/8	9.1 ± *1.6*
4 weeks	8/8	6.7 ± *2.0*	8/8	5.5 ± *2.5*	8/8	15.9 ± *1.7*	8/8	12.4 ± *2.1*
8 weeks	5/8	15.4 ± *1.2*	7/8	8.4 ± *1.8*	8/8	11.9 ± *1.7*	–	–
Aphid +[d]	12/12	0	12/12	0	12/12	0	10/10	0
Aphid -[d]	0/12	–	0/12	–	0/12	–	0/10	–

[a] Number of TuYV-detection/total number of aphids tested across both repeat experiments.
[b] Average difference between amplification time of test sample and aphid positive across both repeat experiments.
[c] Standard error in italics.
[d] For each repeat experiment, a single live viruliferous and non-viruliferous aphid used as positive and negative controls, respectively, at each storage time. Number of TuYV-detection/total number of aphids tested across both repeat experiments and all storage times.

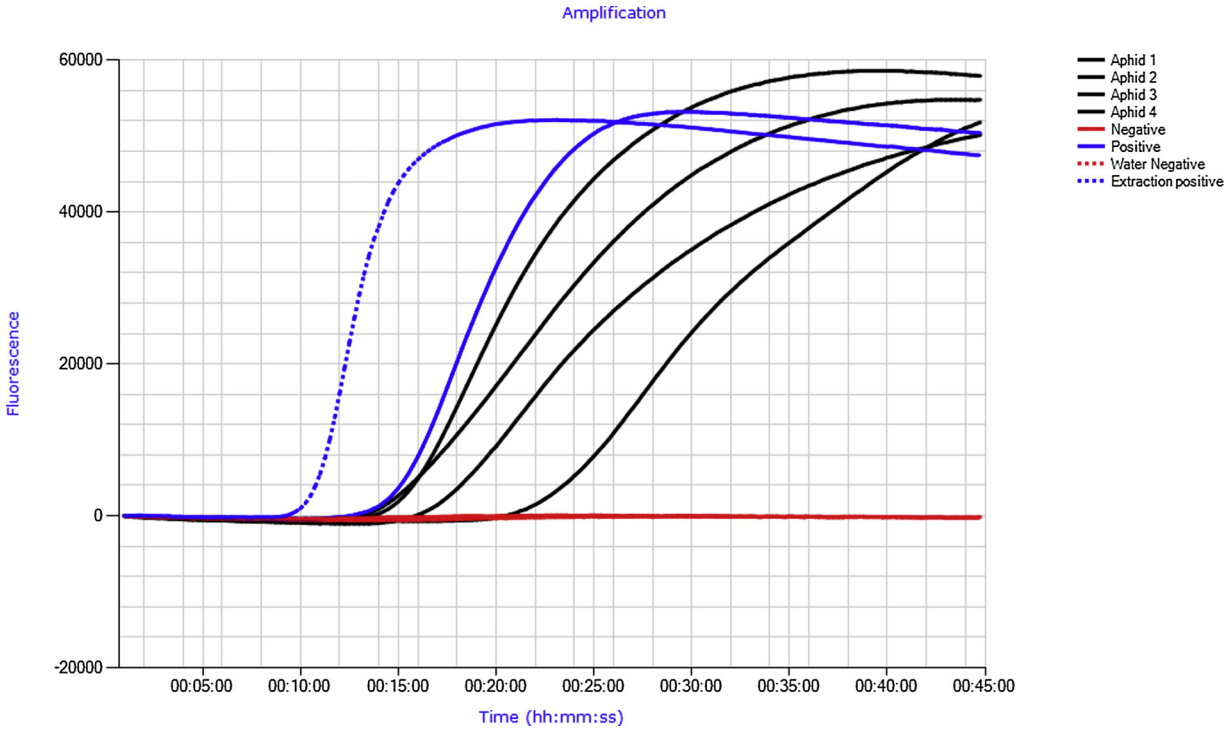

Fig. 2. Reverse transcription loop-mediated isothermal amplification of *Turnip yellows virus* from viruliferous aphids trapped in sticky glue for four weeks[a].
[a]Controls included = live viruliferous (positive) and non-viruliferous (negative) aphid, water negative and total TuYV RNA extraction positive (extraction positive).

3.3. TuYV detection in aphids from trapping and storage substances

Over each storage time tested up to four weeks, TuYV was detected by RT-LAMP in crude extractions of 40/40 aphids taken from 30% ethylene glycol, 39/40 (results from two repeat experiments pooled) from sticky traps and 70% ethanol, and 34/40 from orange oil (Table 3). In aphids taken from 30% ethylene glycol, detection was achieved in 8/8 aphids after all time periods tested, except after 8 weeks (7/8). In aphids taken from sticky traps, detection was achieved in 8/8 aphids after all time periods tested (*e.g.* Fig. 2) except after 2 h (7/8) and 8 weeks (5/8). In aphids taken from 70% ethanol, detection was achieved in 8/8 aphids after all time periods tested except after 1 week (7/8). In aphids taken from orange oil, TuYV was detected in 8/8 aphids after all time periods tested except after 2 weeks (4/8) and 3 weeks (6/8)., Regardless of substance, average amplification time (over the two repeat experiments) generally increased over time. There was no amplification of aphid negative or water controls.

4. Discussion

In this study, using a novel crude extraction method and RT-LAMP assay performed on a portable instrument, a new TuYV diagnostic protocol targeting broadacre rapeseed crops was successfully developed. This protocol provides inexpensive and rapid TuYV detection of viruliferous aphids caught in traps and opportunistic testing of potential host plants. The protocol readily and consistently detected a single TuYV-viruliferous aphid or infected leaf disc, in 99 non-viruliferous aphids or uninfected leaf discs. Moreover, this protocol was used to detect viruliferous aphids stored for eight weeks in sticky trap glue, 30% ethylene glycol and 70% ethanol, substances commonly used in trapping and storage of aphid specimens. The characteristics of this TuYV RT-LAMP assay protocol suggest that it is in-field capable and could be used in epidemiological research and pathogen surveillance programs that greatly improve virus forecasting, decision support and diagnostic services. To the best of our knowledge, this is the first study

utilising LAMP to detect a plant virus inside its aphid vector in crude extractions, and investigating protocol application to field scenarios by testing aphids caught and stored in traps or storage substances.

This RT-LAMP assay protocol provides intrinsic benefits in two important applications over serological assays or PCR as they are commonly used. Firstly, the user-friendly in-field capabilities of LAMP allow opportunistic field diagnostics that the other methods do not. Therefore, with basic training, the protocol could be used by industry professionals, such as agronomists or advisors, as a 'first port of call' diagnostic tool for young symptomatic plants to quickly differentiate between symptoms of TuYV infection and other potential stresses. Key to this is the crude extraction method which is easily performed in-field, significantly reducing total assay time and costs associated with total RNA extraction. To further improve this aspect of the protocol, extraction techniques that are faster and increase purity should be explored as they are developed *e.g.* the equipment-free nucleic acid extraction dipstick method (Zou et al., 2017). Secondly, as a reliable, sensitive and specific assay, it can be used as a rapid and cheaper alternative for use in plant quarantine or diagnostic services when samples numbers are small. In addition RT-LAMP is easier, more user-friendly, requires little technical skills and more than five-fold faster than standard molecular techniques such as RT-PCR that require specialist laboratory equipment. However, serological methods are valuable when high throughput testing is required, and in diagnostic and quarantine applications, provided their limitations are acknowledged (cross reactivity in particular) (Boonham et al., 2014; Duffus and Russell, 1972; Hauser et al., 2000). In these scenarios, this LAMP protocol is not appropriate due to the relatively small number of samples that can be tested in a single run.

This study demonstrated two key characteristics of the RT-LAMP protocol that highlight its suitability for use in aphid trapping programs. Firstly, reliable detection of a single viruliferous aphid in large numbers of non-viruliferous aphids is important when testing aphids caught in field traps. This characteristic facilitates virus detection prior to crop infection even when aphid flights into the crop are high and the ratio of viruliferous migrant aphids is low but sufficient to start an epidemic. In the presence of viruliferous *B. brassicae*, a minor vector, detection of *M. persicae* DNA would likely improve predictions of virus spread risk and add further benefits to this RT-LAMP protocol. Secondly, the assay was able to reliably detect viruliferous aphids that were caught in yellow sticky and 30% ethylene glycol pan traps, and stored in 70% ethanol for 8 weeks at room temperature.This indicates that this assay can provide TuYV detection even when sticky or pan traps are left in the field for over a month, but needs to be validated under field conditions where air temperatures are more variable. Furthermore, detection was not inhibited when aphids came into contact with orange oil, so it can be used to dissolve sticky glue off aphids if needed. Testing aphids taken from traps subjected to field conditions is required to fully field validate this protocol. When aphids were removed after eight weeks, the time to detection when compared to fresh viruliferous aphid positives was, on average, 8 to 15 min slower. To optimize detection of future testing and provide a timely and accurate service, specimens should be tested or transferred from sticky traps to storage preservative, such as ethanol or propylene glycol (Nie et al., 2011; Singh et al., 1995, 1997), within four weeks of being caught. Another circulative, non-propagative polerovirus, PLRV, can be detected by PCR from viruliferous *M. persicae* stored in 70% ethanol at room temperature for seven years (Singh et al., 1997), suggesting similar long-term storage could preserve TuYV-viruliferous *M. persicae* for testing by RT-LAMP if required for epidemiological research purposes, and would be worthwhile investigating.

Previous research suggests LAMP can provide intrinsic benefits when its applications are aimed at (i) increasing robustness of quarantine programs, (ii) improving field surveillance for crop protection programs and epidemiological research (*e.g.* Li et al., 2013; Okuda et al., 2015; Tien Le et al., 2010), (iii) investigating virus-vector relationships (*e.g.* Zhou et al., 2012), (iv) combining with climate-driven epidemiological models for earlier and more accurate disease forecasting for disease management decision support (*e.g.* Kong et al., 2016), and (v) identifying pesticide resistant pathogens or host resistance-breaking strains (*e.g.* Duan et al., 2014; Okuda et al., 2015). Our in-field capable TuYV RT-LAMP assay could be applied to all of these scenarios in the future with the support of laboratory and field research. In particular, surveillance programs consisting of aphid traps to intercept and detect viruliferous migrant aphids upon their incursion into a rapeseed crop, thereby providing an early warning system would improve risk forecasting, decision support delivery to growers and management efficiency, especially with the need for insecticide application. Real-time data obtained from such a system could be hybridized with, and thereby improve and revive, the currently unutilised climate-driven epidemiological model developed for TuYV epidemics in rapeseed in the south-west Australian grainbelt (Maling et al., 2010). Following field validation and deployment of an insect trapping network, use of this diagnostic protocol could help reduce TuYV-induced rapeseed seed yield and quality losses in broadacre systems worldwide.

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