

Comparison of tissue blot immunoassay and reverse transcription polymerase chain reaction assay for virus-testing pulse crops from a South-Eastern Australia survey

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Abstract A virus survey of field pea (*Pisum sativum*) and faba bean (*Vicia faba*) crops in southern NSW and Victoria in October, 2006, provided the opportunity to compare the diagnostic results of a large number of field samples using reverse transcription polymerase chain reaction (RT-PCR) to the accepted reference test of tissue blot immunoassay (TBIA). One hundred unbiased plant samples from each of 21 field pea and three faba bean crops from NSW and ten field pea crops from Victoria were tested for *Alfalfa mosaic virus* (AMV), *Bean yellow mosaic virus* (BYMV), *Cucumber mosaic virus* (CMV), *Pea seed-borne mosaic virus* (PSbMV), *Bean leafroll virus* (BLRV) and *Beet western yellows virus* (BWYV) by TBIA. PSbMV was present at high levels (32–77 %) in 12 out of 21 field pea crops sampled from NSW. BLRV was present at high levels (28–42 %) in faba bean crops from NSW. In Victorian field pea crops, BWYV was most prevalent (4–28 %). Other viruses tested for were present at low incidences or absent. The seed to seedling transmission rate of PSbMV in the field pea seedlots used to sow six of the surveyed NSW pea crops, determined by TBIA, ranged from 0 to 23 % and had a clear effect on the level of PSbMV in the field. Test results for PSbMV and BLRV using TBIA were compared with results using RT-PCR and compared favourably

in giving similar estimates of infection incidence and low costs (both less than A\$1.00 per sample) for detecting these two viruses. It is concluded that TBIA is the preferred technique to get a precise estimate of infection incidence, while RT-PCR is particularly useful to test bulk samples for the presence of viruses with a low incidence and provides an alternative if antiserum is not available.

Keywords Pulse · Virus · Survey · PCR · TBIA

Introduction

South-eastern Australia is now a major producer of temperate pulses, including field pea (*Pisum sativum*), faba bean (*Vicia faba*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*) and narrow-leaved lupin (*Lupinus angustifolius*). Of the fifteen viruses reported to naturally infect temperate pulses in Australia (Latham and Jones 2001; Thomas et al. 2004, 2010; Schwinghamer et al. 2007), the six most important in the production areas of south-eastern Australia are *Alfalfa mosaic virus* (Bromoviridae, genus *Alfamovirus*) (AMV), *Bean leafroll virus* (Luteoviridae, genus *Luteovirus*) (BLRV), *Bean yellow mosaic virus* (Potyviridae, genus *Potyvirus*) (BYMV), *Beet western yellows virus* (Luteoviridae, genus *Polerovirus*) (BWYV), *Cucumber mosaic virus* (Bromoviridae, genus *Cucumovirus*) (CMV) and *Pea seed-borne mosaic virus* (Potyviridae, genus *Potyvirus*) (PSbMV). Regular surveys have been carried out in Victoria, South Australia and New South Wales from the late 1990s to monitor presence of viruses and to quantify differences in virus incidence between seasons, growing environments and varieties (Freeman and Aftab 2001; Freeman et al. 2003a, b; van

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Leur et al. 2003; Aftab et al. 2005; Freeman et al. 2005; Aftab et al. 2009; van Leur et al. 2013a).

Viruses can induce a range of symptoms in plants that can easily be confused in the field with the reaction of plants to abiotic stress factors such as nutritional imbalance, drought, waterlogging, frost and herbicide toxicity. Accurate diagnostic tools are therefore needed to unambiguously identify the presence of specific viruses. Enzyme-linked immunosorbent assay (ELISA) has provided reliable diagnostics for plant viruses over the past decades, but is too costly to process the large numbers of individual plant samples that are needed to make quantitative assessments of virus presence in broad acre field crops. To overcome this, surveyed samples can be pooled and virus incidence can be estimated (Fletcher 1993). Tissue blot immunoassay (TBIA, Makkouk and Comeau 1994; Makkouk and Kumari 1996) is also a serological test, but simpler and cheaper to operate. A large advantage of this technique over ELISA for survey work is that individual plants are blotted and tested, rather than being pooled as commonly occurs with ELISA, thus giving more accurate estimates of virus incidence. However, both ELISA and TBIA rely on the availability of high quality antisera. Although commercial antisera are available for the common pulse viruses they are not available for many other viruses, and this fact led us to consider using polymerase chain reaction (PCR), or more specifically for viruses with a ribonucleic acid (RNA) genome, reverse transcription polymerase chain reaction (RT-PCR) assays in our surveys. The specific reagents used for PCR can be easily reproduced at any time, in contrast to the serological reagents and the availability of commercial high-throughput RNA extraction kits and procedures has made the use of PCR for processing large numbers of plants feasible (Shepherd et al. 2002; Constable et al. 2012).

This paper presents the outcomes of a faba bean and field pea virus survey in south-eastern Australia in 2006, and compares the results and practicality of TBIA and PCR assays for detecting the two most prevalent viruses in survey samples.

Materials and methods

Selection of survey sites and sample collection

A survey of field pea and faba bean crops was made in southern NSW and the Wimmera in Victoria in October 2006. Selection of sites was made by local agronomists. In total, 31 field pea crops (21 in NSW and 10 in Victoria) and three faba bean crops (NSW) were surveyed during the first week of October 2006. An unbiased selection of shoot tips from 100 plants from each crop were collected at 10 m intervals following a “W” pattern in the centre of the crop to determine virus incidence. In addition to this, 20 virus-symptomatic (stunting or yellowing) plants were collected from the sampled crops to determine virus species associated

with symptoms. All shoots were kept in plastic bags on ice during collection and then at 4 °C until they were processed.

Sample blotting and TBIA

The 100 shoot tips from each crop were bundled at random into groups of ten, using a strip of Parafilm, directly after sampling (unbiased samples). The bundles were processed the same day by making a straight cut at the bottom end of the shoot bundle with a razor blade and immediately blotting the cut stem-ends of the shoot bundle onto nitrocellulose membrane (Whatman Protran, 0.45 µm). As the blotted membranes are generally processed only once against one virus-specific antibody, the samples were blotted onto six replicate membranes, each time after a fresh cut to the bottom end of the bundled shoots. Shoot tips of the set of 20 symptomatic plants (symptomatic samples) were blotted individually on a separate set of eight replicate membranes, using the same procedure. The virus positive and negative control samples that were used were blots from plants previously confirmed by serology and/or PCR to be virus infected.

The tissue-blot immunoassay (TBIA) procedure was based on the indirect immunological detection method of Hsu and Lawson (1991), with modifications by Makkouk and Kumari (1996) for pulses. Prior to commencing the processing of blots, a solution of healthy sap was prepared by grinding fresh healthy legume leaves in 20 volumes of phosphate buffer saline (PBS) and strained to remove particulate matter from the healthy solution. This healthy sap solution was used to cross absorb the polyclonal antibodies at a dilution of 1/2000 and the extract was incubated for 2 h at 37 °C before use. The monoclonal antisera were diluted 1/1000 in conjugate buffer (PBS-Tween containing 2 % poly vinyl pyrrolidone+0.2 % ovalbumin). The blotted membranes were placed together in containers and washed three times for 5 mins with phosphate-buffered saline-Tween solution (PBS-Tween- 0.02 M K₂HPO₄, 0.15 M NaCl, pH 7.4, containing 0.05 % Tween 20) on a small shaker. Membranes were blocked using 1 µg/ml polyvinyl alcohol in PBS-Tween and incubated for 1 min at room temperature and washed three times with PBS-Tween as described. Replicate membranes were then placed in separate containers for processing with virus-specific primary antibodies. Diluted virus-specific antiserum was added to each container and membranes were incubated for 1 h at room temperature on a small shaker. Membranes were washed three times in PBS-Tween as described. Goat anti-rabbit or anti-mouse secondary antibodies (for rabbit polyclonal antisera and mouse monoclonal antisera respectively) conjugated to alkaline phosphatase (Sigma-Aldrich Co), were diluted 1/2000 in conjugate buffer, and added to each dish and membranes were incubated for 1 h at room temperature on a small shaker. The membranes were washed three times in PBS-Tween for a final time prior to the addition of the substrate

solution which contained 14 mg nitroblue tetrazolium and 7 mg 5-bromo-4-chloro-3-indolyl phosphate per 40 mL of substrate buffer (0.1 M Tris, 0.1 M NaCl, and 5 mM MgCl₂, pH 9.5). Membranes were submerged in substrate solution for 30–60 min or until colour development was clear in the positive controls and the reaction stopped by immersing membranes in de-ionized water. Membranes were dried and the reaction of the blots examined under a dissecting microscope. Development of a purple colour on the blot indicated a positive result. Blots of phloem-restricted viruses showed as a dark purple staining of the phloem, while viruses that invade all tissues of the host plant produced a dark purple staining of the whole petiole cross section. Negative samples developed no colour.

Both symptomatic and unbiased plant samples were tested for the presence of AMV, BLRV, BYMV, BWYV, CMV and PSbMV. Symptomatic plants were also tested for legume nanoviruses (*Nanoviridae*, genus *Nanovirus*), presumably the Australian species *Subterranean clover stunt virus* (SCSV) and *Tomato spotted wilt virus* (*Bunyaviridae*, genus *Tospovirus*) (TSWV). Type and source of antisera used for the tests are listed in Table 1.

Nucleic acid extraction and RT-PCR

The initial sample preparation and grinding was conducted manually. A 0.3 g sample of each of the unbiased-plant bundles and 3 ml MacKenzie lysis buffer with 1 % sodium metabisulphite (MacKenzie et al. 1997) were placed in extraction bags with an internal membrane (Bioreba®), triturated

with a Homex 6 homogenizer (Bioreba®) and kept on ice while the remaining samples were prepared.

One ml of each extract was transferred to a separate well of a 96-well lysis plate containing 100 µl 20 % N-lauroyl sarcosine. The plate was incubated at 70 °C for 15 min. After lysis, 0.5 vol 96 % ethanol was added and the RNA extraction was carried out in 96-well plates using silica-gel based membrane and spin technology. To bind RNA to the silica membrane, a 450 µl sample was placed in a 96-well, Unifilter® microplate (Whatman®) and spun for 4 min at 6,000 rpm. The supernatant was discarded and the membrane was washed (4 min at 6,000 rpm) twice with 600 µl propanol wash buffer (Corbett Research®) and once with 600 µl 96 % ethanol. After the final wash, the column was dried by spinning for 10 min at 6,000 rpm. RNA was eluted from the silica membrane with 80 µl RNase free water. All liquid transfers were made using multichannel pipettes.

PCR reactions were set up with the Corbett CAS 1200 robot in 25 µl volumes using the Superscript™ III Platinum® one-step RT-PCR system (Invitrogen®) containing 0.1 µM of each primer and 1 µl RNA. BLRV was amplified with the forward and reverse primers BLRV5 and BLRV3 (Ortiz et al. 2005) yielding a 391 bp product. A two-step cycling protocol was used with reverse transcription at 48 °C for 15 min, denaturation at 95 °C for 2 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 2 min with a final hold at 4 °C. PSbMV was amplified using primers PSBCP1 (5' AATGGCGCATTTCAGTGACG 3') and PSBCP2 (5' CCCYTCCAAGCCAAATAGGC 3') designed from the coat protein sequence, yielding a 235 bp product. A three-step cycling protocol was optimised with reverse transcription at 48 °C for 15 min, denaturation at 95 °C for 2 min and 32 cycles of 95 °C for 30 s, 66 °C for 30 s and 72 °C for 30 s with a

Table 1 Virus species and antibodies used in survey of faba bean and field pea crops in south eastern Australia, October, 2006

Virus species		Antibodies used for detection		
Name	Abbrev.	Type	Lot/Batch No	Source ^a
<i>Alfalfa mosaic virus</i>	AMV	PAb	07001	Loewe
<i>Bean leafroll virus</i>	BLRV	MAB	AS-0227/2	DSMZ
<i>Bean yellow mosaic virus</i>	BYMV	PAb	07007	Loewe
<i>Beet western yellows virus</i>	BWYV	PAb	40801	Biorad
<i>Cucumber mosaic virus</i>	CMV	PAb	07108	Loewe
<i>Pea seed-borne mosaic virus</i>	PSbMV	PAb	AS-0129	DSMZ
<i>Legume nanoviruses including Subterranean clover stunt virus</i>	SCSV	MAB	4F2 (nanovirus specific)	BBA
<i>Tomato spotted wilt virus</i>	TSWV	PAb	07501	Loewe

^a Loewe- Biochemica, GmbH, Muhlweg 2a, D-82054 Sauerlach Germany

DSMZ- Leibniz-Institut, DSMZ, GmbH, InhoffenstraBe 7B, 38124 Braunschweig, Germany

Biorad-Phyto-Diagnostics, 3 bd Raymond Poincare, 92430, Marnes-La Coquette, France (now SEDIAG SAS, 3 Boulevard de Beauregard, 21600, Longvic, France)

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Table 2 Incidence (%) of six viruses in field pea and faba bean crops in southern NSW and Victoria, 2006 determined by TBIA^a

Crop	Location	AMV	CMV	BYMV	PSBMV	BLRV	BWYV
NSW 1 pea	Blighty	0	0	1	77	20	63
NSW 2 pea	Collingullie	0	0	0	0	0	2
NSW 3 pea	Marrar	0	0	0	0	0	1
NSW 4 pea	Marrar	0	0	0	0	0	0
NSW 5 pea	Old Junee	0	0	0	0	0	0
NSW 6 pea	Cowra	0	0	0	32	2	1
NSW 7 pea	Cowra	0	0	0	67	2	3
NSW 8 pea	Cowra	0	0	0	22	1	5
NSW 9 pea	Grenfell	0	0	0	66	0	0
NSW 10 pea	Gooloogong	0	0	0	72	50	5
NSW 11 pea	Gooloogong	0	0	0	48	3	3
NSW 12 pea	Gooloogong	0	0	0	30	2	0
NSW 13 pea	Gooloogong	0	0	0	72	6	1
NSW 14 pea	Gooloogong	0	0	0	0	8	1
NSW 15 pea	Gooloogong	0	0	0	0	10	4
NSW 16 pea	Bedgerebong	0	0	0	61	0	1
NSW 17 pea	Wheatlands	0	0	0	2	0	0
NSW 18 pea	Forbes	1	0	0	74	2	2
NSW 19 pea	Forbes	0	0	0	0	1	1
NSW 20 pea	Forbes	0	0	0	0	0	5
NSW 21 pea	Forbes	0	0	0	32	0	0
NSW 22 faba	Deniliquin	1	0	0	0	42 ^b	0
NSW 23 faba	Deniliquin	0	0	1	1	28	0
NSW 24 faba	Blighty	1	0	0	0	42	6
VIC 1 pea	Horsham	0	0	0	0	0	11
VIC 2 pea	Doonen	0	0	0	0	0	10
VIC 3 pea	Doonen	0	0	0	0	0	25
VIC 4 pea	Minyip	0	2	0	2	87	28
VIC 5 pea	Murtoa	0	0	0	0	4	20
VIC 6 pea	Rupanyup	0	1	0	0	2	16
VIC 7 pea	Lubeck	0	0	0	0	2	7
VIC 8 pea	Toolondo	0	0	3	0	0	19
VIC 9 pea	Natimuk	0	0	0	0	2	19
VIC 10 pea	Natimuk	0	5	0	0	0	4

^a Estimate of virus incidence (%) based on number of positive blots (100 blotted samples/crop)

^b Only 9 bundles were blotted for BLRV (90 samples)

final hold at 4 °C. Thermocycling was conducted in an Eppendorf® mastercycler. PCR experiments were performed with positive, negative and no template controls. Products were separated on 1.5 % agarose gels using ElectroFast® high-throughput gel tanks (ABgene).

Determining PSbMV seed-to-plant transmission rates

A subset of the seed used for sowing six of the surveyed NSW pea fields was available to test for PSbMV seed-to-plant transmission. For each seed lot, 160 seeds were sown in trays in an insect-proof, temperature-controlled (16–22 °C) glasshouse during June 2006. Seedlings were tested for the presence of PSbMV by TBIA 2 weeks after emergence.

Estimates of virus incidence

Group testing, involving the pooling of samples from a population and testing as a group for a particular attribute, has been used regularly for estimating virus incidence in plant populations (Eg. Fletcher 1993; Rodoni et al. 1994; Hill et al. 1996). In this study, virus incidences based on RT-PCR were estimated from the number of positive ten-plant pooled samples (bundles) out of ten for each crop by the mathematical method of Fletcher (1993) and assumptions described by Moran et al. (1985). For comparison, infection incidences based on TBIA were estimated using the same mathematical method, but defining positive bundles as ones for which one or more of the ten plants bundled were positive. For TBIA

Table 3 Number of viruses detected by TBIA in plants with symptoms of yellowing and stunting^a in southern NSW

Crop type	No of plants tested	Numbers of infected plants detected by TBIA							
		BYMV	PSbMV	BLRV	BWYV	SCSV ^b	AMV	CMV	TSWV
Fababean	59	5	1	37	9	0	0	0	0
Fieldpea	74	0	54	26	24	1	0	0	0

^a Yellowing and stunting in some plants may have been caused by drought or other factors

^b Nanovirus, presumably SCSV

only, more accurate virus incidences were calculated as the total number of positive plants in the ten bundles representing each crop divided by the total number of plants (100). This method was used to assess virus incidence in the surveyed crops (Tables 2 and 4). Virus incidence estimates based on individual plants were not possible for RT-PCR because shoots in bundles were homogenised into one sample prior to nucleic acid extraction and testing.

Results

Southern NSW faba bean crops

The three irrigated faba bean crops sampled had a high incidence of yellow, severely stunted plants. All had a high incidence of BLRV (28–42 % plants), based on TBIA results, while one crop also had 6 % BWYV. Infections of AMV, BYMV and PSbMV were rare and there were no CMV infections detected in this study (Table 2).

Southern NSW field pea crops

All of the 21 field pea crops surveyed showed severe drought stress, making virus symptoms difficult to observe. BLRV and BWYV occurred in 12 and 15 field pea crops respectively and the virus incidence was low (10 % or less) except for BLRV in two crops (20 and 50 %) and BWYV in one crop (63 %). PSbMV was found in 13 of the 21 field pea crops sampled with high virus incidence (22–77 %) in 12 crops. Infections of CMV, AMV and BYMV were rare in these crops and only occurred at low incidence (5 % or less) (Table 2). The results for the NSW symptomatic samples reflected the general survey results and only one nanovirus-positive pea (presumably SCSV) and no TSWV, CMV or AMV positives were found (Table 3).

For the six surveyed crops for which PSbMV seed-to-plant transmission tests were conducted, there was a very strong relationship between the level of PSbMV in the seed and that found in the crop (Table 4). Of the four crops sown with Kasper seed, three seedlots had greater than 10 % seed transmission (11–23 %) and this led to high levels of PSbMV (32–74 %), whereas the one Kasper seed lot with a low level of PSbMV

(1.4 %) developed little crop infection (2 %). The two crops sown with Excell seed, in which no PSbMV was detected, did not develop PSbMV infections.

Victorian field pea crops

Low levels of BLRV infection (4 % or less) were found in nine of the ten field pea crops sampled. One crop had BLRV infection of 87 %. BWYV was found in all crops and mostly at a greater than 10 % incidence. PSbMV, CMV and BYMV occurred infrequently and with low incidence (5 % or less) and AMV was not detected (Table 2).

Comparison of TBIA and high throughput RT-PCR for estimating incidence of PSbMV and BLRV

Both RT-PCR and TBIA reliably detected the presence or absence of virus in the pooled plant samples and in most cases the incidence of virus in the crops estimated from both sets of results were in agreement (Table 5). At virus incidences over 11 %, (7/10 positive ten-plant samples) these estimates frequently varied from the percentage of individual TBIA-positive plants from a crop (Table 5).

Table 4 Comparison of PSbMV seed transmission and progeny crop infection for six field pea seed lots grown in southern NSW

Crop	Location	Pea variety	Seed transmission (%) ^a	Crop infection incidence (%) ^b
NSW 16 ^c	Bedgerebong	Kasper	23.3	61
NSW 17	Wheatlands	Kasper	1.4	2
NSW 18 ^c	Forbes	Kasper	23.3	74
NSW 19	Forbes	Excell	0.0	0
NSW 20	Forbes	Excell	0.0	0
NSW 21	Forbes	Kasper	11.2	32

^a For each seed lot, 160 seeds were sown and tested 2 weeks after emergence, estimate of virus incidence (%) based on number of positive blots (Approximately 160 blotted samples/seedlot)

^b Estimate of virus incidence (%) based on number of positive blots (100 blotted samples/crop)

^c NSW 16 and NSW 18 were sown with the same seed lot

A comparison of RT-PCR and TBIA for detecting PSbMV and BLRV in ten-plant samples from 24 NSW and 10

Table 5 Incidence of PSbMV and BLRV in field pea (NSW 1–21 and VIC 1–10) and faba bean (NSW 22–24) crops in NSW and Vic. 2006 determined by RT-PCR and TBIA

Crop	PSbMV			BLRV		
	Pooled plants ^a estimate (%) ^b		Individual plants (%) ^c	Pooled plants ^a estimate (%) ^b		Individual plants (%) ^c
	PCR	TBIA	TBIA	PCR	TBIA	TBIA
NSW 1	>21	>21	77	11	11	20
NSW 2	0	0	0	0	0	0
NSW 3	0	0	0	0	0	0
NSW 4	0	0	0	0	0	0
NSW 5	0	0	0	0	0	0
NSW 6	7	15	32	2	2	2
NSW 7	21	21	67	2	2	2
NSW 8	21	21	22	1	1	1
NSW 9	>21	>21	66	1	0	0
NSW 10	>21	>21	72	>21	21	50
NSW 11	>21	>21	48	5	2	3
NSW 12	>21	>21	30	1	1	2
NSW 13	>21	>21	72	4	4	6
NSW 14	0	0	0	9	9	8
NSW 15	1	0	0	9	9	10
NSW 16	21	>21	61	0	0	0
NSW 17	3	3	2	0	0	0
NSW 18	21	>21	74	2	3	2
NSW 19	0	0	0	1	1	1
NSW 20	1	0	0	0	0	0
NSW 21	21	21	32	0	0	0
NSW 22	0	0	0	>21	— ^d	42 ^d
NSW 23	1	1	1	>21	21	28
NSW 24	0	0	0	>21	21	42
VIC 1	0	0	0	0	0	0
VIC 2	0	0	0	0	0	0
VIC 3	0	0	0	0	0	0
VIC 4	1	1	2	>21	>21	87
VIC 5	0	0	0	2	2	4
VIC 6	0	0	0	2	2	2
VIC 7	0	0	0	4	2	2
VIC 8	0	0	0	0	0	0
VIC 9	0	0	0	0	1	2
VIC 10	0	0	0	5	0	0

^a 10 ten-plant samples per crop

^b Estimate of virus incidence (%) based on Fletcher (1993)

^c Estimate of virus incidence (%) based on number of positive blots (100 blotted samples/crop)

^d Only 9 bundles were blotted for BLRV

Victorian field pea and faba bean crops is given in Table 6. Results for the homogenised ten-plant samples prepared for RT-PCR are compared with the same set of ten individual plants bundled and blotted for TBIA (rated positive if one or more plants in the group were positive). Of the 340 ten-plant samples tested for PSbMV, 335 test results agreed using both methods and five tested positive by TBIA and negative by PCR. Of the 339 ten-plant samples tested for BLRV, 329 test results agreed using both methods and ten samples tested negative by TBIA and positive by PCR.

Discussion

Virus presence in field pea and faba bean crops

The high incidence of yellow, severely stunted plants in the three southern NSW faba bean crops correlates with the high level of BLRV infection (range of 28–42 % plants) detected in this study. The incidence of all other viruses tested for in this study was low. The faba bean crops sampled were grown under irrigation in the Deniliquin area and local agronomists indicated that observed symptoms were typical of the large number of dryland faba bean crops in the region which had already died. While BLRV infections in the three sampled crops were very severe, some areas of two of these crops, which were re-sown 4 weeks after the initial sowing, showed very few virus symptoms. This suggests that the infection originated from migrating aphids early in the season and that little further spread of BLRV occurred by vectors colonizing infected crops, possibly due to spraying of crops with insecticide. BLRV was found in 65 % of the faba bean and field pea crops surveyed in southern NSW in 2006. High BLRV incidence (20 % or greater) occurred in all three of the faba bean crops sampled and in only two of the 12 infected pea crops sampled.

This is the only information available on the occurrence of pulse viruses in the surveyed area. Van Leur et al. (2003) surveyed 11 commercial faba bean crops in the Liverpool

Table 6 Comparison of RT-PCR and TBIA for detecting PSbMV and BLRV in 10-plant samples

Virus	Total pooled samples	Agreement		Discrepancy	
		+ve both tests	–ve both tests	+ve RT- PCR –ve TBIA	–ve RT- PCR + TBIA
PSbMV	340	117	218	0	5
BLRV	339	87	242	10	0

Homogenised ten plant samples for RT-PCR, set of ten individual plants for TBIA (rated positive if one or more plants were positive); data from Table 5

Plains region of northern NSW for ten viruses. BYMV was the most frequently found virus with an average over the sampled paddocks of 26 % incidence (range 1–63 %). BLRV and BWYV were found at similar average incidence (14 % and 12 % of plants) and ranges (6–37 % and 1–31 % of plants respectively), while incidences of other viruses were below 1 %. No data on the occurrence of BLRV in southern NSW are available apart from this study, so it is difficult to speculate whether or not the high levels of BLRV we found in 2006 will occur in southern NSW faba bean crops regularly or intermittently. The percentage of crops found to have BLRV in Victoria and South Australia between 2000 and 2004 varied greatly from year to year in both states but BLRV always occurred more frequently in faba beans (22 % of crops) than other pulses (19 % of lentils, 9 % of chickpeas and 4 % of field peas) (Freeman et al. 2005). The incidence of infected plants in faba bean crops ranged from 1 % to 27 % which was greater than all other crops (range 1–2 %) suggesting that the aphid vectors of BLRV prefer colonising the faba beans over other pulse crops, or that the earlier sowing of faba bean exposes it more to migratory aphid flights in autumn (Freeman et al. 2005; Aftab et al. 2005).

All of the pea crops surveyed in southern NSW in 2006 showed severe drought stress, making virus symptoms difficult to distinguish. Except for two crops, the incidence of BLRV was low and as BLRV is not seedborne this was of less concern than the high levels of PSbMV (22–77 % incidence) that were found in 12 of the 21 pea crops sampled. For the six crops for which PSbMV seed-to-plant transmission tests were conducted on the seed sown, there was a very strong relationship between the level of PSbMV in the seed and that found in the crop. Two crops sown with virus-free Excell seed were found to be PSbMV free by the time of sampling. As both Excell and Kasper are highly susceptible to PSbMV (van Leur et al. 2013b) these results demonstrate that PSbMV can be managed by sowing virus-tested seed. Currently, few farmers use seed tested for the presence of PSbMV, even though sowing PSbMV-infected seed leads to significant crop losses (Coutts et al. 2009).

Only field pea crops were surveyed in Victoria in 2006 as most faba bean crops had died due to drought. BWYV was the most common virus and was found in all ten crops, with virus

incidence ranging from 4 % to 28 %. This was a higher incidence range than that found in surveys from 2000 to 2004 in Victoria and South Australia, in which BWYV was found infrequently (about 5 % of crops) and never with an incidence over 6 % (Freeman et al. 2005). In 2006, BLRV was the second most common virus detected in Victorian pea crops with five of the ten crops infected, three of the crops having only 2–4 % incidence and one crop with 87 % incidence. In this survey, infections of pea crops with AMV, BYMV, CMV and PSbMV were very infrequent and at low incidence. Only one crop was infected with PSbMV, at an incidence of 2 %, suggesting that unlike the surveyed pea crops in NSW, seed sown in the sampled Victorian crops had very low levels of PSbMV infection and that little or no aphid activity occurred.

Comparison of diagnostic techniques

TBIA has been used extensively as a rapid, low-cost diagnostic method over many years and is suitable for large-scale surveys (Makkouk and Kumari 1996; Tadesse et al. 1999; Abraham et al. 2000; Najjar et al. 2000; Makkouk et al. 2003; Abraham and Makkouk 2002; Bao et al. 2007; van Leur et al. 2013a). It also has the advantage that samples can be bundled and even blotted onto nitrocellulose membranes in the field and no plant material has to be carried back to the laboratory. Bundling of plant samples into groups of ten reduces the time taken to blot samples, the most labour-intensive stage of the TBIA method, particularly when large numbers of replicate membranes are blotted. The bundling with Parafilm at the point of collection also prevents samples deteriorating during storage over the survey period and for this study ensured that the same material was used for blotting and RNA extractions.

In the past, nucleic acid extractions have been expensive and labour-intensive, making PCR an unsuitable choice of diagnostic method for large-scale surveys. Initial pooling of plant samples into groups of ten and rapid nucleic acid preparation using column technology and a 96-well plate format (Shepherd et al. 2002; Constable et al. 2012) greatly reduces the cost and time required for PCR diagnostics so that it is comparable to TBIA. PCR costs were also reduced by using Whatman plates and preparing the lysis and wash buffers rather than using a commercial extraction kit. The cost per

Table 7 Test cost (materials A\$) and labour required (man hours) for processing 1,920 plants simultaneously for a single test^a

Method	High-throughput units	Sample Preparation	Extraction	Processing	Test cost/virus/plant
PCR	2×96 well plates 192 pooled samples of 10 plants	16 h (grinding) ^b	2 h	4 h (PCR)	A\$ 0.55
TBIA	192 bundles of 10 plants	16 h (bundling and blotting) ^b	0 h	4 h (TBIA)	A\$ 0.16

^a The cost per test for both methods is reduced if multiple virus tests are conducted as the cost of the initial sample preparation/extraction is spread over more than one test

^b Two operators worked for 8 h over 1 day

test for both methods is reduced if multiple virus tests are conducted as the cost of the initial sample preparation/extraction is spread over more than one test.

The limiting step in the TBIA method was the blotting of the samples. The preparation and physical grinding of the samples for RNA extraction was the limiting step in the PCR method, as the high-throughput methodology used for the rest of the method was not available for this step. In Table 7 we attempt to compare labour and material costs for the TBIA and PCR methods, based on our mode of operation. For each method we used two operators working for 1 day to prepare samples and one operator on the second day to test the samples. Our comparison in the table is based on 192 samples (2 microtitre plates and 192 bundles), as no more than two plates could be handled simultaneously during the RNA extraction process. The overall time taken and costs were comparable for both methods. The number of man hours of labour for processing 192 samples by TBIA and PCR were 20 and 22 h respectively. The actual cost of materials (excluding labour) for the PCR method was about four and a half times the cost of TBIA (A\$ 0.55 compared to A\$ 0.16 per individual plant sample) but was still low enough not to be a deterrent to using PCR as a diagnostic method for pooled samples. It should be noted that with both methods a larger number of samples may be able to be tested in a day, with no additional labour costs, depending on the capacity of the laboratory (availability of multiple shakers, PCR machines etc). In this study, after the initial processing of 192 samples by the PCR method, we repeated the process for the remaining 48 samples and were able to complete the processing and testing of our 2,400 plants in 2 days.

Test results for PSbMV and BLRV using TBIA and RT-PCR compared favourably in giving similar estimates of infection incidence and similar costs for detecting these two viruses. Of the 340 ten-plant samples tested for PSbMV, five tested positive by TBIA and negative by PCR and of the 339 ten-plant samples tested for BLRV, ten samples tested negative by TBIA and positive by PCR. The primers in both PCR tests were found to give positive results for the range of virus isolates tested. A negative PCR result associated with a positive TBIA result occurred only for PSbMV and is most likely to be due to samples deteriorating over the survey period, before RNA could be extracted in the laboratory (in our study samples were stored for up to 4 days). In the development and use of both the PSbMV and BLRV PCR tests, primers and protocols were optimised not to cross-react with closely related viruses or with nucleic acid extracted from host tissue (data not shown). A positive PCR result associated with a negative TBIA result occurred only for BLRV, which is a phloem-restricted virus, and is most likely to be caused by blotting being impaired due to a dry positive plant from which RNA could still be extracted.

Group testing involves the pooling of samples from a population and testing as a group for a particular attribute such as

disease, the main benefit being the saving of resources (Hepworth 2005). It is frequently used for estimating virus incidence in plant populations and is described in some detail by Hepworth (2005). The sample pooling method used in this study, described by Fletcher (1993), is appropriate for virus incidences up to 11 %, however for higher virus incidences, further testing of 30 individual plants from a crop is necessary to accurately estimate virus incidence. For the PCR method, this would increase the time taken and cost of the test fourfold (ie 10 initial tests + 30 individual tests = 40 tests) (N.B. In this study, the additional individual PCR tests required to accurately estimate virus in crops with high incidence were not conducted as the additional time and costs were calculated from Table 7). If the PCR method is to be used in future large scale surveys, there is scope to assess a range of sequential pooling strategies to minimize the costs of the procedure.

The ability to use both PCR and TBIA to test for viruses in field samples will greatly enhance the flexibility in approaching surveys. Sample preparation adds a significant labour cost to each procedure. To reduce these bottlenecks, PCR tests on bulk samples (eg 100 plants or one bulk sample per crop) for target viruses would decrease the number of extractions required and could be used as an initial screening test. Further PCR or TBIA tests on ten bundles of ten samples from positive crops to determine virus incidence would decrease the total number of diagnostic tests undertaken. The use of high-throughput RT-PCR was effective for the large-scale diagnosis of viruses in pulse crops and will be particularly applicable when highly specific molecular diagnostic tests are required, such as during response to an incursion of an exotic pathogen, or if virus-specific antisera are not available.

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References

- Abraham A, Makkouk KM (2002) The incidence and distribution of seed-transmitted viruses in pea and lentil seed lots in Ethiopia. *Seed Sci Technol* 30:567–574
- Abraham A, Makkouk KM, Gorfú D, Lencho AG, Ali K, Tadesse N, Yusuf A, Lencho A (2000) Survey of faba bean (*Vicia faba* L.) virus diseases in Ethiopia. *Phytopathol Mediterr* 39:277–282
- Aftab M, Freeman A, Davidson J (2005) Pulse virus surveys in south eastern Australia (2003–2004). In: *Proceedings of the Australasian Plant Pathology Society 15th Biennial Conference*, 24–29 Sept 2005, Geelong, Victoria, p. 338
- Aftab M, Freeman A, Davidson J (2009) Pulse virus surveys from Victoria and South Australia in 2007. In: *Proceedings of the Australasian Plant Pathology Society 17th Biennial Conference*: 29 Sept–1 Oct 2009, Newcastle, NSW, p. 131

- Bao S, Wang X, Zhu Z, Zong X, Kumari S, Freeman A, van Leur J (2007) Survey of faba bean and field pea viruses in Yunnan Province, China. *Australas Plant Pathol* 36(4):1–7
- Constable FE, Connellan J, Nicholas P, Rodoni BC (2012) Comparison of enzyme-linked immunosorbent assays and reverse transcription-polymerase chain reaction for the reliable detection of Australian grapevine viruses in two climates during three growing seasons. *Aust J Grape Wine Res* 18:239–244
- Coutts BA, Prince RT, Jones RAC (2009) Quantifying effects of seedborne inoculum on virus spread, yield losses, and seed infection in the Pea seed-borne mosaic virus - field pea pathosystem. *Phytopathology* 99:1156–1167
- Fletcher JD (1993) Survey of virus diseases in pea, lentil, dwarf and broad bean crops in South Island New Zealand. *N Z J Crop Hortic Sci* 21: 45–53
- Freeman AJ, Aftab M (2001) Surveying for and mapping of viruses in pulse crops in south-eastern Australia. In: *Proceedings of the Australasian Plant Pathology Society 13th Biennial Conference, 24–27 Sept 2001, Cairns, Queensland*, p. 149
- Freeman AJ, Aftab M, Dobson V (2003a) Surveying for viruses in pulse crops in Victoria. In: *Proceedings of the 8th International Congress of Plant Pathology, 2–7 Feb 2003, Christchurch, New Zealand*, p. 261
- Freeman A, Aftab M, Dobson V (2003b) The incidence of seedborne viruses in lentil crops in Victoria and South Australia. In: *Proceedings of the 8th International Congress of Plant Pathology, Christchurch, New Zealand, 2–7 Feb. 2003*, p. 261
- Freeman A, Aftab M, McQueen V, Davidson J (2005) The occurrence of common viruses in pulse crops in south eastern Australia. In: *Proceedings of the 15th Biennial Australasian Plant Pathology Society Conference, Geelong, Sept, 2005*, p. 339
- Hepworth G (2005) Confidence intervals for proportions estimated by group testing with groups of unequal size. *J Agric Biol Environ Stat* 10:478–497
- Hill MF, Giles RJ, Moran JR, Hepworth G (1996) The incidence of chrysanthemum stunt viroid, chrysanthemum B caravirus, tomato aspermy cucumovirus and tomato spotted wilt tospovirus in Australian chrysanthemum crops. *Australas Plant Pathol* 25:174–178
- Hsu HT, Lawson RH (1991) Direct tissue blotting for detection of tomato spotted wilt virus in Impatiens. *Plant Dis* 75:292–295
- Latham LJ, Jones RAC (2001) Incidence of virus infection in experimental plots, commercial crops, and seed stocks of cool season crop legumes. *Aust J Agric Res* 52:397–413
- MacKenzie DJ, McLean MA, Mukerji S, Green M (1997) Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. *Plant Dis* 81:222–226
- Makkouk K, Comeau A (1994) Evaluation of various methods for the detection of barley yellow dwarf luteovirus by the tissue-blot immunoassay and its use for BYDV detection in cereals inoculated at different growth stages. *Eur J Plant Pathol* 100:71–80
- Makkouk K, Kumari S (1996) Detection of ten viruses by the tissue-blot immunoassay (TBIA). *Arab J Plant Prot* 14:3–9
- Makkouk KM, Kumari SG, Shahraeen N, Fazlali Y, Farzadfar S, Ghotbi T, Mansouri AR (2003) Identification and seasonal variation of viral diseases of chickpea and lentil in Iran. *Z Pflanzenkrankh Pflanzenschutz* 110:157–169
- Moran JR, Wilson JM, Garrett RG, Smith PR (1985) ELISA indexing of commercial carnations for carnation mottle virus using a urease-antibody conjugate. *Plant Pathol* 34:467–471
- Najar A, Makkouk KM, Boudhir H, Kumari SG, Zarouk R, Bessai R, Othman FB (2000) Viral diseases of cultivated legume and cereal crops in Tunisia. *Phytopathol Mediterr* 39:423–432
- Ortiz V, Castro S, Romero J (2005) Optimization of RT-PCR for the detection of Bean leaf roll virus in plant hosts and insect vectors. *J Phytopathol* 153:68–72
- Rodoni BC, Hepworth G, Richardson C, Moran JR (1994) The use of a sequential batch testing procedure and ELISA to determine the incidence of five viruses in Victorian cut-flower Sim carnations. *Aust J Agric Res* 45:223–230
- Schwingamer MW, Thomas JE, Parry JN, Schilg MA, Dann EK (2007) First record of natural infection of chickpea by *Turnip mosaic virus*. *Australas Plant Dis Notes* 2:41–43
- Shepherd M, Cross MJ, Stokoe RL, Scott LJ, Jones ME (2002) High-throughput DNA extraction from forest trees. *Plant Mol Biol Report* 20:425a–425j
- Tadesse N, Ali K, Gorfu D, Yusuf A, Abraham A, Ayalew M, Lencho A, Makkouk KM, Kumari SG (1999) Survey for chickpea and lentil virus diseases in Ethiopia. *Phytopathol Mediterr* 38:149–158
- Thomas JE, Schwingamer MW, Parry JN, Sharman M, Shilg MA, Dann EK (2004) First report of tomato spotted wilt virus in chickpea (*Cicer arietinum*) in Australia. *Australas Plant Pathol* 33:597–599
- Thomas JE, Parry JN, Schwingamer MW, Dann EK (2010) Two novel mastreviruses from chickpea (*Cicer arietinum*). *Aust Arch Virol* 155:1777–1788
- van Leur JAG, Makkouk KM, Freeman AJ, Schilg MA (2003) Occurrence of viruses in faba bean on the Liverpool Plains, northern New South Wales. In: *Proceedings of the 8th International Congress of Plant Pathology, 2–7 Feb 2003, Christchurch, New Zealand*, p. 265
- van Leur JAG, Aftab M, Manning W, Bowring A, Riley MJ (2013a) A severe outbreak of chickpea viruses in northern New South Wales, Australia, during 2012. *Australasian Plant Disease Notes*. doi:10.1007/s13314-013-0093-y
- van Leur JAG, Kumari S, Aftab M, Leonforte A, Moore S (2013b) Virus resistance of Australian pea (*Pisum sativum*) varieties. *N Z J Crop Hortic Sci* 41(2):86–101. doi:10.1080/01140671.2013.781039