National Diagnostic Protocol

Red clover vein mosaic virus (RCVMV)

NDP 47 V1

This publication (and any material sourced from it) should be attributed as: Subcommittee on Plant Health Diagnostics (2022). National Diagnostic Protocol for *Red Clover Vein Mosaic Virus* – NDP47 V1. (Eds. Subcommittee on Plant Health Diagnostics) Authors Freeman, A., Tang, J., Maina, S., Lovelock, D.; Reviewers: Tang, J., Maina, S., Lovelock, D. ISBN 978-0-6481144-4-4. CC BY 3.0.

Cataloguing data
Subcommittee on Plant Health Diagnostics (2022). National Diagnostic Protocol for *Red Clover Vein Mosaic Virus* – NDP47 V1. (Eds. Subcommittee on Plant Health Diagnostics) Authors Freeman, A., Tang, J., Maina, S., Lovelock, D.; Reviewers: Tang, J., Maina, S., Lovelock, D. ISBN 978-0-6481144-4-4.

ISBN; 978-0-6481144-4-4

Internet
Report title is available at: https://www.plantbiosecuritydiagnostics.net.au/resources/#

Department of Agriculture, Fisheries and Forestry

Street Address 1: 18 Marcus Clarke Street, Canberra City ACT 2601
Street Address 2: 7 London Circuit, Canberra City ACT 2601
Postal Address: GPO Box 858, Canberra City ACT 2601
Switchboard Phone: 02 6272 3933
Web: http://www.agriculture.gov.au

Inquiries regarding the licence and any use of this document should be sent to: copyright@agriculture.gov.au.

Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs although NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at https://www.plantbiosecuritydiagnostics.net.au/initiatives/national-diagnostic-protocols/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for *Red clover vein mosaic virus* is current as at the date contained in the version control box below.

PEST STATUS	Not present in Australia
PROTOCOL NUMBER	NDP 47
VERSION NUMBER	V1
PROTOCOL STATUS	Endorsed
ISSUE DATE	2022
REVIEW DATE	2027
ISSUED BY	SPHD

The most current version of this document is available from the SPHD website: https://www.plantbiosecuritydiagnostics.net.au/resources/#

Further information

Inquiries regarding technical matters relating to this protocol should be sent to: sphd@agriculture.gov.au

Contents

1 INTRODUCTION

Red clover vein mosaic virus (RCVMV) (genus *Carlavirus*, family *Betaflexiviridae*) was first described in the United States by Osborn in 1937 in red clover (Osborn 1937). Since then, it has been found in other leguminous crops around the world (Bos *et al*. 1972; Edwardson and Christie 1986, 1991; Hagedorn *et al*. 1959; Varma *et al*. 1970; Wetter *et al*. 1959). RCVMV is predominately a virus of temperate pasture species, particularly clovers, but it also infects a number of temperate pulses including *Cicer arietinum* (chickpea), *Lathyrus odoratus* (sweet pea), *Lens culinaris* (lentil), *Pisum sativum* (field pea), and *Vicia faba* (faba bean, broad bean, tick bean).

RCVMV is an obligate plant pathogen. It survives in perennial legume hosts such as clovers or possibly in infected red clover, pea or faba bean seed. Initial infection of a crop occurs when aphid vectors feed on the infected pastures and become infected with the virus and then feed on the crop. Secondary spread occurs within the crop during aphid vector feeding (Fletcher *et al.,* 2016).

RCVMV has been reported to be transmitted by the following aphids: *Acyrthosiphon pisum*, *Aphis fabae*, *Cavariella aegopodii*, *C. theobaldi*, *Myzocallis onomidis*, *Myzus persicae* and *Therioaphis maculate* (Weber and Hampton 1980, Edwardson and Christie 1991).

RCVMV survives between growing seasons of annual pulse hosts in perennial pasture legumes and in seed. Seed transmission has been reported in red clover (*Trifolium pratense*) (Matsulevich 1957, Sander 1959, Brunt *et al*. 1997, Edwardson and Christie 1991, Kraft *et al*. 1998), faba bean (*Vicia faba*) (Sander 1959) and field pea (*Pisum sativum*) (Kraft *et al.* 1998) but is not common.

RCVMV infection has been associated with economic losses such as up to 88% of the grain weight loss of moderately diseased pea (Khan and Singh 1997a) and up to 100% yield losses in chickpea depending on infection time (Larsen and Miklas 2000).

1.1 Primary host range

RCVMV has a wide host range among temperate pulses and annual and perennial legume pasture species. Where RCVMV occurs, it has been found to be common in red clover, and sweet clover as well as cultivated legumes (Hanson and Hagedorn 1961, Fletcher *et al*. 2016). A full list with references is included in Appendix 8.1.

2 TAXONOMIC INFORMATION

Order: Tymovirales
Family: *Betaflexiviridae*
Subfamily: *Quinvirinae*
Genus: *Carlavirus*
Species: *Red clover vein mosaic virus*
Acronym: RCVMV

Synonyms

- *Marmor trifolii*
- *Pea streak virus* 1
- *Trifolium virus* 2
- *Wisconsin pea stunt virus*

Virus strains

Isolates of RCVMV are serologically similar (Varma *et al*. 1970, Wetter *et al*. 1962). Bos *et al*. (1972) described a biologically highly deviating strain of RCVMV but found that it could not be distinguished serologically from other RCVMV strains.

Serological relationships

RCVMV is most closely serologically related to *Pea streak virus* (PSV) (Bos *et al.* 1972, Bos 1973). The two viruses had been previously described as strains of the one virus (Varma 1970), but studies by Wetter *et al*. (1962), Bos *et al.* (1972) and Veerisetty and Brakke (1977) confirmed that they were separate viruses.

Genetic relationships

There are only two available genomes of RCVMV: a New Zealand RCVMV sequence (GenBank accession number KR108251) from clover (Fletcher *et al*. 2016), and chickpea isolate from United States (GenBank accession number FJ685618) (Larsen *et al*. 2009). Both share 96% nucleotide (nt) identity. There is also one RCVMV partial sequence of isolate P42-PRI (accession FJ560905) (231 bp), a non-functional replicase gene from the Netherlands. Analysis of these sequences indicates that the coat protein (CP) of RCVMV has 77-78.3% nt identity with the CP of the putative virus species called *Red clover carlavirus* 1 (GenBank accessions MG596238- MG596241). This level of identity for the CP is above the taxonomic demarcation cut-off for *Carlavirus* species and suggests they are strains of the same species. However, there are significant differences in the replicase region, and they only share 57.8-58.5% nt identity and some parts of the replicase are much less conserved when RCVMV and *Red clover carlavirus* 1 are compared, particularly between nucleotide positions 1350 and 4103 of the RCVMV genome. This difference for the replicase is well below the taxonomic demarcation cut-off for *Carlavirus* species and therefore RCVMV and *Red clover carlavirus* 1 should be considered as distinct. Therefore, it is important to obtain sequence of the replicase in order to differentiate RCVMV and other accepted and putative *Carlavirus* species.

Although RCVMV was found to be serologically related to *Pea streak virus* (PSV), their genomes only share at 52% nt identity and the nt identity of CP and replicase regions, which are used to differentiate species, are below the demarcation cut-off for the genus *Carlavirus*.

3 DETECTION

Studies have associated RCVMV with several symptoms such as vein mosaic or vein chlorosis and plant stunting depending on host species and virus strain. Mild and latent infections have also been reported to occur (Bos *et al*. 1972; Graves and Hagedorn 1956; Hagedorn *et al*. 1959; Wetter *et al*. 1959; Fletcher *et al.* 2016). In a mixed infection scenario, symptoms expression may vary.

3.1 Symptom description

RCVMV generally causes vein mosaic (Figures 1 and 2), vein chlorosis and clearing (Figure 3), curling resulting in apical rosetting (Figure 4) and stunting. Stem necrosis can develop, with a rapid wilt and collapse of the plants. A full list of symptoms for all host plants is included in Appendix 8.1.

3.1.1 Clovers

RCVMV causes a characteristic chlorosis of leaf veins, veinlets and tissue immediately adjacent to the veins in red clover (Khan *et al*. 1978). Vein mosaics (Figures 1 and 2) are a common symptom of RCVMV in many clover species (Sander 1959) although Gibbs *et al*. (1966) found that white clover plants were symptomless when infected with RCVMV unless also infected with *Clover yellow vein virus* (CYVV). CYVV on its own, does not cause obvious symptoms in clovers unless in combination with RCVMV or *White clover mosaic virus* (Gibbs *et al*. 1966).

RCVMV is the most common virus in red clover, alsike clover and sweet clover (Hanson and Hagedorn 1961).

3.1.2 Pulses

The first report of RCVMV in pulses was associated with a disease called Wisconsin pea stunt which was characterised by severe plant stunting, tight apical rosetting (Figure 4), leaves of reduced size, often wrinkled and folded upward showing marked vein clearing (Figure 3) (Hagedorn and Walker 1949). Similar symptoms have since been reported in faba beans, chickpeas and lentils (Sander 1959, Gibbs *et al*. 1966, Larsen *et al*. 1996, Larsen and Myers 1998).

Rubio-Huertos and Bos (1973) reported that in most pea cultivars, the first symptoms of systemic infection were usually systemic vein clearing and leaf curling although some cultivars developed necrotic stem streaking sometimes followed by irregular yellowing and premature plant death. Bos *et al*. (1972) isolated a new highly deviating strain of RCVMV which caused necrotic stem streaking in peas but found that although readily transmitted to 30 pea cultivars, it was latent in most cultivars.

RCVMV is reported to cause stunting in faba beans (Sander 1959), and inoculated plants have been reported to show chlorotic lesions or general chlorosis, tip mottle and abscission (Gibbs *et al*. 1966). Common vetch (*Vicia sativa*) is reported to develop necrotic local lesions, which sometimes become systemic (Stuteville 1964, Varma 1970).

Larsen *et al*. (1996) reported a disease of chickpeas caused by RCVMV, with symptoms including severe stunting, mosaic, proliferation of axillary buds, malformation of leaves and branches and reduced flower and pod numbers. Larsen and Myers (1998) found lentil plants with mixed infections of RCVMV and *Pea*

enation mosaic virus (PEMV), many of which developed symptoms typical of PEMV, but others exhibited severe stunting, proliferation of axillary branches and general chlorosis or death. Glasshouse inoculations of peas, faba beans, chickpeas and lentils with this RCVMV isolate resulted in mild systemic mosaic symptoms in all species.

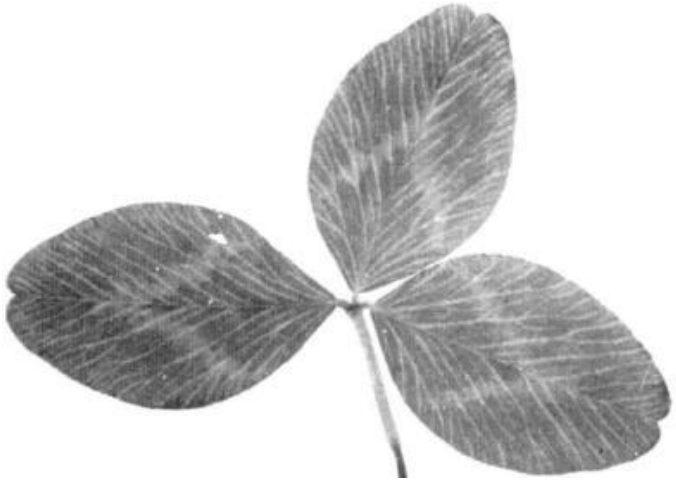

Figure 1 Mild vein mosaic in leaf of red clover (*Trifolium pratense*) (Varma 1970).

Figure 2 Mild vein mosaic in leaf of red clover (*Trifolium pratense*) (University of Illinois https://ipm.illinois.edu/diseases/rpds/307.pdf).

Figure 3 Pea (*Pisum sativum*) infected with RCVMV exhibiting vein chlorosis and banding, USA. (Courtesy R. O. Hampton)

Figure 4 Necrosis and rosetting of pea (*Pisum sativum*) cv. Big Ben (Varma 1970).

4 IDENTIFICATION

The traditional methods of virus identification, including microscopy and mechanical transmission tests can be used for preliminary identification of the presence of a virus, but are not reliable for a definitive identification of RCVMV.

Lists of indicator plant species and their reactions to RCVMV infection are given in Sander (1959), Gibbs *et al*. (1966), Varma (1970), Bos *et al*. (1972) and Brunt *et al*. (1997), with Bos *et al*. (1972) also including photographs of symptoms. A table of the indicator plants and reactions in included in Appendix 8.2.

ELISA tests can support identification of RCVMV but are less sensitive than the molecular techniques now available (Fletcher *et al*., 2016; Larsen and Myers 1996).

4.1 Electron microscopy

Direct examination of plant sap using the transmission electron microscope (sap dip) can be used to detect virus particles. Immunosorbent electron microscopy (trapping or decoration) with virus-specific antibodies enables trapping of particles of the target virus and offers a more definitive test result. However as this is a serological test, the accuracy depends on the quality of the antiserum, sample preparation and operator experience.

Key references: Ball (1974), Milne (1986), Roberts (1986).

RCVMV is a carlavirus with a straight filamentous capsid approximately 645 nm in length (600-700nm) and a width of 12 nm. The axial canal is distinct and 3.5 nm in diameter and the basic helix is obvious (ICTV dB Management 2006).

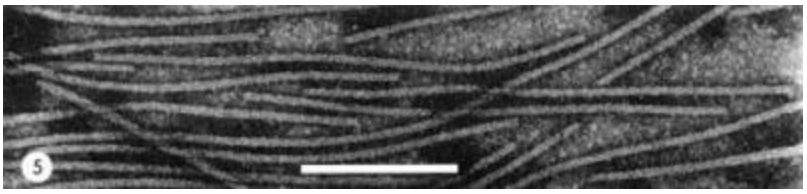

Figure 5 RCVMV particles stained with phosphotungstate. Bar represents 200 nm (Varma 1970).

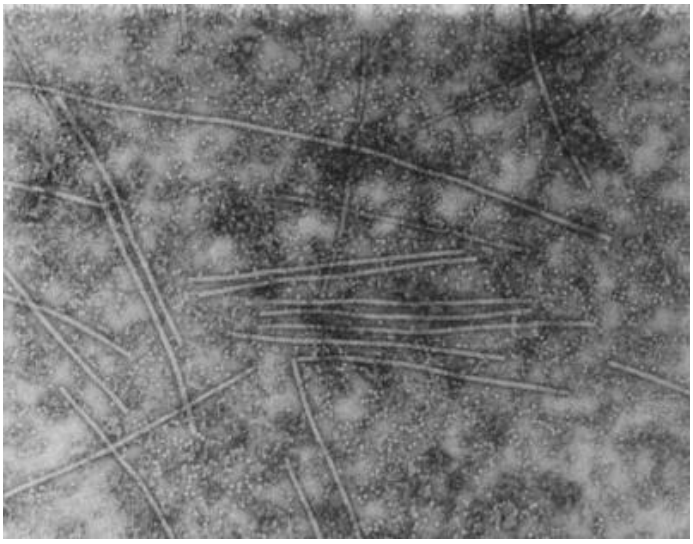

Figure 6 Electron micrograph of RCVMV (Brunt *et al*. 1997).

4.1.1 General items required

1. Samples - leaves, shoots or washed roots.
2. Transmission electron microscope grids: copper 400 mesh, Formvar coated, then carbon coated.
3. Glass microscope slides, waxed glass microscope slides, plastic wells, pasteur pipettes, filter papers, fine forceps.
4. Distilled water, 0.1M sodium phosphate buffer, pH 7.0.
5. Freshly prepared stains: 2% phosphotungstic acid (PTA) or 2% uranyl acetate (UA) dissolved in distilled water, adjusted to pH 7.0 with NH_3.
6. Freshly prepared dilutions of appropriate virus antibodies in 0.07 M sodium phosphate buffer, pH 6.5.

4.1.2 Methods

Sap dip (negative staining) method

1. Using the scalpel blade, cut approximately 3 mm^2 of the test plant material and place it on a clean microscope slide (if the test material has any suspicious virus symptoms, take the tissue from this area).

2. Place a 3 mm diameter drop of PTA next to the piece of plant material and thoroughly crush the plant material into the PTA. If necessary, add an extra drop of PTA.

3. Pick up a coated grid with forceps and touch it, coated side down, onto the drop of PTA and plant sap mixture.

4. After 2-3 seconds, drain the excess droplet of the grid by touching its edge with a piece of torn filter paper.

5. Allow the grid to dry for approximately 2 minutes then observe grids for virus particles using an electron microscope.

Immunosorbent transmission electron microscopy trapping method

1. Pipette 30 µL drops of antiserum diluted 1:100, 1:1,000 and 1:10,000 in normal saline onto waxed glass slides.

2. Float a carbon-coated grid, film-side down, on each drop and incubate for 2-3 hours at 37°C.

3. Wash grids five times in normal saline or place grids in 0.1 M sodium phosphate buffer, pH 7.0, in plastic wells, agitate at intervals for 10 minutes, transfer to a second plastic well containing buffer and leave for a further 10 minutes and then drain.

4. Extract plant material in normal saline at 1:5 wt/vol and then centrifuge at 8,000g for 10 minutes.

5. Float grids on 30 µL drops of sample sap extract and incubate for 2 hours at room temperature or for 3-36 hours at 4°C.

6. Wash grids five times in normal saline or place grids in plastic wells, containing normal saline, agitate at intervals for 10 minutes, transfer to a second plastic well containing normal saline and leave for a further 10 minutes and then drain.

7. Stain grids with 2% PTA and/or UA by floating grids on the stain for 10 minutes then drain grids by touching the edge with torn filter paper.

8. Observe grids for virus particles using a transmission electron microscope.

Decoration method

1. As an additional step, just prior to examination of the prepared grid using the electron microscope, in either of the above procedures, add a drop of suitably diluted antiserum to the prepared grids, incubate for 3 hours at 37°C and drain.

2. Observe grids using an electron microscope for antibody halos surrounding virus particles. Such halos indicate the specific binding of the virus-specific antibody to the trapped virus particles on the grid and therefore provide evidence of the true identity of the virus, based on the specificity of the antiserum used.

4.2 Enzyme-linked immunosorbent assay (ELISA)

RCVMV ELISA kits are available through Creative Diagnostics or AC Diagnostics. The test should be conducted following the manufacturer's instructions supplied with the kit. This kit comes with a positive control, providing permits are in place. There are no cross reactions to other viruses associated with this kit.

4.3 Molecular detection

4.3.1 Total RNA extraction

Total RNA can be extracted from fresh or dried leaf tissue. A range of nucleic acid extraction methods may be used, from commercial kits to methods published in scientific journals. The following nucleic acid extraction kits have been used successfully for the detection of RCVMV at Plant Health and Environment Laboratory (PHEL) of New Zealand.

RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany)

Follow the manufacturer's instructions provided within the kit. Alternatively use an already validated kit.

Thermo Kingfisher (Thermo Scientific, Waltham, MA, USA)

Thermo Kingfisher mL semi-automated nucleic acid extraction machine can be used in conjunction with an InviMag® Plant DNA Mini Kit (Invitek GmbH, Berlin, Germany), as per the manufacturer's instructions. Other automated extraction protocols can also be used, provided they have been validated.

4.3.2 Primers

For the detection of RCVMV, the following primers need to be used.

Primer	Sequence (5'-3')	Genes targeted	PCR product size
RCVMV-5217F[1]	TGCACTGAATCGGATTATGA	RdRp of RCVMV	512 bp
RCVMV-5728R[1]	GCATAAGACACTTCAATAGCAT		
Nad5-S[2]	GATGCTTCTTGGGGCTTCTTGTT	Nad5 of plant DNA	181 bp
Nad5-AS[2]	CTCCAGTCACCAACATTGGCATAA		

1. Fletcher *et al.* 2016; 2. Menzel *et al.* 2002.

The primers RCVMV-5217F/5728R are specific for RCVMV detection and produce a 512bp amplicon while Nad5-S/AS are used as an internal control to ensure the RT-PCR competency of RNA extracts and produce a 181bp amplicon (Figure 7).

All assays should include a positive control and a water control, without nucleic acid. A synthetic positive control for RT-PCR using the primer pair RCVMV-5217F/5728R has been developed. A 400bp product is amplified (Figure 8). This can be used where viral RNA is unavailable.

4.3.3 RT-PCR reagents and cycling conditions

A one-step RT-PCR may be done in a 20µl volume using either one of the reagents shown in the following table, or other available validated commercial kits.

SuperScript III one-step RT-PCR system with Platinum Taq polymerase (Invitrogen)		GoTaq® Green Master Mix (Promega, Madison, Wisconsin, USA) with SuperScript III reverse transcriptase (Invitrogen)	
Nuclease-free water	4.2 µl	Nuclease-free water	3.5 µl
2 x reaction mix	10.0 µl	2 x GoTaq master mix	10.0 µl
5 µM forward primer	1.0 µl	5 µM forward primer	1.0 µl
5 µM reverse primer	1.0 µl	5 µM reverse primer	1.0 µl
10 mg/ml BSA	1.0 µl	10 mg/ml BSA	1.0 µl
SuperScript III /PlatinumTaq Mix	0.8 µl	Dithiothreitol (DTT)	1.0 µl
		SuperScript III	0.25 µl
		RNasinPlus (Promega)	0.25 µl
RNA template	2.0 µl	RNA template	2.0 µl

Cycling conditions are as below:

50ºC, 30 min

94ºC, 5 min

94ºC, 30 s
54ºC, 30 s } x 40
72ºC, 45 s

72ºC, 5 min

4.3.4 Agarose gel electrophoresis

Four to six microlitres of the PCR reaction is subject to electrophoresis on a 1.5% agarose-TAE gel in 1x TAE buffer stained with SYBR™ DNA Gel Stain (Invitrogen) at 90V for 50 minutes. The sizes of amplified fragments are determined by comparison to a 100bp DNA ladder (Promega, Madison, Wisconsin, USA). The expected product sizes are 181bp for the Nad5 internal control and 512bp for RCVMV (Figure 7), while the synthetic control (400bp) for RCVMV was verified at Agriculture Victoria (Figure 8).

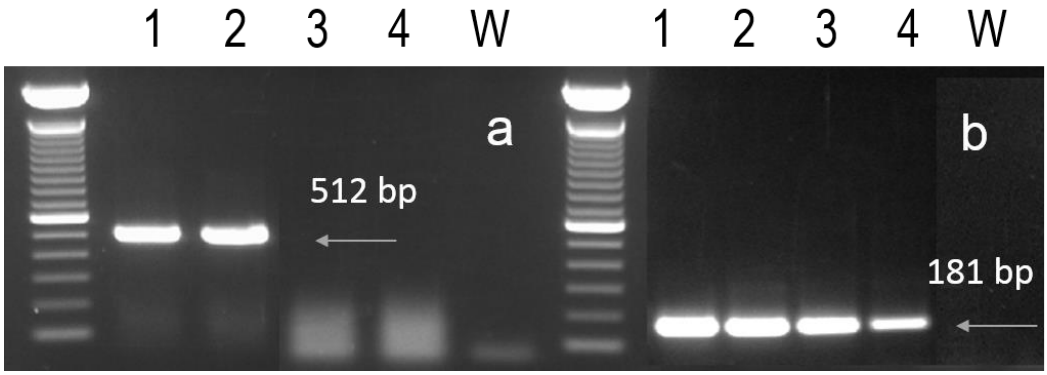

Figure 7 An electrophoretic gel showing bands generated by the primers RCVMV-5217F/5728R (a) and Nad5-S/AS (b). Samples are: 1 & 2, RCVMV positive controls; 3 & 4, healthy host plants; W, water control (no template control).

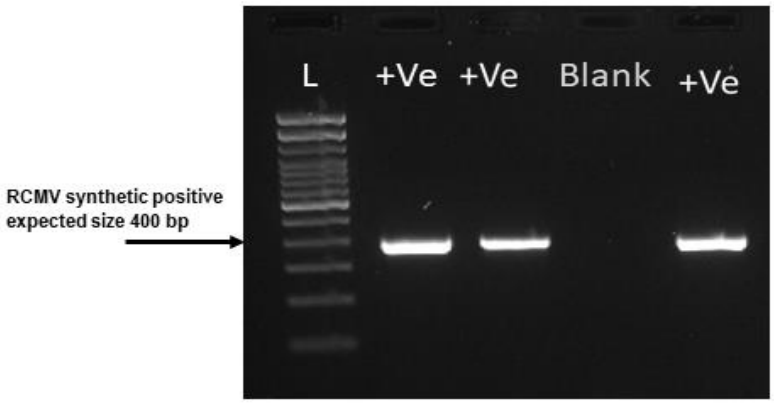

Figure 8 Gel electrophoresis of a synthetic positive for RCVMV at a size of 400bp (512bp for a true positive).

4.3.5 Sequencing

Following the manufacturer's instructions (QIAquick PCR Purification Kit, Qiagen or similar), the PCR product can be cleaned directly from the RT-PCR tube or excised from the gel. The product along with the appropriate primers can then be sent to a sequencing facility (e.g. Micromon, Monash University) if it cannot be completed on site. The resulting sequences can then be compared to RCVMV isolates on GenBank, including the type species (NC_012210.1). Although *in silico* assessment suggests that there not be any off-target amplification from the assay, any amplicons (~±50nt) should be Sanger sequenced to confirm the identity of the amplificon.

5 CONTACTS FOR FURTHER INFORMATION

Supplies of the antiserum and dried virus culture have been obtained from the ATCC and stored in the locked quarantine -18°C storage facility at the DPI-Horsham pulse post-entry quarantine station. If further antisera is required, it can be purchased from overseas – an import permit is required. Shane King, Manager Horsham Pulse Quarantine Program, holds a current permit to import the antiserum and virus-infected tissue.

Australia

Solomon Maina
Agriculture Victoria
110 Natimuk Road
Horsham, Victoria, 3400
Phone: +613 4344 3111
Email: solomon.maina@agriculture.vic.gov.au

Shane King
Agriculture Victoria
110 Natimuk Road
Horsham, Victoria, 3400
Phone: +613 5362 0786
Email: shane.king@agriculture.vic.gov.au

New Zealand

Joe Tang
Plant Health & Environment Laboratory
Ministry for Primary Industries
231 Morrin Road, St Johns, Auckland 1072
PO Box 2095 Auckland 1140
Email: Joe.Tang@mpi.govt.nz

6 ACKNOWLEDGEMENTS

The document was originally developed by Dr Angela Freeman, Victoria DPI. It was reviewed by J Tang (PHEL, MPI NZ) who included the molecular protocol. It has been updated with additional information in 2020 and verified by Dr. Solomon Maina and Dr. David Lovelock.

7 REFERENCES

Alconero R. (1983) Viruses infecting six species of perennial clover (*Trifolium* spp.) in field evaluations of plant introductions and cultivars. *Plant Disease* **67**, 1270–1271.

Al-Shahwan IM, Farooq T, Al-Saleh MA, Abdalla OA, Amer MA. (2016) First report of Red clover vein mosaic virus infecting alfalfa in Saudi Arabia. *Plant Disease* **2,** 539.

Al-Shahwan IM, Abdalla OA, Al-Saleh MA, Amer MA (2017) Detection of new viruses in alfalfa, weeds and cultivated plants growing adjacent to alfalfa fields in Saudi Arabia. Saudi J Biol Sci 24(6):1336–1343

Baggett JR, Kean D. (1992) 'Oregon Giant' edible pod pea. *HortScience* **27**, 853–854.

Ball EM. (1974) *Serological tests for the identification of plant viruses*. American Phytopathological Society.

Bos L, Maat DZ, Markov M. (1972) A biologically highly deviating strain of red clover vein mosaic virus, usually latent in pea (*Pisum sativum*), and its differentiation from pea streak virus. *Netherlands Journal of Plant Pathology* **78**, 125–152.

Bos L. (1973) *Pea streak virus*. CMI/AAB Descriptions of Plant Viruses. No. 112.

Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ, Watson L, Zurcher EJ. (eds) (1997) Plant viruses online: Descriptions and lists from the VIDE database. http://biology.anu.edu.au/Groups/MES/vide/ (1996 onwards) Version: 16th January 1997.

Dellavalle G, Masenga V, Lisa V. (1994) Cucumber mosaic, a potyvirus and a carlavirus from *Dimorphotheca sinuata*. *Acta Horticulturae* **377**, 91–98.

Edwardson JR, Christie RG. (1986) Viruses Infecting Forage Legumes, Volume I. Agricultural Experiment Stations, *Institute of Food and Agricultural Sciences*, University of Florida, Gainesville

Edwardson JR, Christie RG (1991) *Handbook of viruses infecting legumes*. CRC Press, Inc., Florida.

Fletcher J, Tang J, Blouin A, Ward L, MacDiarmid R, and Ziebell H. (2016) Red clover vein mosaic virus—A novel virus to New Zealand that is widespread in legumes. *Plant Disease*. **100**:890-895.

Gibbs AJ, Varma A, Woods RD. (1966) Viruses occurring in white clover (*Trifolium repens* L.) from permanent pastures in Britain. *Annals of Applied Biology* **58**, 231–240.

Hagedorn DJ, Walker JC. (1949) Wisconsin pea stunt, a newly described disease. *Journal of Agricultural Research* **78**, 617–626.

Hanson EW, Hagedorn DJ. (1961). Viruses of red clover in Wisconsin. Agronomy journal 53: 63-67

Khan MA, Maxwell DP, Smith RR. (1978) Inheritance of resistance to red clover vein mosaic virus in red clover. *Phytopathology* **68**, 1084–1086.

Khan AT, Singh RN. (1997a) Effect of pea stunt disease on flowering, podding, grain setting and yield. *Indian Phytopathology* **50**, 282–284.

Khan AT, Singh RN. (1997b) Effect of pea stunt disease on shoot development in field pea. *Indian Phytopathology* **50**, 285–289.

Kraft JM, Larsen RC, Inglis DA. (1998). Diseases of pea. In 'The pathology of food and pasture legumes'. (Eds DJ Allen and JM Lenne) pp. 325-370. (CABI & IRCISAT, New York).

Larsen RC, Kaiser WJ, Wyatt SD. (1996) First report of a virus disease of chickpea caused by a strain of red clover vein mosaic carlavirus. *Plant Disease* **80**, 709.

Larsen RC, Wyatt SD, Druffel K. (1997) Partial nucleotide sequencing of red clover vein mosaic carlavirus (RCVMV) 3' terminus. *Phytopathology* **87**, S56.

Larsen RC, Myers JR. (1998) First report of red clover vein mosaic carlavirus naturally infecting lentil. *Plant Disease* **82**, 1064.

Larsen RC, Miklas PN. (2000) Effect of red clover vein mosaic carlavirus infection on 478 seed production and biomass yield in chickpea. Phytopathology 91, S186.

Larsen RC, Wyatt SD, Druffel KL. (2009) The complete nucleotide sequence and genome organization of red clover vein mosaic virus (genus *Carlavirus*). Archives of virology. 5, 891-8944.

Matsulevich BP. (1957) The effects of clover mosaic on the productivity of red clover (Translated title). *Agrobiologiya* **2**, 75–79.

McLaughlin MR, Ensign RD. (1989) Viruses detected in forage legumes in Idaho. *Plant Disease* **73**, 906–909.

McLaughlin MR, Boykin DL. (1988) Virus diseases of seven species of forage legumes in the south-eastern United States. *Plant Disease* **72**, 539–542.

Menzel W, Jelkmann W, Maiss E. (2002) Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J.Virol. Methods 99, 81–92.

Milne RG. (1986) New developments in electron microscope serology and their possible applications. In 'Developments in Applied Biology I: Developments and Applications in Virus Testing' (Eds RAC Jones and L Torrance) pp. 179–191. (Association of Applied Biologists, Wellesbourne, Warwickshire).

Musil M, Smrz J, Vacek V. (1977) The occurrence of virus diseases in a collection of red clover (*Trifolium pratense* L). *Ochrana Rostlin* **13**, 183–188.

Osborn HT. (1937) Vein-mosaic of red clover. *Phytopathology* **27**, 1051–1058.

Rahman F, Peaden RN. (1993) Incidence of viruses on alfalfa in western North America. *Plant Disease* **77**, 160–162.

Rahman F, Soberg RL. (1988) *Survey of viruses of alfalfa in the Western United States and Canada*. NAAIC, Beltsville, Maryland, USA.

Roberts IM. (1986) Practical aspects of handling, preparing and staining samples containing plant virus particles for electron microscopy. In 'Developments in Applied Biology I: Developments and Applications in Virus Testing' (Eds Jones RAC, Torrance L) pp. 213–243. (Association of Applied Biologists, Wellesbourne, Warwickshire).

Rubio-Huertos M, Bos L. (1973) Light and electron microscopy of red clover vein mosaic virus in pea (*Pisum sativum*). *Netherlands Journal of Plant Pathology* **79**, 84–103

Sander E. (1959) Biological properties of red clover vein mosaic virus. *Phytopathology* **49**, 749–754.

Staniulis J, Zitikaite I. (1989) Red clover vein mosaic virus in Lithuania. (3. Purification and serology). *Lietuvos TSR Mokslu Akademijos Darbai, C (Biologijos Mokslai)* **2**, 3–9.

Stuteville DL. (1964) *Virus diseases of red clover*. University of Wisconsin Dissertation Abstracts 25: 4, 2162.

Varma A. (1970) *Red clover vein mosaic virus*. CMI/AAB Descriptions of Plant Viruses. No. 22.

Varma A, Gibbs AJ, Woods RD. (1970) A comparative study of red clover vein mosaic virus and some other plant viruses. *Journal of General Virology* **8**, 21–32.

Veerisetty V, Brakke MK. (1977) Differentiation of legume carlaviruses based on their biochemical properties. *Virology* **83**, 226–231.

Weber KA, Hampton RO. (1980) Transmission of two purifies carlaviruses by the pea aphid. *Phytopathology* **70**, 631–633.

Wetter C, Quantz L, Brandes J. (1962) Vergleichende untersuchungen uber das rotkleeadernmosaik-virus und das erbsenstrichel virus. *Phytopath. Z.* **44**, 151–169.

Zitikaite I, Staniulis J. (1989) Red clover vein mosaic virus in Lithuania (2. Physical and Morphological properties of the virus). *Lietunos TSR Mokslu Akademijos Darbai, C (Biologijos Mokslai)* **1**, 3–11.

8 APPENDICES

8.1 Host range of RCVMV

Host	Reference
Cicer arietinum (chickpea)	Larsen *et al*. (1996) Larsen *et al*. (1997) Larsen *et al*. (2009)
Dimorphotheca sinuata (african daisy)	Dellavalle *et al*. (1994)
Lathyrus odoratus (sweet pea)	Sander (1959)
Lens culinaris (lentil)	Larsen and Myers (1998)
Medicago sativa (lucerne)	McLaughlin and Boykin (1988) Rahman and Soberg (1988) Rahman and Peaden (1993) Al-Shahwan *et al.* (2016)
Sonchus oleraceus (Common sowthistle)	Al-Shahwan *et al.* (2017)
Cucurbita maxima (Sqash)	Al-Shahwan *et al.* (2017)
Solanum tuberosum (Potato)	Al-Shahwan *et al.* (2017)
Phaseolus vulgaris (french bean)	Zitikaite and Staniulis (1989) Fletcher *et al.* (2016)
Pisum sativum (field pea)	Sander (1959) Staniulis and Zitikaite (1989) Zitikaite and Staniulis (1989) Edwardson and Christie (1991) Baggett and Kean (1992) Larsen *et al*. (1996) Khan and Singh (1997a and b) Fletcher *et al.* (2016)
Trifolium alpestre (purple globe clover)	Alconero (1983)
T. ambiguum (Kura clover)	Alconero (1983)
T. hybridum (alsike clover)	Sander (1959) McLaughlin and Boykin (1988)
T. incarnatum (crimson clover)	Sander (1959) McLaughlin and Boykin (1988)
T. pratense (red clover)	Osborn (1937) Hagedorn and Walker (1949) Sander (1959) Stuteville DL (1964) Musil *et al*. (1977) Khan *et al*. (1978)

Host	**Reference**
	McLaughlin *et al*. (1989) Zitikaite and Staniulis (1989) Edwardson and Christie (1991) Brunt *et al*. (1997)
Trifolium repens (white clover)	Sander (1959) Gibbs *et al*. (1966) Weber and Hampton (1980) McLaughlin *et al*. (1989) McLaughlin and Ensign (1989) McLaughlan *et al*. (1992) Sherwood (1997)
T. subterraneum (subterraneum clover)	McLaughlin and Boykin (1988)
T. vesiculosum (arrow leaf clover)	McLaughlin and Boykin (1988)
Vicia faba var *major* (broad bean)	Sander (1959) Edwardson and Christie (1991) Fletcher *et al.* (2016)
Vigna unguiculate (cowpea)	Al-Shahwan *et al.* (2016)

8.2 Indicator plant species and reaction to RCVMV

Indicator Species	Symptoms	Reference
Chenopodium album	Local lesions on inoculated leaves. Not systemic.	Bos *et al*. (1972)
C. amaranticolor	Some isolates cause chlorotic local lesions on inoculated leaves. Not systemic.	Gibbs *et al*. (1966) Varma (1970) Bos *et al*. (1972)
C. quinoa	Some isolates cause chlorotic local lesions on inoculated leaves. Not systemic.	Gibbs *et al*. (1966) Varma (1970) Bos *et al*. (1972)
Gomphrena globosa	Purple spreading local lesions. Systemic in plants kept in dark for 2 days before inoculation.	Varma (1970)
Melilotus alba	Systemic vein clearing and mild mottle.	Varma (1970)
Nicotian glutinosa	Mosaic	Al-Shahwan *et al.* (2016)
Pisum sativum	Stunting of plant, veinal chlorosis and curling of young leaves. Pinpoint or ring-like necrotic lesions followed by wilting and death of inoculated leaves.	Gibbs *et al*. (1966) Varma (1970) Bos *et al*. (1972)
Trifolium dubium	Systemic vein clearing and mild mottle.	Varma (1970)
T. hybridum	Systemic vein clearing and mild mottle.	Varma (1970)
T. pratense	Vein mosaic and mottle in young leaves.	Varma (1970) Fletcher *et al.* (2016)
T. repens	Symptomless.	Gibbs *et al*. (1966) Fletcher *et al.* (2016)
Vicia faba	Chlorotic local lesions on inoculated leaves followed by abscission. Chlorotic mottle in tip leaves which may become cupped then abscised.	Gibbs *et al*. (1966) Varma (1970)
V. sativa	Brown necrotic local lesions. Sometimes systemic.	Varma (1970)

9 DIAGNOSTICS PROCEDURES TO SUPPORT SURVEILLANCE

9.1 Introduction

RCVMV is seedborne in faba beans, field peas and red clover and non-persistently transmitted by aphids in all affected crops (Kraft *et al*. 1998; Osborn 1937; Sander 1959; Weber and Hampton 1980). Introduction to new regions and primary transmission into leguminous crops such as faba beans, field peas and red clover is most likely to occur through virus contaminated seeds (Fletcher *et al.* 2016).

Secondary spread within the crop and to other susceptible crops and weeds occurs through aphid transmission from infected plants to primary and alternative hosts and transmission from remnant contaminated seed from previous crops to seedlings and subsequent aphid transmission to other hosts. In perennial crops, such as red clover, secondary spread could lead to increasing and high prevalence in subsequent years.

Disease surveillance will assist identification of RCVMV in the field. However, similar symptoms associated with other biotic and abiotic factors or mixed infections with other viruses leading to different symptomatology could confound a symptoms-based diagnosis. Therefore, a laboratory-based confirmation of RCVMV infection using RT-PCR or ELISA is required and both are amenable to larger sample numbers required for surveillance (Table 1). There are currently no in-field surveillance methods for detection of RCVMV.

Table 1 Diagnostic tools for the detection of RCVMV.

Method	**Identification level**	**Identification Confidence**	**Deployment (Field/Lab)**	**Required Time**	**Throughput (No. of samples)**
Morphological (Symptoms) (Section 3)	To Genus	Low (<90%)	Field/Laboratory	<1 d - >1 d	Low (1-10)
Microscopic Examination (Section 4.1)	To Genus	Medium (90-99%)	Laboratory	<1 week	Low (1-10)
RT-PCR (Section 4.3)	To Species/Strain	High (99%+)	Laboratory	>1 d	High (100 samples)
ELISA (Section 4.2)	To Species	Medium (90-99%)	Laboratory	<1 week	Very High (1000 samples)

9.2 Sampling

9.2.1 Crop inspection

Crops should be monitored for symptoms associated with RCVMV infected plants:

- Clover: Chlorosis of leaf veins and veinlets, including vein mosaics; chlorosis of leaf tissue immediately adjacent to the veins (Figures 1, 2 section 3) (Khan *et al.* 1978).
- Pulses: Peas, faba beans, chickpeas and lentils may show tight apical rosetting (Figure 4), leaf curling, small leaves that may be wrinkled, folded upward and systemic marked vein clearing (Figure 3) (Hagedorn and Walker 1949). Similar symptoms have since been reported in (Sander 1959, Gibbs *et al*. 1966, Larsen *et al*. 1996, Larsen and Myers 1998). Some pea cultivars develop necrotic stem streaking followed by irregular yellowing and premature plant death. Faba beans might show chlorotic lesions or general chlorosis, tip mottle and abscission (Gibbs *et al*. 1966). Common vetch (*Vicia sativa*) may show necrotic local lesions, which sometimes become systemic (Stuteville 1964, Varma 1970).
- Some cultivars may be symptomless and some strains of RCVMV may be latent or cause mild symptoms in the field (Bos *et al.* 1972, Fletcher *et al*. 2016).

Due to the short distance over which aphids are likely to transmit the virus, the closest relative host crops should be systematically sampled in a 'W' pattern moving away from the infected crop.

9.2.2 Field sampling

Where possible collect symptomatic plants. Field sampling should involve picking virus like (3-4) young leaves for (PCR) or young petioles put in storage bags and labelled clearly followed by storing them in cooler box or bulk of 10 samples for ELISA testing. Information such as symptomatic hosts, asymptomatic hosts, host stage growth should be recorded accurately. Fresh material should be handed carefully in cool temperatures to avoid degradation of plant material which consequently might yield poor quality/degraded RNA. Seed testing should include 400 randomly selected seeds and preferably >2000 seeds should be tested.

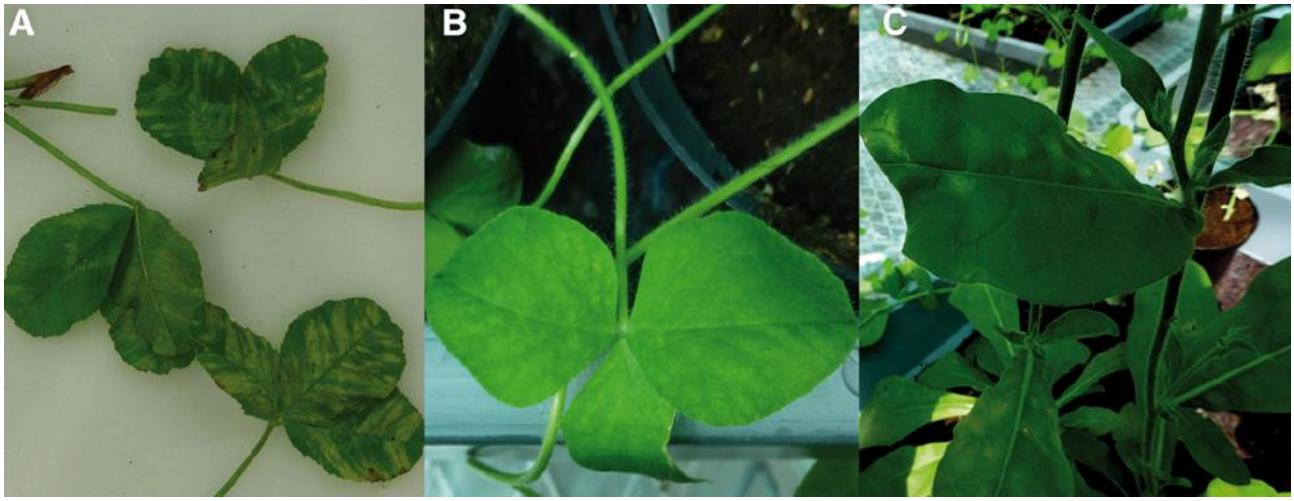

Figure 1 Symptoms of *Red clover vein mosaic virus* (RCVMV, NZ isolate) on different host plants. A, Symptoms of interveinal chlorosis (RCVMV) and later found to be infected with other two viruses, *Alfalfa mosaic virus* (AMV), and *White clover mosaic virus* (WClMV). B, RCVMV symptoms on *Trifolium incarnatum* displaying mild mottling. C, RCVMV symptoms on *Nicotiana occidentalis* showing chlorotic ring spots (Fletcher *et al*. 2016).

9.3 In Field Tests

There are no field based tests currently available for RCVMV.

9.4 Laboratory Tests

Laboratory methods for the detection of RCVMV include Direct DA-ELISA and Reverse transcription polymerase chain reaction (RT-PCR; sections 4.2 and 4.3).

9.5 Acknowledgements

This section was written and prepared by Solomon Maina and David Lovelock, Agriculture Victoria.

9.6 References

Bos L, Maat DZ, Markov M (1972) A biologically highly deviating strain of red clover vein mosaic virus, usually latent in pea (*Pisum sativum*), and its differentiation from pea streak virus. *Netherlands Journal of Plant Pathology* **78**, 125–152.

Fletcher J, Tang J, Blouin A, Ward L, MacDiarmid R, and Ziebell H. (2016) Red clover vein mosaic virus—A novel virus to New Zealand that is widespread in legumes. *Plant Disease*. **100**:890-895.

Gibbs AJ, Varma A, Woods RD. (1966) Viruses occurring in white clover (*Trifolium repens* L.) from permanent pastures in Britain. *Annals of Applied Biology* **58**, 231–240.

Hagedorn DJ, Walker JC (1949) Wisconsin pea stunt, a newly described disease. *Journal of Agricultural Research* **78**, 617–626.

Khan MA, Maxwell DP, Smith RR. (1978) Inheritance of resistance to red clover vein mosaic virus in red clover. *Phytopathology* **68**, 1084–1086.

Kraft JM, Larsen RC, Inglis DA (1998) Diseases of pea. In 'The pathology of food and pasture legumes'. (Eds DJ Allen and JM Lenne) pp. 325-370. (CABI & IRCISAT, New York).

Larsen RC, Kaiser WJ, Wyatt SD (1996) First report of a virus disease of chickpea caused by a strain of red clover vein mosaic carlavirus. *Plant Disease* **80**, 709.

Larsen RC, Myers JR. (1998) First report of red clover vein mosaic carlavirus naturally infecting lentil. *Plant Disease* **82**, 1064.

Osborn HT. (1937) Vein-mosaic of red clover. *Phytopathology* **27**, 1051–1058.

Sander E (1959) Biological properties of red clover vein mosaic virus. *Phytopathology* **49**, 749–754.

Varma A, Gibbs AJ, Woods RD (1970) A comparative study of red clover vein mosaic virus and some other plant viruses. *Journal of General Virology* **8**, 21–32.

Weber KA, Hampton RO (1980) Transmission of two purifies carlaviruses by the pea aphid. *Phytopathology* **70**, 631–633.