

National Diagnostic Protocol

Cherry leaf roll virus (CLRV)
for cherry and walnut strains



NDP 10 V2

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- 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.

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

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Document status

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Further information

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1 INTRODUCTION

Cherry leaf roll virus (CLRV), a member of genus *nepovirus*, was first reported in 1933 in English walnut (*Juglans regia*: Schuster & Miller 1933) and Sweet cherry (*prunus avium*). Since then CLRV has been reported throughout the world infecting many deciduous trees, shrubs, small fruits, ornamentals and herbaceous plants.

Walnut blackline disease is caused by the walnut strain of CLRV and results in a fatal necrosis of the graft between susceptible, infected scions of the English/Persian walnut (*Juglans regia*) and hypersensitive, resistant rootstocks. Leaf roll and tree decline were observed in sweet cherry trees infected with CLRV.

1.1 Transmission

CLRV has no natural vector and is readily transmitted by grafting and by seed and pollen in several host species. There is evidence that at least some strains may be transmitted in pollen to the plant pollinated (Jones 1985). CLRV is seed-borne in most natural and herbaceous hosts tested, including American elm, *Betula* spp., *Prunus* spp., rhubarb, *Sambucus* spp. and walnut. The elm mosaic and birch strains are transmitted both through pollen and through the ovule to the seed of their natural hosts. Some reports suggest that CLRV can be transmitted by water (Buttner 2011)

Although CLRV shows many features of nepoviruses, it appears not to be transmitted by nematodes. Transmission of cherry isolates by the soil-inhabiting nematodes *Xiphinema coxi*, *X. diversicaudatum* and *X. vuittenezi*, has been reported (Fritzsche and Kegler 1964). However, more recent studies have not been able to confirm this report (Cooper and Edward 1980; Jones *et al.* 1981). Nor were these strains transmitted by *X. americanum*, *X. bakeri*, *Longidorus elongates*, *L. leptcephalus*, *L. macrosoma* or *Paralongidorus maximus* (Cooper and Edward 1980; Jones *et al.* 1981). The elm mosaic strain is not transmitted by *X. americanum* or by the aphid *Myzus persicae* (Ford *et al.* 1972). CLRV has been detected in the bug *Cleidocerys resedae* but its role in transmission to seedlings requires further investigation (Werner *et al.* 1997).

1.2 Host range

The natural host range of CLRV is wide and varied, with each of the natural host isolates having a wide experimental host range in more than 36 plant families (Schmelzer 1966; Hansen and Stace-Smith 1971; Horvath 1979). Some natural hosts and symptoms of CLRV are listed in Table The full host range is available on many web sources, including plant viruses online, CAB abstracts, NCBI sequence data or Plantwise Technical Factsheet (NZ).

Susceptible host species are found in the following plant families: *Amaranthaceae*, *Apiaceae*, *Apocynaceae*, *Betulaceae*, *Campanulaceae*, *Caprifoliaceae*, *Caryophyllaceae*, *Chenopodiaceae*, *Commelinaceae*, *Compositae*, *Cornaceae*, *Cruciferae*, *Cucurbitaceae*, *Juglandaceae*, *Labiatae*, *Leguminosae-Papilionoideae*, *Malvaceae*, *Oleaceae*, *Pedaliaceae*, *Polemoniaceae*, *Polygonaceae*, *Ranunculaceae*, *Rosaceae*, *Rutaceae*, *Scrophulariaceae*, *Solanaceae*, *Tropaeolaceae*, *Ulmaceae*.

The following species were susceptible to experimental CLRV infection: *Antirrhinum majus*, *Apium graveolens*, *Atriplex hortensis*, *Berteroa incana*, *Beta macrocarpa*, *Beta vulgaris*, *Betula*, *Capsicum*

frutescens, Catharanthus roseus, Celosia argentea, Celosia cristata, Chenopodium amaranticolor, Chenopodium foetidum, Chenopodium murale, Chenopodium quinoa, Commelina, Cornus florida, Cucumis melo, Cucumis sativus, Cucurbita maxima, Cucurbita pepo, Delphinium elatum, Glycine max, Gomphrena globosa, Helianthus annuus, Juglans regia, Lathyrus odoratus, Lavatera trimestris, Lobelia erinus, Momordica balsamina, Nicotiana benthamiana, Nicotiana clevelandii, Nicotiana debneyi, Nicotiana megalosiphon, Nicotiana occidentalis, Nicotiana rustica, Nicotiana tabacum, Ocimum basilicum, Olea europaea, Petunia x hybrida, Phaseolus vulgaris, Phlox drummondii, Physalis floridana, Physalis peruviana, Pisum sativum, Prunus avium, Prunus cerasifera, Prunus persica, Ptelea trifoliata, Raphanus sativus, Rheum rhaponticum, Rubus idaeus, Rumex obtusifolius, Sambucus racemosa, Senecio vulgaris, Sesamum indicum, Spinacia oleracea, Stellaria media, Tinantia erecta, Torenia fournieri, Tropaeolum majus, Ulmus americana, Zinnia elegans.

1.3 Effect on hosts

Symptoms vary with the hosts (Table 1). Trees infected with CLRV will produce yields of late-ripening fruit and inevitably the tree will die in four or five years.

Table 1. Symptoms of cherry leaf roll virus on some hosts

Botanical name	Common name	Symptoms	Source
<i>Betula pendula</i>	Silver birch	Chlorotic ringspot, leaf patterns, yellow vein netting.	Schmelzer 1972a; Cooper and Atkinson 1975
<i>Cornus florida</i>	Flowering dogwood	Chlorotic ringspot, leaf patterns, yellow vein netting.	Waterworth and Lawson 1973
<i>Fagus sylvatica</i>	Common or European Beech	Severe leaf rolling, leaf yellowing and premature leaf loss	Werner <i>et al</i> 1997
<i>Juglans regia</i>	English walnut	Leaf pattern and black line, terminal shoot dieback	Savino <i>et al.</i> 1977; Cooper and Edward 1980; Mircetich <i>et al.</i> 1980
<i>Prunus avium</i>	Sweet cherry	Leaf rolling and death. Flower pedicels shortened.	Cropley 1961; Schimanski <i>et al.</i> 1975
<i>Ptelea trifoliata</i>	Hop tree	Yellow spotting	Schmelzer 1972b
<i>Rubus</i> spp.	Bramble spp	Chlorotic ringspot, leaf patterns, yellow vein netting.	Cropley and Tomlinson 1971; Jones 1976; Jones and Wood 1978.
<i>Sambucus nigra</i>	Black elder	Chlorotic ringspot, leaf patterns, yellow vein netting.	Schimanski and Schmelzer 1972; Hansen and Stace-Smith 1971
<i>Solanum acaule</i>	wild potato	Chlorosis, necrotic lesions	Crossin <i>et al</i> 2010
<i>Tropaeolum majus</i>	Nasturtium	Leaf mosaic, deformation, vein necrosis	Marais <i>et al</i> 2010
<i>Ulmus americana</i>	American elm	Chlorotic mosaic, ring pattern and dieback	Mayhew and Epstein 1971
<i>Delphinium elatum</i>	English larkspur	Symptomless	Ahmed and Bailiss 1975
<i>Olea europaea</i>	Olive	Symptomless	Savino and Gallitelli 1981
<i>Rheum rhaponticum</i>	Rhubarb	Symptomless	Tomlinson and Walkey 1967
<i>Rumex obtusifolius</i>	Bitter dock	Symptomless	Walkey and Cooper 1973

2 TAXONOMIC INFORMATION

Genomic organisation of CLRV is poorly understood, but morphology and particle composition is typical for nepoviruses, which are classified within the family of Secoviridae.

Order:	Picornavirales
Family:	Secoviridae
Subfamily:	Comovirinae
Genus:	Nepovirus
Species:	<i>Cherry leaf roll virus</i>

Common names: elm mosaic, ash mosaic virus, sambuccus ringspot, yellow net virus, cherry leaf roll, walnut blackline disease.

2.1 Taxonomic description

CLRV has a bipartite single-stranded positive-sense RNA genome, estimated to be about 15 kb in length. RNA-1 and RNA-2 are estimated to be about 8.2 and 6.8 kb respectively (Murant *et al.* 1981) and both RNAs are separately encapsidated in isometric particles (Fig 1). (Jones 1985). Particles are isometric, c. 28 nm in diameter, angular in outline. A proportion of the particles is penetrated by negative stain and these presumably correspond to the T component.

The genomic RNAs have a genome-encoded protein (VPg), covalently linked at their 5' terminus and are polyadenylated at their 3' terminus (Brooks *et al.* 1995; Hellen and Cooper 1987). CLRV belongs to the subgroup C nepoviruses and are characterized by a large, separately encapsidated RNA-2 with a long (1.2 to 1.6 kb) 3' non-coding region which is almost identical to that of RNA-1 (Rebenstorf *et al.* 2006).

Original taxonomic description: Schuster and Miller (1933).

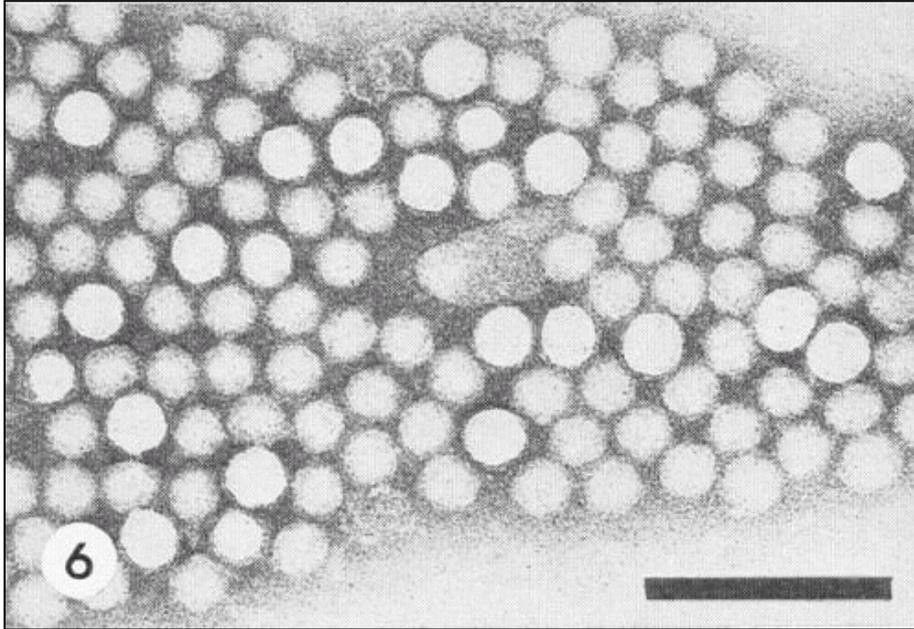


Figure 1 Virus particles from a purified preparation in phosphotungstate. Bar represents 100 nm. (Jones 1985)

2.2 Strains of the disease

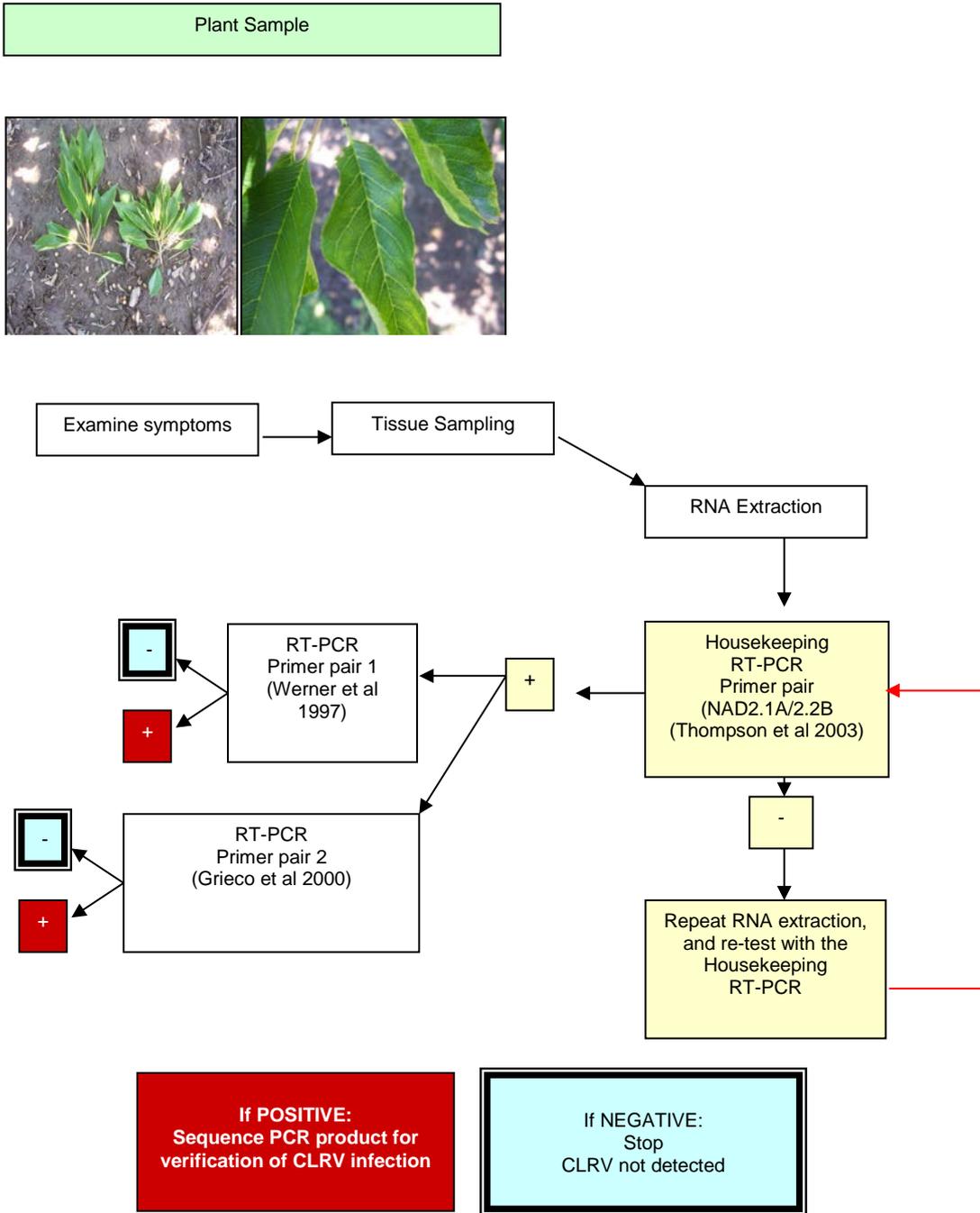
Many strains have been reported. Isolates from different natural host species are serologically distinguishable from each other but most isolates from a single host species are not. However, recent reports suggest that mix diversity was observed in birch leaf roll disease Rumbou *et al.* (2016). Isolates that cannot be differentiated serologically may differ in virulence both in natural and experimental hosts. The following are the most studied strains:

- Type (cherry) strain (Cropley 1961)
- Elm mosaic strain (Varney and Moore 1952)
- Rhubarb strain (Tomlinson and Walkey 1967)
- Golden elderberry strain (Hansen and Stace-Smith 1971)
- Red elder ringspot strain (Schmelzer 1972*b*)
- Dogwood ringspot strain (Waterworth and Lawson 1973)
- Birch strain (Cooper and Atkinson 1975)
- Walnut ringspot and walnut yellow vein strains (Savino *et al.* 1977)
- Blackberry and red raspberry strains (Jones 1985; Cropley and Tomlinson 1971; Jones and Wood 1978)

The walnut strain of CLRV is the causative agent of blackline disease, a fatal necrosis of the graft union of English walnut trees.

3 DETECTION

3.1 Diagnostic flow chart



3.2 Symptoms

Symptoms associated with CLRV can be difficult to diagnose, as the expression of the symptoms is dependent on host species, CLRV strain and climatic conditions. Diagnosis which is based on symptoms alone is not advised. Symptoms should be used as a guide only.

Foliar symptoms of CLRV on cherries include yellowing and chlorosis of leaves; leaf rolling; and bunching of shoots (Figures 2-10). CLRV causes blackline disease in walnuts; this symptom is clearly demonstrated at the graft union of walnut trees (Figures 11-13). A healthy graft union is depicted in . Necrosis at the graft union can lead to dieback of branches and a general decline of the CLRV-infected tree.

Infected experimental hosts mainly show symptoms of chlorotic or necrotic local lesions and a systemic necrosis or mosaic on the leaves (Figures 15-17). These symptoms can vary depending on the species of experimental host used, the strain of CLRV and greenhouse conditions. Local and systemic lesions are symptomatic of CLRV infection on *Chenopodium amaranticolor* and *C. quinoa*.

Other pathogens that cause similar symptoms include:

- *Armillaria mellea* - causes Armillaria root rot in many plant species
- *Pseudomonas mors-prunorum* - causes bacterial canker in stone fruit trees
- *Pseudomonas syringae* - bacterial canker
- Western X disease phytoplasma



Figure 2. Cherry tree infected with CLRV showing yellowing and low vigour foliage with shortened and bunchy shoots, USA (Source: B. Rodoni).



Figure 3. Rolling of cherry leaf margins caused by CLRV, USA (Source: B. Rodoni).



Figure 4. Chlorosis/yellowing and puckering of CLRV-infected cherry leaves, USA (Source: B. Rodoni).



Figure 5. Shortening of internodes of a cherry shoot infected with CLRV, USA (Source: B. Rodoni).



Figure 6. Light green veins and general chlorosis of a CLRV-infected cherry leaf, USA (Source: B. Rodoni).



Figure 7. Rough margins of the midvein of a cherry leaf infected with CLRV, USA (Source: B. Rodoni).



Figure 8. Chlorosis and curling of leaf margin of a CLRV infected cherry Leaf (right) compared to a healthy cherry leaf (left), USA (Source: B. Rodoni).



Figure 9. Veins are coarse and whitish with dark green areas along the midrib on the lower leaf surface and enlarged, yellow-green veins. These symptoms are more evident on small, older leaves. Photo: Washington State University.

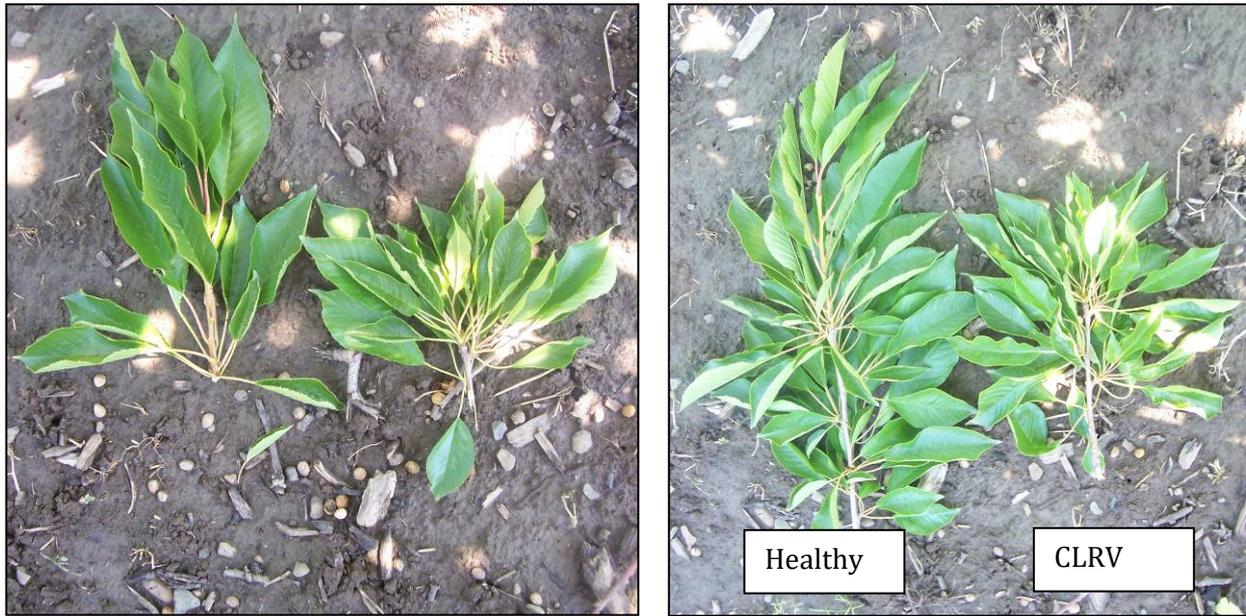


Figure 10. Comparisons of a healthy cherry shoot compared to a CLRV-infected cherry shoot. Note the increased bunchiness (shortened internodes) of the CLRV-infected shoot and the rolling of the CLRV-infected leaves, USA (Source: B. Rodoni).



Figure 11. Death of an infected walnut tree. (Picture courtesy of John M. Mircetich, USA)

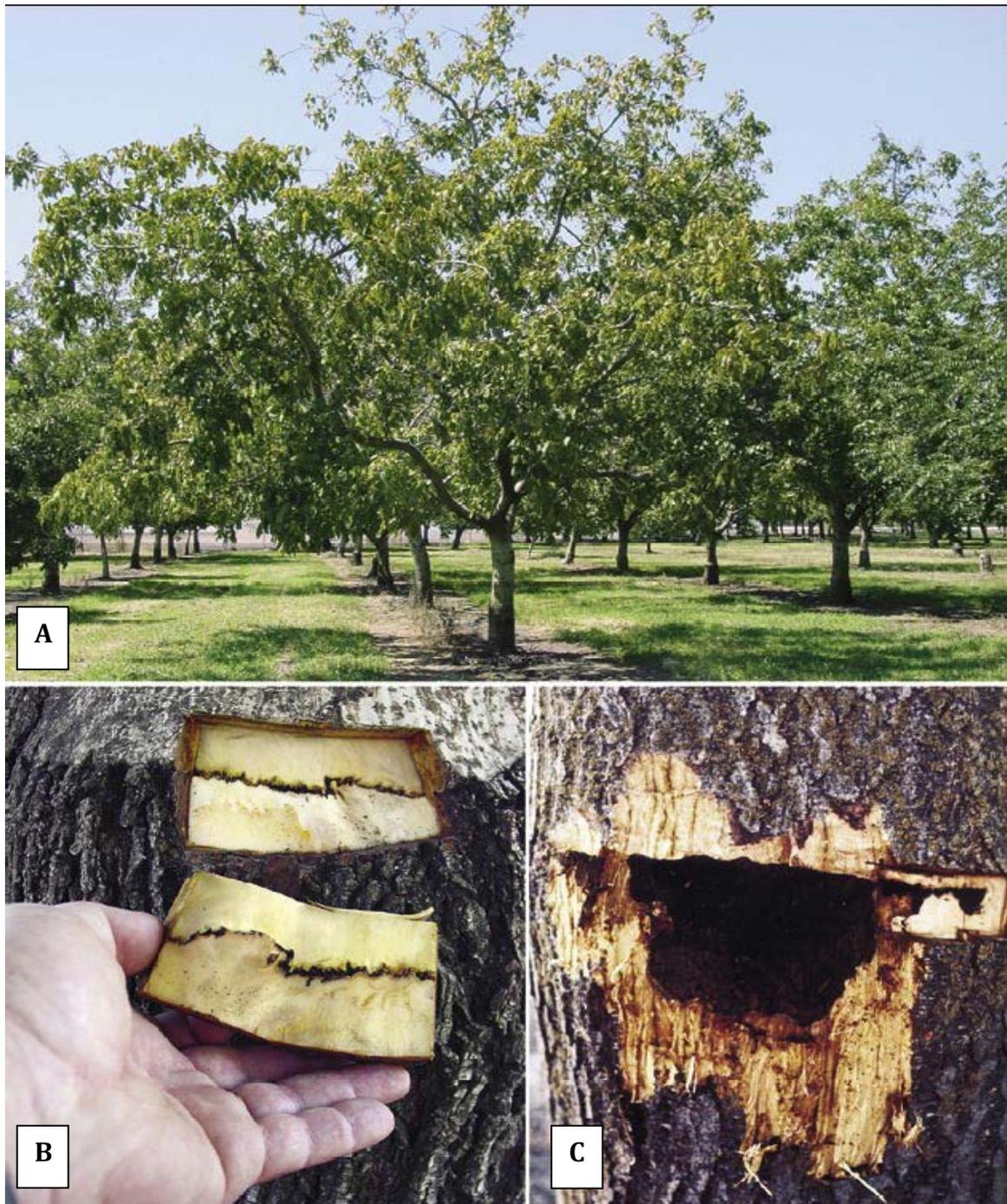


Figure 12. (a) Walnut trees with blackline disease caused by CLRV decline in vigour, leading to dieback of branches; (b) Blackline symptoms on CLRV-infected trees on black walnut, and, (c) 'Paradox' rootstocks are slowly girdled by the death of rootstock tissue at the graft union. (Pictures courtesy of Grant Joseph A; Mcgranahan Gale H; California agriculture (Calif. agric.) ISSN 0008-0845). Trees on English rootstocks develop no graft union symptoms and escape the debilitating effects of blackline infection.



Figure 13. Typical symptom on walnut tree. Bark has been removed to show the line of black dead tissue at the graft union (Used with permission from University of California Statewide IPM Program. Copyright, for use see <http://www.ipm.ucdavis.edu/GENERAL/copyright.html>). Source: John Mircetich, University of California



Figure 14. A healthy walnut tree. The bark was removed to show the absence of dark discoloration at the graft union (Used with permission from University of California Statewide IPM Program. Copyright, for use see <http://www.ipm.ucdavis.edu/GENERAL/copyright.html>). Source: Jack Kelly Clark, University of California



Figure 15. CLRV symptoms showing local necrotic lesions with concentric rings in *Nicotiana tabacum* cv. White Burley. (Photo: National Vegetable Research Station, Wellesbourne, Warwickshire, England.)

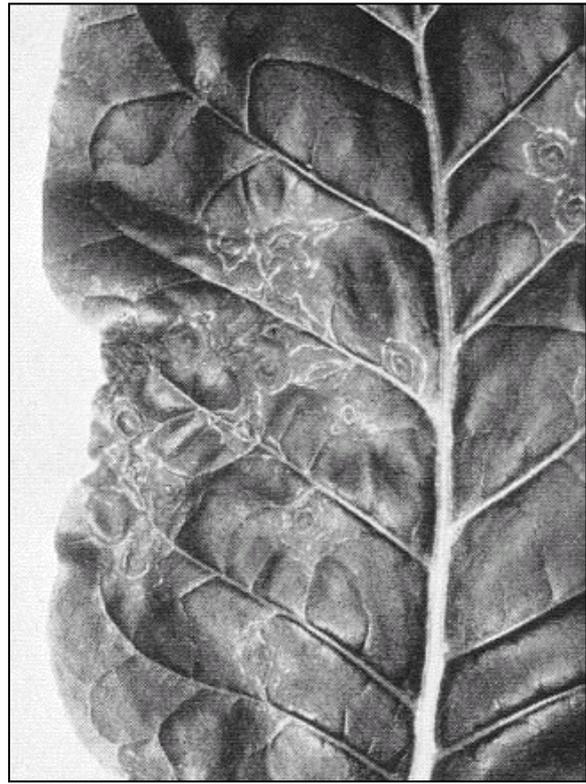


Figure 16. Systemic symptoms in *Nicotiana tabacum* cv. White Burley. (Photo J. A. Tomlinson National Vegetable Research Station, Wellesbourne, Warwickshire, England)

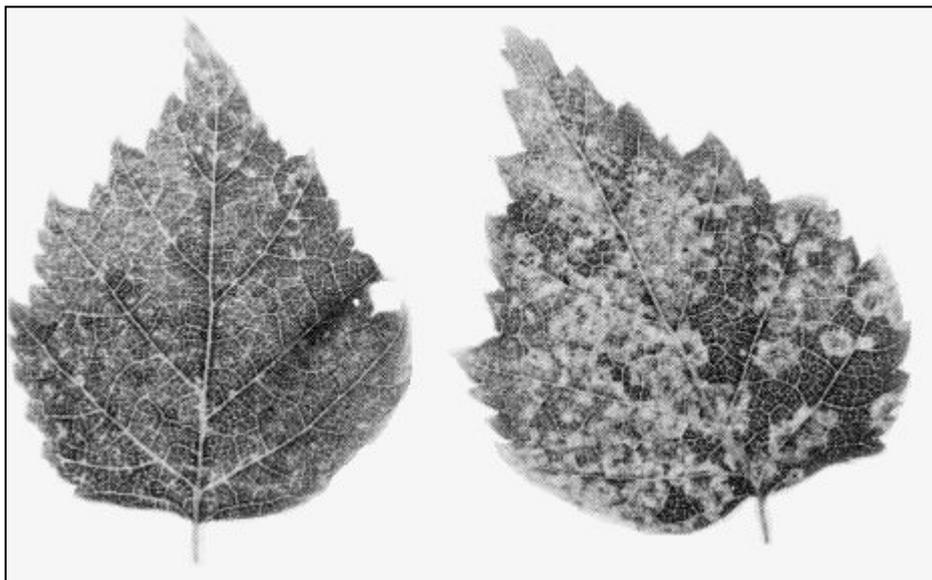


Figure 17. Systemic chlorotic mottle, ringspots and line patterns in leaves of *Betula pendula* following mechanical inoculation. (Photo: J.I. Cooper.)

3.3 Sample collection

The distribution of the virus in a tree can be irregular; however, the detection from flowers, young leaves, old leaves, fruit, dormant wood, and roots is possible. The time of year when the sample is taken is also critical and can greatly affect the test results. Optimal sampling is during spring, or early summer, but can vary from season to season depending on the weather conditions.

CLRV has been reported to be easily detectable throughout the year in tissue of male inflorescences, leaf buds, leaves, single seeds and cortical tissues of young twigs (Werner *et al.* 1997). The virus can also be detected in roots, meristems and within tubules in pollen, ovules and mature seeds (Walkey and Webb 1968; 1970; Jones *et al.* 1973).

3.3.1 Methods

- Leaves and shoots showing CLRV symptoms should be collected. However, it should be kept in mind that many infections may be without symptoms.
- Select leaves from the middle of the branch, and leaves should be selected from branches located from all around the tree.
- Expanded leaves should be sampled from the lower middle third of the current year's growth (i.e. towards the middle of the branch). Ideally collect 100 mm lengths of the shoot/branch stem to maximise tissue selection for RNA extraction.
- Collected leaves and budwood should be stored at 4°C or at -20°C during shipping and prior to testing.

4 IDENTIFICATION

4.1 Morphological methods

4.1.1 Indicator plants and culture maintenance

Nicotiana tabacum cv. White Burley or Xanthi-*nc* and *Chenopodium amaranticolor* are good local lesion hosts (Jones 1985) (Table 2). *Nicotiana rustica* or *N. tabacum* cvs White Burley and Xanthi-*nc* are suitable for maintaining virus cultures.

Table 2 Diagnostic hosts for CLRV

Host species	Symptoms
<i>Chenopodium amaranticolor</i> , <i>C. quinoa</i>	Chlorotic or necrotic local lesions (Fig. 19); systemic mottle, necrosis and malformation
<i>Cucumis sativus</i>	Chlorotic local lesions, occasional systemic mosaic
<i>Nicotiana rustica</i> , <i>N. tabacum</i> cvs White Burley, Xanthi- <i>nc</i>	Necrotic local lesions and rings; systemic necrotic or chlorotic rings (Figs 16, 17, 20).

Note: Mechanical transmission methods are not reliable for virus identification since more than one virus can cause the same symptoms and symptom development on indicator plants can be affected by environmental factors.

4.2 Biochemical methods

4.2.1 Serology

In gel double diffusion tests the virus produces one band of precipitate and in spring it can be detected by this means in cherry sap (**Figure 18**). Enzyme-linked immunosorbent assay (ELISA) has been used to detect infection in tissues of natural and experimental hosts (Jones 1985).

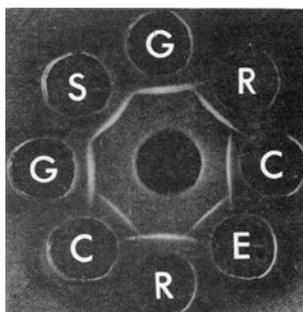


Figure 18. Agarose gel double diffusion plate.

4.3 Molecular methods

4.3.1 Total RNA extraction

Equipment required

1. 2-20 μ L, 20-200 μ L, and 200-1000 μ L micropipettes and sterile tips
2. Autoclave
3. Autoclave bags
4. Balance (at least 2 decimal places)
5. Disposable gloves
6. Microcentrifuge
7. Sterile microcentrifuge tubes
8. Paper towel
9. RNeasy® Plant Mini Kit (Qiagen™) (Kingsnorth *et al.* 2003)
10. Sharps container
11. Sterile scalpel blades and scalpel blade handle
12. Water bath or heat block set at 70°C
13. Weigh boats
14. Plastic disposable pasteur pipettes
15. An autoclaved mortar and pestle or a Homex tissue macerator and Homex bags (Bioreba AG/BioSys)

Reagents required

1. MacKenzie Extraction buffer (MacKenzie *et al.* 1997)

Chemical	Amount	Final Concentration
Guanidine thiocyanate ($\text{CH}_5\text{N}_3\cdot\text{CHNS}$)	23.64 g	4 M
3M Sodium acetate ($\text{C}_2\text{H}_3\text{NaO}_2$)	3.33 mL	0.2 M
0.5M EDTA ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$)	2.5 mL	25 mM
PVP-40 (Polyvinylpyrrolidone)	1.25 g	2.5% (w/v)

Add sterile distilled water to final volume of 50 mL

Store at room temperature

Please note, fresh MacKenzie buffer should be prepared every 3-6 months

2. 100% β -mercaptoethanol ($\text{C}_2\text{H}_6\text{OS}$)
3. 20% **N-Lauroylsarcosine solution (w/v)**
4. 100% Ethanol

Method

The RNA extraction method is based on that described by MacKenzie *et al.* (1997). All steps are carried out at room temperature and are as follows:

1. Weigh out 200 mg of bark/phloem tissue from the petiole and shoot stems. (Leaf material can be used also)
2. Place sample in mortar/homex bag.
3. Add 1980 μL of MacKenzie buffer.
4. Add 20 μL of β -mercaptoethanol in the fumehood.
5. Homogenise in fume hood.
6. Pipette 1.0 mL of the slurry into a labelled microcentrifuge tube (you may need to cut the end of the pipette tip if the slurry is too thick).
7. Add 100 μL of 20% Sarkosyl to each tube and mix.
8. Incubate tubes at 70°C for 10 minutes.
9. Spin tubes in microcentrifuge for 1 minute at 13,000 rpm.
10. Using approximately 500 μL of the homogenate, continue with step 4 of the “RNeasy® Plant Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi” and follow as per manufacturer's instructions. (p75 of the RNeasy® Plant Mini Handbook , p52 of the online catalogue April 2006)

4.3.2 Detection of CLRV in total RNA extracts using RT-PCR.

For the detection of CLRV, total RNA extracts should be assayed using the primers and annealing temperatures listed in **Table 3**. Each primer is used as a stock solution at a concentration of 10 μM .

Primers

Two RT-PCR primers are recommended for the detection of CLRV.

1. CLRV5-CLRV3 primer pair described by Werner *et al.* (1997) amplifies a 416 bp product of the CLRV3'end UTR region.
2. CLRV-1-CLRV-2 primer pair described by Grieco *et al.* (2000) amplifies a 431 bp region of the CLRV coat protein gene.

The Nad2.1A/Nad2.2b primer pair as described by Thompson *et al.* (2003) is used as an internal PCR control to: a) determine the quality of the RNA extract and b) determine whether the RNA extract contains inhibitors which may interfere with the activity of the reverse transcriptase and Taq DNA polymerase enzymes. It has a product size of 188 bp.

The Primer sequences and PCR conditions used for each primer set are provided in **Table 3**.

Table 3. Primers required for the detection of Cherry leaf roll virus

Primer	Sequence (5'-3')	PCR Cycling Conditions	Product size
CLRV-5 ¹	5'-TGGCGACCGTGTAACGGCA-3' sense	1 cycle [48°C 30 min], 1 cycle [94°C 4 min], 35 cycles [94°C 30 sec, 55°C 45 sec, 72°C 45 sec], 1 cycle [72°C 7 min]	416 bp
CLRV-3 ¹	5'-GTCGGAAAGATTACGTAAAAGG-3' antisense		
CLRV-1 ²	5'-TTGGCGACCGTGTAACGGCA-3'	1 cycle [48°C 30 min], 1 cycle [94°C 4 min], 35 cycles [94°C 30 sec, 55°C 30 sec, 72°C 30 sec], 1 cycle [72°C 7 min]	431 bp
CLRV-2 ²	5'-GTCGGAAAGATTACGTAAAAGG-3'		
Nad2.1a	GGACTCCTGACGTATACGAAGGATC	1 cycle [48°C 45 min], 1 cycle [94°C 2 min], 35 cycles [92°C 30 secs, 55°C 45 secs, 72°C 45 secs], 1 cycle [72°C 7 mins]	188 bp
Nad2.2b	AGCAATGAGATTCCCAATATCA T		

¹ Werner *et al.* (1997); ² Grieco *et al.* (2000); ³ Thompson *et al.* (2003)

Equipment required

1. 0-2 µL, 2-20 µL, 20-200 µL, and 200-1000 µL micropipettes and sterile tips
2. 0.2 or 0.5 mL sterile PCR tubes
3. Bulb spinner or centrifuge
4. Disposable gloves
5. Freezer
6. Gel electrophoresis tanks, rigs and racks
7. DNA Molecular Weight markers
8. Ice
9. Sterile microcentrifuge tubes to store reagents
10. Microwave
11. Power pack
12. Thermocycler
13. UV transilluminator with camera

PCR Controls

1. Positive control - RNA extract from plant tissue infected with CLRV.

- Alternatively a “plasmid control” that has the target CLRV sequence cloned into the plasmid

2. Negative plant control - RNA extract from uninfected plant tissue of the same species as that used for the positive control.
3. Negative buffer control - an aliquot of the RT-PCR “Master Mix” without template.
4. The house keeping RT-PCR, using primers Nad2.1a – Nad2.2b, indicates the potential for false negative results. The generation of a band confirms the presence of RNA in the extract, and that the RNA extract does not contain inhibitors. Failure to produce an amplicon of expected size (188 bp) indicates that either dilution of the RNA extract or re-extraction of RNA from the sample is required.

RT-PCR reagents

1. One-step RT-PCR kit (Invitrogen® SuperScript™ One-Step RT-PCR with Platinum® Taq, Catalogue No. 12574-026, is recommended)

2. Nuclease-free water

3. 5x TBE Buffer

Per 1 litre

Tris (C ₄ H ₁₁ NO ₃)	54 g
Boric acid (H ₃ BO ₃)	27.5 g
0.5M EDTA [(CH ₂ .N(CH ₂ .COOH).CH ₂ COONa] ₂ .2H ₂ O) pH 8.0	20 mL

Store at room temperature.

4. 1% Agarose gel with ethidium bromide

Use a 1% DNA grade agarose gel made with 0.5x TBE solution, and stained with 0.03 µg/mL Ethidium bromide.

5. 1x TE Buffer

Per 100 mL

1 M Tris-HCl (pH 8.0)	1 mL
0.5 M EDTA	200 µL

Adjust pH to 8.0± 0.2. Store at room temperature.

6. 6x loading dye

Per 100 mL

1 x TE	10 mL
Glycerol (Sigma 200-289-5)	50 mL
Bromophenol blue (Sigma 263-653-2)	0.2%

Store at room temperature.

Method

This method is to be repeated for each set of the three primer pairs listed in **Table** Use one-step RT-PCR reagents as specified by the manufacturer. Some volumes outlined below may vary depending on

the buffer and enzyme concentrations specified by the manufacturer. Ensure that the final volume of the RT-PCR reaction is 25 µL by altering the volume of nuclease-free water accordingly.

1. Label sterile PCR tubes

2. Prepare "Master Mix" on ice in a sterile microcentrifuge tube.

The "Master Mix" usually contains buffer, forward and reverse primers, RT/Taq and nuclease-free water.

Prepare the "Master Mix" according to the RT/Taq manufacturer's recommendations.

Ensure that the final volume for each reaction is 24 µL.

Add 24 µL of "Master Mix" to each PCR tube.

3. Add 1µL of each template (total RNA extract) to each corresponding PCR tube.

4. Cycle the tubes using the RT-PCR conditions listed in Table 3.

5. At completion of the RT-PCR, mix 10 µL of each reaction with 2 µL of 6x gel loading dye, and load samples into a 1% agarose gel containing 0.03 µg/mL ethidium bromide.

6. Electrophorese in 0.5 x TBE at 100V for 45 minutes or until the bromophenol blue front has migrated half way down the length of the gel.

7. Visualise and photograph gel on UV transilluminator.

8. Criteria for determination of a valid assay: 188 bp product from samples containing plant extracts, no product in no template and negative plant extract controls, product of the expected size in positive control.

Criteria for positive sample result: Product of the expected size in test sample, obtained from a valid assay.

4.3.3 DNA Sequencing of PCR Products

Equipment required

1. 0-2 µL, 2-20 µL, 20-200 µL, and 200-1000 µL micropipettes and tips
2. 0.2 or 0.5 mL PCR tubes
3. 1.5 or 2 mL centrifuge tubes to store reagents
4. Bulb spinner or centrifuge
5. Freezer
6. Ice machine
7. Latex gloves
8. PC with Internet access
9. QIAQuick® PCR Purification Kit (Qiagen™)
10. Thermocycler
11. UV illuminator

Reagents

ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems (www.appliedbiosystems.com))

Forward and Reverse primers (As per section 4.3.2).

Sterile dH₂O.

Method

PCR products are cleaned using the QIAquick Spin kit (Qiagen) as per manufacturer's instructions. The purified PCR products are prepared for sequencing with ABI Big Dye (Roche), as per the manufacturer's instructions. Sequencing is out-sourced to Monash University, Melbourne University or similar provider. The DNA sequences are compared against sequences on the GenBank database using the program BlastN (Altschul *et al.* 1997), to confirm if the positive PCR product sequence is from CLRV.

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7.2 Useful websites

CLRV descriptions – Plant Viruses on line

<http://sdb.im.ac.cn/vidе/dеscr198.htm>

Cherry leafroll virus (CLRV) - Washington State University

http://cpcnw.wsu.edu/fruit-trees__trashed/fruit_trees_research/apricot-chloriotic-leaf-roll/cherry-leaf-roll-clrv/

Descriptions of Plant Viruses - Cherry leaf roll virus.

<http://www.dpvweb.net/dpv/showdpv.php?dpvno=306>