A STUDY OF LUTEOVIRUSES INVOLVED IN POTATO LEAFROLL DISEASE

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Abstract

In total, 801 samples of potato leafroll disease were collected and tested for potato leafroll virus (PLRV) and beet western yellows virus (BWYV) in 1986, 1987, and 1988 using triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) and virus-specific monoclonal antibodies. The samples represented 32 cultivars and originated in eight Canadian provinces and 12 American states. None of the samples tested positive for BWYV, whereas 772 (96.4%) tested positive for PLRV. Neither PLRV nor BWYV could be recovered, with aphid transfers to indicator hosts, from 28 of the 29 samples that tested negative for both viruses. PLRV was recovered from one sample that originally tested negative by TAS-ELISA; the indicator plant tested positive for PLRV by TAS-ELISA.

Nucleic acid spot hybridization (NASH) using random primed and cloned cDNA probes was compared with double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and TAS-ELISA, and aphid transmission tests for detection and identification of PLRV and BWYV in 165 potato leafroll disease samples. All of the samples tested negative for BWYV with each of the assay procedures. PLRV was detected in all of the samples with TAS-ELISA, NASH with a cloned cDNA probe for PLRV, and with aphid transmission to ground cherry (*Physalis*

pubescens). Both DAS-ELISA and NASH using random primed cDNA produced one false-negative result. Shepherd's purse (*Capsella bursa-pastoris*) was a host for 72% (119/165) of the PLRV isolates.

The susceptibility of potato to BWYV was tested by inoculating Russet Burbank with three isolates of BWYV from Canada and four from the United States. Two of the isolates were in a mixed infection with PLRV. None of the isolates were transmitted by *Myzus persicae* to virus-free potato plants, either by themselves or in association with PLRV.

Common weeds were surveyed in the potato-producing areas of British Columbia for PLRV and BWYV. In total, 10,098 weed samples, representing 98 species in 22 plant families, were collected and tested by TAS-ELISA from 1986 to 1989. BWYV was detected in 1% of the samples; the hosts were: chickweed, common groundsel, heart-podded hoary cress, hedge mustard, little western bittercress, prickly lettuce, shepherd's purse, and wild radish. PLRV was detected in three volunteer potato plants, two samples of shepherd's purse, and one black nightshade plant. The low incidence of PLRV in plants other than potato indicates that weeds are of minor importance in the epidemiology of potato leafroll disease in British Columbia.

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List of Abbreviations

A_{260} - absorbance at 260 nm

A_{280} - absorbance at 280 nm

A_{405} - absorbance at 405 nm

AAT - a selection medium for growing hybridoma cultures

ai - active ingredient

ATP (dATP) - adenosine triphosphate (deoxy adenosine triphosphate)

BALB/c - a mouse line used for producing hybridomas

BLRV - bean leafroll virus

bp - base pair

BMYV - beet mild yellowing virus

BMYV-324 - a sugar beet isolate of BMYV from England

BW/PL-5.2 - a mixed infection of BWYV and PLRV in ground cherry

BWYV - beet western yellows virus

BWYV-12H - a common groundsel isolate of BWYV from British Columbia, Canada

BWYV-210E - a common groundsel isolate of BWYV from British Columbia, Canada

BWYV-40-8003 - a *Malva* isolate of BWYV from Tasmania

BWYV-150 - a broccoli isolate of BWYV from California, USA

BWYV-B10 - a beet isolate of BWYV from Beltsville, USA

BWYV-BC - sugar beet isolate of BWYV from British Columbia, Canada

BWYV-CA - a *Malva* isolate of BWYV from Oceanside, California, USA

BWYV-D3 - an isolate of BWYV from the state of Washington, USA

BWYV-MP4BF - a crucifer isolate of BWYV from Germany

BWYV-MY4 - a *Malva* isolate of BWYV from California, USA

BWYV-NETH - a lettuce isolate of BWYV from the Netherlands

BWYV-RB2 - a broccoli isolate of BWYV from California, USA

BWYV-RY-7 - a broccoli isolate of BWYV from California, USA

BWYV-RY-1-R - a broccoli isolate of BWYV from California, USA

BWYV-SAL(9) - a lettuce isolate of BWYV from Germany

BYDV - barley yellow dwarf virus

BYDV-MAV-NY - the New York type-strain of BYDV-MAV that is transmitted specifically by *Sitobion (=Macrosiphum) avenae*

BYDV-RPV-IL - a RPV isolate of BYDV that is specifically transmitted by *Rhopalosiphum padi*

BYDV-RPV-NY - the New York type-strain of BYDV-RPV that is specifically transmitted by *Rhopalosiphum padi*

BYDV-RPV-T - a RPV isolate of BYDV from Tasmania that is specifically transmitted by *Rhopalosiphum padi*

C - Celsius

cDNA - complementary DNA

CLRV - carrot redleaf virus

cm (mm) - centimeter (millimeter)

CsCl - cesium chloride

DAS-ELISA - double antibody sandwich enzyme-linked immunosorbent assay

DNA - deoxyribonucleic acid

DMEM - Dulbecco's Modified Eagle Medium

ds - double-stranded

ds-RNA - double stranded RNA

EDTA - ethylenediaminetetraacetic acid

ELISA - enzyme-linked immunosorbent assay

EM - electron microscopy microscopy

FCS - fetal calf serum

FOX-NY - a myeloma cell line used to produce hybridomas

g (mg,µg,pg) - gram (milligram,microgram,picogram)

g - gravity

GPA - green peach aphid (*Myzus persicae*)

GRAV - groundnut rosette assistor virus

hr - hour

IgG - immunoglobulin G

IgM - immunoglobulin M

IPTT752 - a mixed infection of BWYV and PLRV from turnip in the state of Washington, USA

ISEM - immunosorbent electron microscopy (=immuno EM)

ISDV - Indonesian soybean dwarf virus

kb - kilobase

kDa - kilodalton

l (ml,µl) - liter (milliliter,microliter)

LYV - legume yellows virus

m (cm,mm,nm) - meter (centimeter,millimeter,nanometer)

M (mM) - molar (millimolar)

MAb - monoclonal antibody

MAV - see BYDV-MAV

MiAV - Michigan alfalfa virus

M-MLV - Moloney murine lukemia virus

MYV - Malva yellows virus

min - minute

M_r - molecular weight

NaCl - sodium cloride

NaOH - sodium hydroxide

NASH - nucleic acid spot hybridization

nt - nucleotide

ORF - open reading frame

PAV - a vector nonspecific isolate of BYDV (BWYV-PAV)

PBS - phosphate-buffered saline

PEG - polyethylene glycol

PeLRV - pea leafroll virus

PLRV - potato leafroll virus

PLRV-BC - an isolate of PLRV from British Columbia

PLRV-ID - an isolate of PLRV from Idaho

PLRV-ORE - an isolate of PLRV from Oregon

PLRV-ST4 - a severe isolate of PLRV from British Columbia

polyA - polyadenylate; an oligonucleotide formed entirely of adenosine residues

Pst 1 - a restriction enzyme isolated from *Providencea stuartii* that cuts at the recognition sequence GTGCAG

pUC9 - a plasmid cloning vector

PVP - polyvinylpyrrolidone

RBF/Dn - a mouse line used in the production of hybridomas

RNA - ribonucleic acid

RMV - a vector specific strain of BYDV (BYDV-RMV) that is transmitted by *Rhopalosiphum madis*

RPV - a vector specific strain of BYDV (BYDV-RPV) that is transmitted by *Rhopalosiphum padi*

RGV - rice guillaume virus

RY-1-R - see BWYV-RY-1-R

RYV - radish yellows virus

$s_{w,20}$ - sedimentation coefficient - the rate of sedimentation per unit centrifugal field measured in Svedberg units (S) and corrected to sedimentation in water at 20 C

SCLRV - subterranean clover redleaf virus

SDI - serological differentiation index

SDS - sodium dodecyl sulfate

SDV - soybean dwarf virus

SDV-AP - an isolate of SDV that is transmitted by *Acyrthosiphon pisum*

SDV-AS - an isolate of SDV that is transmitted by *Aulacorthum solani*

SGV - a vector specific strain of BYDV (BYDV-SGV) that is transmitted by *Schizaphis graminum*

SMYEV - strawberry mild yellow edge virus

ss - single-stranded

SSC - a buffer containing 0.015 M sodium chloride and 0.015 mM trisodium citrate

SSPE - a buffer containing 0.15 M sodium chloride, 0.01 M sodium phosphate, and 1 mM EDTA

SYV - Solanum yellows virus

SYV-21 - isolate 21 of SYV from Beltsville, USA

SYV-44 - isolate 44 of SYV from Beltsville, USA

TAS-ELISA - triple antibody sandwich enzyme-linked immunosorbent assay

TNDV - tobacco necrotic dwarf virus

Tris-HCl - tris(hydroxymethyl)aminomethane - hydrochloric acid

TuYV - turnip yellows virus

TYTV - tomato yellow top virus

$\mu Em^{-2}s^{-1}$ - microergs per square meter per second

UV - ultraviolet

vol - volumn

VPg - genome-linked protein

X-gal - 5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Chapter 1

Introduction

1.1 Luteoviruses

The luteovirus group was first recognized by the International Committee on Taxonomy of Viruses in 1975 (Shepherd *et al.*, 1976). The group name is from the Latin "luteus", meaning yellow, because infected plants tend to show yellowing symptoms (Casper, 1988). Other characteristic symptoms are reddening, leaf rolling or curling, and brittleness, although some luteoviruses are latent in many hosts (Waterhouse, Gildow, and Johnston, 1988). Luteoviruses occur worldwide in many important crops and can cause substantial economic losses; barley yellow dwarf virus (BYDV), beet western yellows virus (BWYV), and potato leafroll virus (PLRV) are the most important members of this group (Rochow and Duffus, 1981).

Luteovirus taxonomy is in a state of flux. The number of definitive members has changed several times, from three in 1975 to 15 in 1982 (Matthews, 1982) and then back to 14 in 1985 (Francki, Milne, and Hatta, 1985). The definitive members of the group are listed in Table I and closely related strains and synonyms are listed under each member. The classification I have presented in Table I lists nine of the ten definitive

members selected by Waterhouse *et al.* (1988). The tenth, BYDV-RPV, is grouped as a strain of BWYV as suggested by Casper (1988) because of its strong serological relationship to BWYV (Rochow, 1984; Rochow and Duffus, 1978). This grouping is also supported by investigations of cytopathological effects (Gill and Chong, 1979a,b) and nucleic acid hybridization studies showing homology between BWYV and the RPV isolate of BYDV (Martin and D'Arcy, 1990). The number of definitive luteoviruses will likely decrease again in the future when a more complete evaluation of the group can be made using multiple traits, including host range, serology, nucleic acid homologies, and vector relationships (Martin *et al.*, 1990; Waterhouse *et al.*, 1988).

Casper (1988) and Waterhouse *et al.* (1988) have recently reviewed the properties of the luteovirus group. Luteoviruses have isometric particles about 25 nm in diameter, will sediment as a single component between 104 and 118 S, and have a buoyant density of about 1.40 g/cm^3 in CsCl. The coat protein subunits are composed of a single polypeptide of M_r about 24 x 10^3. The genome is a single molecule of positive sense, single-stranded RNA of M_r approximately 2.0 x 10^6 with a genome-linked protein (VPg), M_r about 7.0 x 10^3 for PLRV and 17.0 x 10^3 for BYDV, and no polyA tail (Mayo *et al.*, 1982; Murphy, D'Arcy, and Clark, 1989). The virions have high A_{260}/A_{280} ratios of between 1.6 and 1.9, an RNA content of about 28-30%, and are strongly immunogenic.

Table I. Definitive members of the luteovirus group[a]

Members	Reference
Barley yellow dwarf (BYDV) MAV	Rochow (1984)
BYDV isolates PAV and SGV	
Bean leafroll (BLRV)	Ashby (1984)
Pea leafroll (PeLRV)	Ashby & Johnstone (1985)
Legume yellows (LYV)	Ashby (1984)
Michigan alfalfa (MiAV)	Thottappilly *et al.* (1977)
Beet western yellows (BWYV)	Rochow & Duffus (1981)
BYDV isolates RPV and RMV	Rochow (1984)
Beet mild yellowing (BMYV)	Govier (1985)
Malva yellows (MYV)	Duffus (1972)
Radish yellows (RYV)	Duffus (1960, 1972)
Rice giallume virus (RGV)	Osler (1984)
Turnip yellows (TuYV)	Duffus (1972)
Carrot red leaf (CRLV)	Waterhouse & Murant (1982)
Groundnut rosette assistor (GRAV)	Casper *et al.* (1983)
Indonesian soybean dwarf (ISDV)	Rajeshwari & Murant (1988)
Potato leafroll (PLRV)	Harrison (1984)
Solanum yellows (SYV)	Milbrath & Duffus (1978)
Tomato yellow top (TYTV)	Thomas (1984)
Soybean dwarf virus (SDV)	Tamada & Kojima (1977)
Subterranean clover red leaf (SCRLV)	Ashby & Johnstone (1985)
Strawberry mild yellow edge (SMYEV)	Martin & Converse (1985)
Tobacco necrotic dwarf (TNDV)	Kubo (1981)

[a]Waterhouse, Gildow, & Johnstone, 1988.

Luteoviruses are transmitted by aphids in the persistent (circulative and nonpropagative) manner (Eskandari, Sylvester, and Richardson, 1979). Generally, luteoviruses have a high degree of vector specificity; each being transmitted efficiently by one or a few species of aphid. Aphids acquire luteoviruses while feeding in the phloem tissue of infected plants (Leonard and Holbrook, 1978). Using electronic monitoring, Leonard and Holbrook (1978) demonstrated that the green peach aphid (GPA), *Myzus persicae* Sulz., could aquire PLRV in as little as 1-2 min once the stylets had penetrated and remained in the sieve elements. Ultrastructural studies indicate that luteovirus particles pass through the hindgut by cellular transport into the aphid's haemocoel where they then circulate in the haemolymph (Gildow, 1985). Following endocytosis into the aphid's accessory salivary gland and transport within coated vesicles through the salivary gland secretory cells, the virus particles are released by exocytosis into the salivary duct (Gildow, 1985). Transmission probably occurs during the egestion phase of feeding (Leonard and Holbrook, 1978). Efficient transmission of most luteoviruses requires acquisition and inoculation feeding periods of 24 hr each. A minimum latent period (defined as the time from the start of the acquisition feeding until the aphid can infect plants with the virus) is usually between 12 and 24 hr (Duffus, 1977).

None of the luteoviruses has been transmitted by mechanical inoculation or by seed (Rochow and Duffus, 1981)

Until recently, little was known about the molecular biology of luteoviruses because the particles are phloem-limited, scarce, and difficult to purify. Miller *et al.* (1988a) determined the complete nucleotide sequence of a BYDV-PAV serotype and its genome organization was deduced from the open reading frames (ORF's). The cistron closest to the 5' end encodes the putative RNA-dependent RNA polymerase, the coat protein cistron is near the middle of the genome, and there is a possible translational readthrough of the coat protein ORF to produce a 69 kd protein.

The nucleotide sequence of the genomic RNA (5641 nt) of BWYV has recently been determined and its genomic organization proposed (Veidt *et al.*, 1988). BWYV has six long ORF's and a cluster of three of these, including the coat protein cistron, have extensive amino acid sequence homology with the corresponding ORF's of BYDV-PAV (Veidt *et al.*, 1988; Miller *et al.*, 1988b). The putative polymerase regions, in contrast, are distinct from one another; the BWYV RNA polymerase ORF resembles that of southern bean mosaic, whereas that of BYDV-PAV appears to share common ancestry with carnation mottle virus (Veidt *et al.*, 1988; Miller, Waterhouse and Gerlach, 1988). Kozak (1986) suggested that RNA viruses may evolve by exchanging functional

sequence modules with one another; this mechanism could explain the juxtaposition of similar and different ORF's in BWYV and BYDV-PAV (Veidt *et al.*, 1988). It is now clear that recombination between viral RNA molecules is not a rare event, indeed it is now considered to be one of the major mechanisms of RNA virus evolution (Goldbach and Wellink, 1988). Mayo *et al.* (1989) reported the nucleotide sequence of PLRV; its genome organization is very similar to that of BWYV. Significant amino acid sequence homology exists between all of the analogous ORF's in BWYV and PLRV except in the first ORF. The genome organization of BWYV and PLRV is distinct from BYDV-PAV by having an additional ORF at the 5' end; there is no corresponding ORF in the BYDV-PAV genome (Martin *et al.*, 1990). The sequences of four different isolates of PLRV have been compared by Keese *et al.* (1990). Although the isolates originated from widely separated locations (Australia, Canada, Netherlands, and Scotland), they were closely related with more than 93% sequence homology. It appears there is little diversity in PLRV isolates worldwide.

Subgenomic RNA's have been detected in nucleic acid extracts from PLRV-infected potato (M_r 1 x 10^6) and from BYDV-PAV infected oats (*c.* 0.8 and 2.8 kb) (Barker, Mayo, and Robinson, 1984; Gerlach, Miller, and Waterhouse, 1987). Gildow, Ballinger, and Rochow (1983) reported five species of double-stranded RNA (ds-RNA) in tissue infected with BYDV-RPV and four

from tissue infected with BYDV-MAV. Falk and Duffus (1984) found five ds-RNA's in nucleic acid extracts from plants infected with BWYV. Incomplete translation of PLRV and SDV RNA *in vitro* and the presence of subgenomic RNA in plants infected with these viruses suggests that luteoviruses may be translated via subgenomic messenger RNA species (Waterhouse, Gildow and Johnstone, 1988). A recent review on the evolution and molecular biology of luteoviruses (Martin *et al.*, 1990) suggests several different gene expression strategies for this group including internal initiation, translational frameshifting, and subgenomic RNA for expression of ORF's in the 3' half of the genome.

1.2 Luteoviruses infecting potato

PLRV, the causal agent of potato leafroll disease, occurs worldwide and is economically the most important virus affecting potato crops (Harrison, 1984). Recently Duffus (1981a,b) has reported that BWYV is a common and major component in the potato leafroll disease complex in North America. Isolates were recovered from potatoes with leafroll symptoms from widely separated locations including British Columbia, California, Maine, Oregon, and Wisconsin. They reacted serologically in a similar manner to BWYV isolates obtained from beets, crucifers, and composites but they differed in specific serological

reactions. In a similar study, Duffus and Johnstone (1982a) concluded that the leafroll syndrome in Tasmania was essentially the same as it is in North America. They suggested that, since strict quarantine measures had been enforced in Tasmania since the early 1930's, BWYV had already spread with the movement of potato tubers throughout the world before the establishment of certification schemes and quarantine restrictions. For example, Kyrikou, Close, and Ashby (1983) found potatoes to be infected in the field with a New Zealand isolate of BWYV.

The properties of BWYV and PLRV are compared in Table II. It is clear from this comparison that these viruses are similar. The most apparent difference between them is their host range. BWYV has the widest host range of the luteoviruses, and causes stunting and chlorosis in a wide range of dicotyledonous species including many economic hosts such as broccoli, broad bean, cabbage, cauliflower, chick pea, clover, crambe, cucumber, flax, mustard, oilseed rape, pea, pepper, pumpkin, radish, spinach, soybean, sugar beet, sunflower, swede, table beet, tomato, turnip, and watermelon. Over 150 species in 23 dicotyledonous families are susceptible to BWYV (Duffus, 1960,1964,1972,1977; Duffus and Johnstone, 1982b) as are at least a few monocotyledonous ones (Ashby and Johnstone,1985; Duffus and Rochow, 1978).

Table II. Comparison of the properties of beet western yellows virus (BWYV) and potato leafroll virus (PLRV)

	BWYV	PLRV
Main host families	Amaranthaceae[6] Chenopodiaceae Compositae Cruciferae Leguminosae Solanaceae	Amaranthaceae[7] Solanaceae
Main aphid vector	*Myzus persicae*	*M. persicae*
Serological properties[8]		
Close serological relationship SDI 0-3	BMYV TuYV MYV BYDV-RPV	TYTV SYV TNDV
Moderate serological relationship SDI 4-6	SDV GRAV	BWYV GRAV SDV
Distant serological relationship SDI > 6	BLRV CRLV PLRV SMYEV	BLRV CLRV
Particle properties		
Sedimentation coefficient ($S_{20,w}$)	114[1]	115[2]
Diameter (nm)	25[1]	24[2]
Protein subunits M_r ($x10^3$)	24[1]	26.3[3]
$A_{260/280}$	1.65[1]	1.78[2]
Density in CsCl (g/cm^3)	1.42[1]	1.39[3]
Genome properties		
ssRNA M_r ($x10^6$)	1.9[1]	2.0[3]
size (nt)	5641[4]	5883[5]

[1] Hewings & D'Arcy, 1983
[2] Takanami & Kubo, 1979
[3] Rowhani & Stace-Smith, 1979
[4] Veidt *et al.*, 1988
[5] Keese *et al.*, 1988
[6] Duffus, 1960,1964
[7] Natti, Kirkpatrick & Ross, 1953
[8] Waterhouse, Gildow & Johnstone, 1988

In contrast, most of the known hosts of PLRV (about 20 species) are in the Solanaceae (Harrison, 1984). A few species in the Amaranthaceae, Cruciferae, Portulacaceae, and Nolanaceae are susceptible to PLRV (Natti, Kirkpatrick, and Ross, 1953; Thomas,1984; Tamada, Harrison and Roberts, 1984).

The question of which viruses are responsible for causing potato leafroll disease is of more than theoretical interest. North American seed potato certification programs are based on an extensive testing program to ensure that the elite stock is virus-free (Stace-Smith, 1987; Martin, 1987). Currently the reliance is on serological testing using antiserum prepared against PLRV. The occurrence of BWYV isolates in potato stocks raises serious questions as to the validity of any currently used serological indexing procedures (Duffus, 1981a). Further, BWYV has a very wide host range, and many common weeds in the Cruciferae and Compositae serve as overwintering sources of BWYV (Wallis, 1967a,b). It is possible that the extensive natural host range of BWYV could act as a reservoir of inoculum in potato-producing and even in isolated seed potato-producing areas (Duffus, 1981a).

1.3 Definition of the problem

Duffus (1981a) used three lines of evidence to demonstrate the occurrence of BWYV in potato plants affected with potato leafroll disease:

(1) Indicator hosts for BWYV and PLRV. He used two indicator hosts, ground cherry (*Physalis floridana* Rydb. =*P. pubescens* L.) and shepherd's purse (*Capsella bursa-pastoris* [L.] Medic.). These two were chosen because PLRV and BWYV cause similar reactions in ground cherry, whereas shepherd's purse is a commonly used indicator for BWYV. At the time Duffus (1981a,b) reported his work, shepherd's purse was not known to be a host for PLRV. Duffus found that five potato sources with leafroll symptoms had naturally occurring isolates that infected shepherd's purse.

(2) Serological tests. Virus isolates from infected shepherd's purse were clarified by low-speed centrifugation and pelletted by ultracentrifugation. Pellets were resuspended in buffer to approximately 3.0% of the original volume of sap. The resulting virus samples were incubated for 0.5 hr at 37 C with equal volumes of antisera prepared against strains of BWYV, legume yellows luteovirus (LYV), and healthy

shepherd's purse sap as a control. The incubated mixtures were subjected to density-gradient centrifugation and analyzed photometrically. The reduction or elimination of virus antigen in the scanning patterns was considered a positive test for BWYV. Duffus found that virus isolates from potato leafroll-affected plants reacted serologically the same as other BWYV isolates and LYV, but not with antisera to healthy shepherd's purse. He also found that antiserum to PLRV reacted with all BWYV strains tested. Duffus maintained that the reciprocal reaction, BWYV antiserum against PLRV isolates, was negative in all tests except the distant relationship demonstrated by Roberts, Tamada, and Harrison (1980) but he did not include this control in his experiment.

(3) Host range studies. Duffus found that the host range of one isolate from leafroll-affected potato (P5) was similar to that of other isolates of BWYV isolated from crucifers and composites.

Duffus' conclusion that BWYV is widely distributed in North America is supported by the work of Sibara and Slack (1985b) who reported that 65% of 519 potato leafroll-affected plants were infected with both BWYV and PLRV, 4% with BWYV alone, and 16%

with PLRV alone. Neither BWYV nor PLRV was detected in 15% of the symptomatic samples and they suggested that a significant component of the potato leafroll complex was not detected by their antisera. These samples represented 18 cultivars originating from Wisconsin, Maine, Minnesota, Nebraska, and North Dakota. Gallenberg, Zitter, and Jones (1987) reached similar conclusions when they detected BWYV in potato leafroll samples from nine states and provinces in North America.

In contrast to evidence that supports Duffus' work are several studies that question the importance or involvement of BWYV as a causal agent of potato leafroll disease. Clarke, Powelson and Beraha (1983) failed to detect BWYV in Russet Burbank potatoes affected with net necrosis. Foliage sampled from plants with leafroll symptoms also were negative for BWYV but were positive for PLRV. Marco (1984) reported that isolates of BWYV from Israel were not able to infect potato or several other members of the Solanaceae. Barker (1986) tested several potato cultivars for susceptibility to two British isolates of BWYV. Neither isolate, either by itself or in association with PLRV, was transmitted by GPA to virus-free potato plants. Barker concluded that although local strains do not present any identified risk to the potato crop in Britain, tests for BWYV are already included in quarantine procedures for potatoes imported into Britain. The results of ISEM (immunosorbent electron microscopy) studies indicate that BMYV, a strain of

BWYV, is closely related serologically to Scottish isolates of PLRV (Roberts and Harrison, 1979). Richter *et al.* (1983) compared PLRV and BMYV serologically by agar gel double diffusion, ELISA, and immuno-EM decoration. Nine antisera prepared against PLRV and three against BMYV were tested by agar gel double diffusion against both viruses. In each case a cross-reaction was obtained, titers being lower with the homologous than the heterologous virus by a factor of 3-5, indicating a distant serological relation. Decoration tests performed in the same way gave equivalent results. Reactions were weaker for the heterologous reaction than for the homologous one. However, in ELISA no cross-reaction was found with 1-100 µg/ml of the heterologous virus, whereas the detection limit for the homologous virus was 10 ng/ml. Marco (1985) found in ISEM tests that PLRV and BWYV could be easily detected by coating grids with either homologous or heterologous antiserum. He also demonstrated that PLRV and BWYV could be trapped on ELISA plates with either homologous or heterologous antiserum. Both results clearly indicate serological cross-reactivity between PLRV and BWYV. Tamada, Harrison, and Roberts (1984) suggest that cross-reactions between BWYV, BMYV, and PLRV in ELISA may account for at least some of the serological evidence that BWYV occurs in potato. The results of other tests with BWYV antiserum in which no relationship with PLRV (Duffus and Gold, 1969) or only a weak relationship was found (Roberts *et al.* 1980) may reflect antigenic variation in BWYV or possibly

the unavailability, at the time, of high-titered antisera (Tamada *et al.*, 1984).

In reaching his conclusions, Duffus (1981a,b) has made a number of assumptions which might be questioned:

> (1) Duffus assumed that virus isolates that are aphid transmitted from leafroll-affected potato to shepherd's purse must be BWYV and not PLRV because shepherd's purse was not known to be a host for PLRV at that time. Thomas (1981) found that an Australian isolate of tomato yellow top virus (TYTV), a strain of PLRV (Harrison, 1984; Casper, 1988), had a host range that was mainly restricted to the Solanaceae, but also infected shepherd's purse asymptomatically. Further, an isolate from leafroll-affected potato, which caused typical leafroll symptoms in potato and symptomless infection in shepherd's purse, reacted in gel diffusion tests with PLRV antiserum (Thomas, 1981). Syller (1985) found that six PLRV isolates, representing a range of symptom severity on both ground cherry and potato, all infected shepherd's purse but the frequency of successful infection depended on both the isolate and the source plant. His work also showed that the concentration of PLRV in potato is dependent on both the virus isolate and on

the susceptibility of the cultivar. Syller concluded that it is not necessary to assume the existence of two or more viruses, even closely related ones, in order to explain why the virus could be transmitted to shepherd's purse from some potato plants but not from others. Barker (1986) succeeded in transmitting a virus from leafroll-affected potatoes to shepherd's purse, but the virus was PLRV not BWYV. Fox *et al.* (1990) reported transmission of an isolate of PLRV to, and recovery from, two wild crucifers, shepherd's purse and *Sisymbrium altissimum* L. (Jim Hill or tumble mustard).

(2) The serological test Duffus used to confirm that the isolates transmitted to shepherd's purse were BWYV was based on the assumption that BWYV antiserum does not cross-react with PLRV even though in the reciprocal test PLRV antiserum cross-reacts with BWYV. The technique he used involved incubating semi-purified virus and antiserum mixtures, subjecting the mixture to density gradient centrifugation, and scanning the gradient photometrically. A positive assay was based on the reduction or elimination of the virus peak in the scanning patterns of density gradient columns. While this test would provide evidence that a virus-antiserum interaction had

occurred, it may not distinguish between a serologically related antigen and a serologically specific antigen (Stace-Smith, 1987). Since BWYV and PLRV are serologically related (Marco, 1985; Harrison, 1984; Thomas, 1984), a reduction in the scanning pattern in a virus-antiserum mixture where PLRV is incubated with an antiserum prepared against BWYV would be expected. Duffus' results could be interpreted as showing that isolates of PLRV, serologically related to BWYV, can be transmitted by aphids from potato to shepherd's purse (Stace-Smith, 1987). This last statement is supported by some of Duffus' own work; Milbrath and Duffus (1978) demonstrated that solanum yellows virus (SYV), now known to be a strain of PLRV (Casper, 1988; Waterhouse *et al.*, 1988), is serologically related to BWYV and BYDV-RPV using infectivity neutralization and ELISA tests.

(3) Host range study. Duffus (1981a) used shepherd's purse, infected with an isolate of BWYV (P-5) from potato as a source plant for the host range study, not the original infected potato plant. Because shepherd's purse is a good host for many strains of BWYV and is very attractive to the vector GPA, it is possible that the shepherd's purse may have become

infected with BWYV from a source other than potato. Transmission of a virus from potato directly to a host range typical of BWYV would have been more convincing. Casper (1983) did not succeed in attempts to transmit a virus from leafroll-affected potatoes to the hosts of BWYV and concluded that BWYV is probably not important as a cause of potato leafroll disease in Germany.

1.4 Objectives

The role of BWYV in potato leafroll disease has not been unequivocally determined. The effectiveness of control programs for this disease will be threatened if BWYV proves to be an important component of potato leafroll disease. Consequently, a study was undertaken to determine the importance of BWYV as a cause of potato leafroll disease in Canada and the United States.

The objectives of this research were:

(1) to produce monoclonal antibodies (MAbs) that react with BWYV but do not cross-react with PLRV;

(2) to determine the incidence of BWYV and PLRV in potato leafroll disease samples from several locations in Canada and the United States using virus-specific MAbs;

(3) to produce nucleic acid probes (cDNA) for BWYV and PLRV and compare nucleic acid hybridization methods with ELISA for detection of BWYV and PLRV;

(4) to determine the susceptibility of potato to several isolates of BWYV from Canada and the United States;

(5) and, to determine the importance of weeds as reservoirs of BWYV and PLRV in the potato-producing areas of British Columbia.

Chapter 2

Production of monoclonal antibodies

2.1 Introduction

Potato leafroll, caused by potato leafroll virus (PLRV), is a major disease of potato (Banttari, Ellis, and Khurana, 1990) and is responsible for high yield losses throughout the world wherever potatoes are grown (Peters and Jones, 1981). In 1981 Duffus (1981a) reported that beet western yellows virus (BWYV) is a common and important component of a virus complex responsible for this disease. Seed potato certification programs that rely on antisera prepared against PLRV to confirm the diagnosis of potato leafroll disease may not detect plants infected with BWYV (Marco, 1985; Stace-Smith, 1987). In the past, polyclonal antisera prepared against luteoviruses in rabbits often have had relatively high levels of nonspecific reaction (Clarke, Converse and Kojima, 1980; Martin and Stace-Smith, 1984) and those reactions can lead to false-positive results in ELISA (Gunn and Pares, 1988). Variability in the quality of polyclonal antisera can lead to disagreements among investigators working with the same antigen in different laboratories (Halk and DeBoer, 1985). Often, high quality reference or diagnostic polyclonal antisera are not produced in sufficient quantities for general distribution. Hybridoma

technology, introduced by Köhler and Milstein (1975), revolutionized antibody production and eliminated many of the problems associated with polyclonal antiserum. This technology provides a means to produce an unlimited, uniform supply of antibody of a required specificity (Goding, 1986). The objective of this research was to produce MAbs that could be used in an ELISA procedure to detect and differentiate BWYV and PLRV.

2.2 Materials and methods

2.2.1 Virus isolates and purification

The source of the PLRV isolate used for purification was a single tuber of the potato cultivar Russet Burbank infected with a severe strain of the virus (Wright and MacCarthy, 1963). This isolate was characterized by Rowhani and Stace-Smith (1979) and will be called PLRV-BC. PLRV-BC was propagated in *Physalis pubescens* L. (=*P. floridana* Rybd.) inoculated by viruliferous GPA in a growth chamber (21 C, 105 $\mu Em^{-2}s^{-1}$ fluorescent and incandescent light) for a 72 hr inoculation access period. The aphids were killed by spraying the plants with pirimicarb (0.25 g ai/l, Chipman Inc., Winnipeg, Manitoba) and the plants were moved to a greenhouse (15-25 C) for 4 to 6 weeks. Leaf tissue was harvested and used immediately or frozen at -80 C until

used. A sugar beet isolate of BWYV from British Columbia (MacCarthy, 1969) was used for virus purification and will be referred to as BWYV-BC. *P. pubescens* was inoculated with BWYV-BC and propagated in a separate growth chamber and greenhouse using the same procedure as for PLRV-BC. The luteovirus isolates used to determine the specificity of MAbs, and their sources are listed in Table III.

Both PLRV and BWYV were purified using a modification of the procedure used by D'Arcy *et al.* (1983). Virus-infected tissue was frozen in liquid nitrogen and crushed into small pieces with a wooden pestle. The frozen tissue was then transferred to a stainless steel blender jar, pre-cooled with liquid nitrogen, and ground to a fine powder. The powdered tissue was added to 0.1 M phosphate buffer pH 6.0 (2 ml/g of tissue) containing 0.1% 2-mercaptoethanol, 0.2% (w/v) Ultrazym 100 (Schweizerische Ferment AG, Basel, Switzerland), and 0.02% sodium azide. The mixture was stirred slowly overnight at 4 C.

Triton X-100 (1% v/v) was added and the preparation was stirred for 2 hr at room temperature followed by the addition

Table III. Luteovirus isolates used to determine specificity of monoclonal antibodies.

Virus isolate	Origin	Host	Source
BWYV-B10	Beltsville, USA	beet	J.E. Duffus[1]
BWYV-RB2	California, USA	broccoli	J.E. Duffus
BWYV-MY4	California, USA	*Malva sp.*	J.E. Duffus
BWYV-RY-1-R	California, USA	broccoli	J.E. Duffus
BWYV-150	California, USA	broad bean	J.E. Duffus
BWYV-RY-7	California, USA	broccoli	J.E. Duffus
BWYV-MP4BF	Germany	crucifer	J.E. Duffus
BWYV-SAL(9)	Germany	lettuce	J.E. Duffus
BWYV-NETH	Netherlands	lettuce	J.E. Duffus
BWYV-40-8003	Tasmania	ground cherry	G. Johnstone[2]
BWYV-BC	B.C., Canada	sugar beet	H. MacCarthy[3]
BMYV-324	England	sugar beet	J.E. Duffus
TuYV	England	turnip	J.E. Duffus
BYDV-RPV-NY	New York, USA	oats	B.W. Falk[4]
BYDV-MAV-NY	New York, USA	oats	B.W. Falk
BYDV-RPV-IL	Illinois, USA	oats	C. D'Arcy[5]
BYDV-RPV-T	Tasmania	oats	G. Johnstone
SDV-AS	Tasmania	clover	G. Johnstone
SDV-AP	Tasmania	clover	G. Johnstone
SYV-21	Illinois, USA	*Solanum sp.*	J.E. Duffus
SYV-44	Illinois, USA	*Solanum sp.*	J.E. Duffus
PLRV-BC	B.C., Canada	potato	N.S. Wright[3]
PLRV-ST4	B.C., Canada	potato	N.S. Wright
PLRV-ORE	Oregon, USA	potato	O. Gutbrod[6]
PLRV-ID	Idaho, USA	potato	R.G. Clarke[7]

[1] USDA, ARS, Salinas, California, USA.
[2] Tasmania Department of Agriculture, Hobart, Australia.
[3] Agriculture Canada, Vancouver Research Station, Vancouver, B.C., Canada.
[4] University of California, Davis, California, USA.
[5] University of Illinois, Urbana, Illinois, USA.
[6] Oregon State University, Corvallis, Oregon, USA.
[7] Idaho Crop Improvement Association, Idaho Falls, Idaho, USA.

of 1/10 vol of chloroform:butanol (1:1) with continued stirring for 30 min at room temperature. The emulsion was clarified by centrifugation at 13,200 x *g* for 20 min. Solid NaCl and polyethylene glycol 6000 (PEG) were added to the supernatant to give 0.4 M and 8% (w/v) respectively and the mixture was stirred for 1 hr at 4 C. The precipitate was recovered by centrifugation at 13,200 x *g* for 30 min and the pellets were resuspended with a glass homogenizer in 1/10th the original vol of 0.05 M phosphate buffer pH 7.0. The suspension was clarified by low speed centrifugation as above. The virus particles were pelleted by high speed centrifugation at 184,000 x *g* for 90 min through a 10 ml 20% sucrose cushion. The pellets were resuspended in 0.05 M phosphate buffer pH 7.0. Following one additional cycle of low and high speed centrifugation, the viruses were further purified on 10-40% linear sucrose density gradients, prepared by the freeze-thaw method of Davis and Pearson (1978), centrifuged in a Beckman SW 41 rotor at 178,000 x *g* for 90 min. Gradients were scanned photometrically with an ISCO UA-5 absorbance monitor and the fractions containing the virus were collected. The fractions were combined and diluted twofold with 0.05 M phosphate buffer pH 7.0 and then centrifuged at 290,000 x *g* for 2 hr. The pellets were resuspended in a small vol of 0.05 M, phosphate buffer pH 7.0 and stored at 4 C. The virus concentration was estimated from the UV-absorption spectra (220-320 nm, Hewlett-Packard Model 5451A spectrophotometer) assuming an extinction coefficient of $A_{260}^{0.1\%}$

= 8.6 based on the value calculated for PLRV by Takanami and Kubo (1979).

2.2.2 Antibodies

The rabbit antiserum against PLRV used in this study was prepared by Rowhani and Stace-Smith (1979). A rabbit antiserum against a Californian isolate of BWYV from broccoli (RY-1-R) was a gift from Dr. J. Duffus (USDA, ARS, Salinas, California). MAb (371A), specific for PLRV, was a gift from Dr. R. Martin (Vancouver Research Station, Agriculture Canada, Vancouver, B.C.).

Immunoglobulins were purified from antisera by ammonium sulfate precipitation and DEAE-cellulose column chromatography as described by Clarke, Lister, and Bar-Joseph, (1986). The immunoglobulins were adjusted to approximately 1 mg/ml (A_{280} =1.4) and stored in aliquots of 0.5 ml at -20 C.

2.2.3 Hybridoma production, screening and isotyping

Two immunization procedures were used: (1) RBF/Dn mice (Jackson Laboratories, Inc., Bar Harbour, Maine) were immunized with three injections of purified BWYV in 0.05 M phosphate

buffer pH 7.0. The first injection, 50 µg of BWYV emulsified with Freund's incomplete adjuvant, was given subcutaneously. The second and third injections of 50 µg each were administered intraperitoneally at 4 and 6 weeks following the first injection. Three days following the third injection, the mice were killed by CO_2 asphixiation and the spleens were removed aseptically. (2) BALB/c mice were immunized with three injections of virus. The first injection was 50 µg each of purified BWYV and PLRV emulsified with Freund's incomplete adjuvant and given subcutaneously. The second injection was 50 µg of PLRV given 4 weeks later intraperitoneally in 0.05 M phosphate buffer pH 7.0. The third and final booster, 6 weeks after the first, was 50 µg of BWYV given intraperitoneally in phosphate buffer. Three days later the mice were sacrificed and the spleens were harvested as above.

The same fusion protocol was used for both immunization procedures. FOX-NY myeloma cells (Hyclone Laboratories, Inc., Logan, Utah) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco Canada Inc., Burlington, Ontario) supplemented with 1 mM pyruvate and 2 mM L-glutamine and 10% fetal calf serum (FCS). Spleen cells were fused with myeloma cells (spleen cell to myeloma cell ratio approximately 10:1) in 50% polyethylene glycol 4000 (BDH Chemicals Canada Ltd., Vancouver, B.C.) as described by Kannangara, Wieczorek and Lavender (1989). All myelomas and hybridomas were grown at 37 C in an atmosphere of

10% CO_2. The cell fusion mixture was dispensed into five 96-well culture plates (Nunc, Denmark) and incubated overnight in nonselective media (DMEM containing 20% FCS) with mouse thymocytes as feeder cells. The microcultures were then fed with AAT selection media [7.5 x 10^{-5} M adenine, 8 x 10^{-7} M aminopterin, and 1.6 x 10^{-5} M thymidine (Taggart and Samloff, 1983)]. After 10 days, culture fluids from the hybridomas were screened for anti-BWYV and anti-PLRV antibody production by an indirect triple antibody sandwich ELISA [(TAS-ELISA), Martin and Stace-Smith, 1984] described in detail below. Culture fluid from the hybridomas were also screened for antibodies against healthy plant sap. Hybridoma cell lines which produced antibodies that tested positively with either BWYV or PLRV and negatively against healthy potato and ground cherry sap were cloned twice by limiting dilution, grown in cell culture, retested, and the positive cultures were stored under liquid nitrogen.

For production of antibodies in ascitic fluid, BALB/c mice were primed intraperitoneally with 2,6,10,14-tetramethylpentadecane (Pristane; Aldrich Chemical Co., Milwaukee, Wisconsin) 10 days before intraperitoneal injection of approximately 10^7 hybridoma cells. Some BALB/c mice were also immunosuppressed by injection of cyclophosphamide (0.025 g/g animal weight, Sigma Chemical Co., St. Louis, Missouri) 24 hr before injection of the hybridoma cells. Ascitic fluid was

collected 10 to 20 days later by inserting an 18-gauge needle into the peritoneal cavity of mice showing pronounced abdominal swelling. After low speed centrifugation at 8,700 x *g* for 20 min, to remove cellular debris, the ascitic fluid supernatant was mixed with an equal vol of saturated ammonium sulfate and stored at 4 C until required.

The MAb isotypes were determined by double antibody sandwich ELISA (DAS-ELISA) using a mouse hybridoma sub-isotyping kit (Calbiochem, Behring Diagnostics, La Jolla, California). Subcloned hybridoma supernatant fluids were tested using the manufacturer's protocol.

2.2.4 ELISA

An indirect triple antibody sandwich ELISA (TAS-ELISA) procedure was used for screening hybridomas for antibody production and for testing antibody specificity (Martin and Stace-Smith, 1984). All reagents were used at 100 µl per well in flat-bottomed Lindbro microtiter plates (Flow Laboratories, Mississauga, Ontario) except for the blocking steps, which were 300 µl per well. Plates were coated overnight at 4 C with polyclonal immunoglobulin homologous to the antigen to be tested, diluted to 1 µg/ml in phosphate-buffered saline [(PBS) 127 mM NaCl, 2.6 mM KCl, 8.5 mM Na_2HPO_4, 1.1 mM KH_2PO_4]. Plates

were blocked with 0.2% BLOTTO [BLOTTO (Johnson *et al.*, 1984) is 10 g nonfat dried milk made up to 100 ml with distilled water and 0.02% sodium azide as a preservative] in PBS for 30 min at room temperature.

Leaf tissue from infected and healthy plants was ground in a sap extractor (Erich Pollähne, F.G.R.) while adding grinding buffer (PBS/ 0.05% Tween 20/ 2% PVP/ 0.2% BLOTTO) drop-wise onto the bevelled rollers (1 ml per 0.1 g leaf tissue). Isolates provided by Dr. J. E. Duffus (Table 3) as freeze-dried cultures were ground with a mortar and pestle (0.02 g/ml grinding buffer). Extracts were incubated in the wells overnight at 4 C. Plates were then washed three times for 20 sec with PBS containing 0.05% Tween 20 (PBS-T) and then blocked again as above. Homologous or heterologous MAbs, from culture fluid supernatants, were diluted 1:1 in PBS-T containing 0.2% BLOTTO (PBS-T-BLOTTO) and incubated in the wells for 3 hr at room temperature. Plates were washed as above and conjugate, rabbit anti-mouse alkaline phosphatase (Jackson Immunoresearch Laboratories, Inc., Avondale, Pennslyvania), diluted 1:5,000 in PBS-T-BLOTTO was incubated in the wells for 3 hr at room temperature. After washing as above, substrate (ρ-nitrophenyl phosphate, Sigma 104-105, Sigma Chemical Co.) at 0.5 mg/ml in 10% (v/v) diethanolamine pH 9.8 was added to the wells and incubated for 2 hr at room temperature, then overnight at room temperature. The absorbance of each well was read at 405 nm

(A_{405}) in a Titertek Multiscan MCC plate reader (Flow Laboratories). ELISA reactions were scored +++ for readings of 1.0 and greater, ++ for readings of 0.5 to 1.0, and + for readings of less than 0.5 but greater than three times the mean of the readings of the healthy control plants. Readings below this threshold were scored - for negative (Voller, Bidwell, and Bartlett, 1977).

2.3 Results

2.3.1 Production of hybridomas

From the first immunization protocol and fusion two hybridoma clones secreting BWYV-specific antibodies were obtained. Clones 510H and 112E produced ascitic fluids in BALB/c mice, after immunosupression with cyclophosphamide, with titres of 10^{-8} and 10^{-4} respectively in TAS-ELISA. Clone 510H produced between 3-8 ml of ascitic fluid per mouse whereas clone 112E tended to produce solid tumors and less than 1 ml of ascites per mouse.

The fusion from the second immunization protocol resulted in 18 wells of 420 with hybridomas testing positive for production of antibody to either BWYV or PLRV, or both, but not

to healthy plant sap from potato or *P. pubescens*. Seven of these hybridomas were successfully cloned and tested further. The isotypes of the MAbs are presented in Table IV.

Table IV. Monoclonal antibody subclasses

Monoclonal antibody	Isotype	Homologous antigen
510H	IgG2a	BWYV-BC
112E	IgG1	BWYV-BC
13CD	IgM	BWYV-BC
15CD	IgM	BWYV-BC
31CC	IgM	BWYV-BC
43GB	IgM	BWYV-BC
43BC	IgM	BWYV-BC/PLRV-BC
26BE	IgG1	PLRV-BC
41BC	IgM	PLRV-BC

2.3.2 Monoclonal antibody specificity

The specificity of the MAbs was determined in TAS-ELISA using 25 luteovirus isolates (Table III) and the results are given in Table V. Sixteen of the isolates are BWYV [BWYV includes isolates identified by the synonyms (Table I) beet mild yellowing virus (BMYV), turnip yellows virus (TuYV), and the RPV isolate of barley yellow dwarf (Casper, 1988)]. Two MAbs, 510H and 112E, reacted with all of the BWYV isolates tested and four others (13CD, 15CD, 31CC, and 43GB) detected all of the BWYV isolates except the RPV isolate of BYDV. MAbs 26BE and 41BC

Table V. Specificity of monoclonal antibodies[#]

	Monoclonal antibodies									
Antigen	510H	112E	13CD	15CD	31CC	43GB	43BC	26BE	41BC	371A*
BWYV-BC	+++	++	++	+	++	++	+++	-	-	-
BWYV-RY-1-R	+++	+++	+++	+	++	++	+++	-	-	-
BWYV-RY-7	+++	+++	+++	+++	++	++	+++	-	-	-
BWYV-SAL(9)	+++	+++	+++	+++	+++	+++	+++	-	-	-
BWYV-NETH	+++	+++	+++	+	+++	+++	+++	-	-	-
BWYV-150	++	++	++	+	+	+	++	-	-	-
BWYV-40-008	+++									
BWYV-B10	+	+	++	+	+	+	+	-	-	-
BWYV-RB2	+++	+++	+++	+	++	+++	+++	-	-	-
BWYV-MP4	++	++	+	+	+	+	+	-	-	-
BWYV-MY4	++	+	+	+	+	++	+	-	-	-
BMYV-324	+++	++	+	+	+	+	+	-	-	-
TuYV	+++	+++	+++	+	+++	+++	+++	-	-	-
BYDV-RPV-NY	++	++	-	-	-	-	-	-	-	-
BYDV-RPV-T	++									
BYDV-MAV-NY	-	-	-	-	-	-	-	-	-	-
BYDV-PAV-IL	-	-	-	-	-	-	-	-	-	-

Table V. Continued

	Monoclonal antibodies									
Antigen	510H	112E	13CD	15CD	31CC	43GB	43BC	26BE	41BC	371A*
PLRV-BC	-	-	-	-	-	-	+	++	+++	+++
PLRV-ST4	-	-	-	-	-	-	+	++	+++	+++
PLRV-ORE	-	-	-	-	-	-	+	++	+++	+++
PLRV-ID	-	-	-	-	-	-	+	+++	+++	+++
SYV-21	-	-	-	-	-	-	+	+	+	+
SYV-44	-	-	-	-	-	-	+	++	++	++
SDV-AS	-	-	-	-	-	-	-	-	-	-
SDV-AP	-	-	-	-	-	-	-	-	-	-

Reactions (A_{405}) after overnight incubation of substrate
+++ = 1.0 or more
++ = 0.5 - 1.0
+ = less than 0.5
- = negative

*MAb 371A was produced by Martin and Stace-Smith (1984)

reacted with all of the PLRV isolates screened including two isolates of a strain of PLRV called solanum yellows virus (SYV). One MAb (43BC) reacted with an epitope common to BWYV and PLRV. None of the MAbs reacted with the other luteoviruses nor with healthy plant sap.

2.4 Discussion

Although the serological relationships among the luteoviruses are complex (Waterhouse *et al.*, 1988), MAbs provide a tool to discriminate among them. Several clear advantages of MAbs over polyclonal antisera have been reported (Halk and DeBoer, 1985):

1. the availability of an unlimited supply of uniform antibody specific for a single epitope;
2. MAbs can be produced from a small amount of antigen, even if the antigen is impure;
3. hybridomas can be stored in liquid nitrogen, ensuring a continuous supply of antibody over time;
4. MAbs obviate the qualitative and quantitative antibody content encountered in the use of different batches of polyclonal antiserum;
5. and, specific MAbs may reveal serological relationships between antigens that were previously unrecognizable using polyclonal antiserum.

The objective of this study, to produce MAbs that would be suitable for detecting and identifying BWYV and PLRV in plant tissue using ELISA, was achieved. The TAS-ELISA procedure has two advantages over DAS-ELISA: (1) a single anti-mouse conjugate can be used to detect all mouse MAbs and (2) the specificity of

the MAb is not altered by the conjugation of an enzyme (Koenig, 1978).

MAbs 510H and 112E detected all the BWYV strains tested including the RPV isolate of BYDV that is now recognized as a strain of BWYV (Casper, 1988; D'Arcy, Torrance, and Martin, 1989). Four other MAbs detected all the BWYV strains except BYDV-RPV. Any of these six MAbs would be suitable for screening potato samples for BWYV because the host range of BYDV-RPV does not include dicotyledonous species (Rochow and Duffus, 1978). MAb 510H was selected for further large scale testing because of its broad specificity for BWYV, detecting 16 isolates from six countries on four different continents, and because of its high titer in ascites fluid. Two MAbs, 26BC and 41BC, detected all of the PLRV isolates tested including the two strains of PLRV named solanum yellows virus (SYV). None of the MAbs detected either strain of soybean dwarf virus (SDV) or the MAV and PAV isolates of BYDV. Because the specificity of MAbs 26BC and 41BC appeared to be the same as MAb 371A produced by Martin and Stace-Smith (1984), 371A was chosen for further testing because it was already available as ascitic fluid and it had been widely tested against many isolates of PLRV.

Clone 43BC produced antibody that detected an epitope common to both BWYV and PLRV. This epitope has also been reported by D'Arcy *et al.* (1989), and it may provide an

explanation for the confusion concerning the presence of BWYV in potato affected with potato leafroll disease. In Duffus' work demonstrating the presence of BWYV in potato (Duffus, 1981a) it had been assumed that BWYV antiserum did not cross-react with PLRV. If some of the antibodies in his BWYV polyclonal antiserum were against an epitope common to both viruses, then the antigen scanning pattern analysis used by him, would not be specific for BWYV. Since a positive test was based on the reduction or elimination of the virus peak, this assay would have been evidence that an antigen-antibody interaction had occurred, but the interaction may have involved an epitope not unique to BWYV.

The results of this study show that the MAbs described here can be used effectively in a routine diagnostic TAS-ELISA for the detection and identification of BWYV and PLRV in plant tissue.

Chapter 3

Detection and identification using monoclonal antibodies

3.1 Introduction

Potato leafroll disease is one of the most important diseases of potato and it occurs worldwide wherever potatoes are grown (Peters and Jones, 1981). The disease is usually considered to be caused by PLRV (Barker, 1986) but in North America (Duffus, 1981a,b) and in Tasmania (Duffus and Johnstone, 1982) BWYV has been isolated from plants with typical potato leafroll disease symptoms.

The conclusion that BWYV occurs in potato (Duffus, 1981a,b) was made on the basis of two assumptions: that shepherd's purse (*Capsella bursa-pastoris*) is a host for BWYV but it is not a host for PLRV, and that BWYV antiserum does not cross-react with PLRV. Both assumptions are challenged by more recent evidence in the scientific literature (Stace-Smith, 1987). Several researchers have demonstrated that PLRV can indeed infect shepherd's purse (Fox *et al.*, 1990; Syller, 1985; Thomas, 1984) and others have reported that BWYV antiserum does cross-react with PLRV in some serological tests (Marco, 1985; Richter *et al.*, 1983).

Immunological techniques are among the most important tools for the detection and identification of plant viruses (Halk, 1986). Recent advances in methodology, including ELISA and monoclonal antibody (MAb) production, have greatly improved the sensitivity, specificity, and ease of luteovirus diagnosis (D'Arcy *et al.*, 1989). BWYV is potentially a greater threat to seed potato production than PLRV because it has a much wider host range. Many common weeds are hosts of BWYV and they may serve as virus reservoirs in seed production areas. The objective of this study was to determine if BWYV is an important component of a complex causing potato leafroll disease in Canada and the United States using virus specific MAbs. A preliminary report has been published (Ellis and Wieczorek, 1988).

3.2 Materials and methods

3.2.1 Collection of samples

Most of the samples were collected in the seed potato certification winter test plots near Homestead, Florida and Oceanside, California. With the assistance of seed potato officials from each listed state or province (Table VI), foliage samples were collected from plants with secondary leafroll symptoms such as leafrolling of the lower leaves, chlorosis, and stunting (Fig. 1). The samples were placed in labelled plastic

Fig. 1. Secondary symptoms of potato leafroll virus from tuber-borne infection.

bags and shipped to Vancouver in coolers. Some of the samples were provided by seed certification officials at Oregon State University, and by Food Production and Inspection Branch officials of Agriculture Canada. In sum, 801 samples, representing 32 cultivars, originating in 8 Canadian provinces and 12 American states were collected and tested in 1986, 1987, and 1988 (Table VI).

Table VI. Origins of potato leafroll disease samples

Province or state	Number of samples		
	1986	1987	1988
Canada			
Alberta	2		4
British Columbia	20		8
Manitoba	2		
New Brunswick	6		63
Nova Scotia			8
Ontario	8		3
Prince Edward Island	8		10
Quebec	9		
United States			
California		9	
Colorado		39	6
Idaho		209	96
Maine		26	22
Michigan		7	18
Minnesota			7
Nebraska			20
North Dakota		6	16
Oregon		86	
Utah			3
Wisconsin		12	64
Wyoming			4

3.2.2 Virus isolates and purification

As in section 2.2.1

3.2.3 Antisera and monoclonal antibodies

An antiserum against BWYV-BC was produced by immunizing a young, White New Zealand rabbit with 1 mg of purified virus. The first injection, 100 µg of virus emulsified with an equal volume of Freund's complete adjuvant, was administered intramuscularly in a hind leg. The second injection of 200 µg of virus was given intravenously 10 days later. The next three injections of approximately 300, 200, and 100 µg were emulsified with Freund's incomplete adjuvant and injected intramuscularly in a hind leg at 2-week intervals. Blood was collected at 2-week intervals following the last injection. A booster injection of 100 µg of virus was given after the second bleeding. The antiserum titer was determined by agar gel double diffusion tests as described by Rowhani and Stace-Smith (1979).

The PLRV antiserum used was a gift from Dr. R. Stace-Smith (Vancouver Research Station, Agriculture Canada).

The immunoglobulins were purified from the PLRV antiserum, adjusted to 1 mg/ml, and stored in 0.5 ml aliquots at -20 C as

described in section 2.2.2. The BWYV antiserum was purified by affinity chromatography using Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals AB, Sweden). Immunoglobulins (IgG) were adsorbed to the column in 0.1 M sodium phosphate buffer pH 7.0. The column was then washed with 10 vol of the same buffer to remove any unbound material. The immunoglobulins were eluted with 1.0 M acetic acid, immediately neutralized with 1.0 M Tris-HCl pH 9.0, then precipitated by adding an equal vol of saturated ammonium sulfate and incubating overnight at 4 C. The immunoglobulins were collected by centrifugation at 12,100 x *g* for 20 min. The pellet was resuspended in PBS, adjusted to approximately 1 mg/ml (A_{280} = 1.4) and stored in aliquots of 0.5 ml at -20 C.

MAb 371A, specific for PLRV, was provided by Dr. R. Martin (Vancouver Research Station, Agriculture Canada). BWYV-specific MAb 510H described in section 2.3.2 was selected for routine detection of BWYV because of its broad specificity to BWYV isolates.

3.2.4 ELISA

Each sample was tested separately for PLRV and BWYV using virus-specific MAbs, 371A and 510H respectively, using the indirect TAS-ELISA protocol described in detail in section

2.2.4. Each sample was tested twice. Reactions were considered positive when the A_{405} readings were greater than three times the mean of the readings for five healthy controls (Voller *et al.*, 1977).

The sensitivity of the TAS-ELISA was determined by testing a tenfold dilution series of purified PLRV-BC and BWYV-BC. Each virus was serially diluted in virus-free potato sap diluted 1:9 in grinding buffer. Duplicates of each virus dilution, from 1 µg/ml to 1 pg/ml, were tested as above.

3.2.5 Aphid transmission tests

Mature wingless GPA were used in attempts to transmit virus from potato to the test species *Physalis pubescens* and *Capsella bursa-pastoris*. The potato leaves were placed on moistened filter paper in petri dishes. The aphids were transferred from plant to plant with a camel-hair brush. About 50 nonviruliferous aphids, reared on Chinese cabbage (*Brassica pekinensis* Rupr.), were placed on the leaf. After a 48 hr acquisition feeding, about 20 aphids were transferred to each of the test species. The plants were infested with aphids at the 2-to 4- true leaf stage (Lutman and Tucker, 1987) and kept caged in small plastic cylinders covered on top with fine wire mesh. After a 48 hr inoculation access feeding, the aphids were killed

by spraying the plants with pirimicarb at 0.25 g ai/l (Chipman Inc.). The plants were moved to a greenhouse (15-20 C) for 6 weeks and then rated for development of symptoms. The test plants were also screened for PLRV and BWYV using TAS-ELISA as described above.

3.3 Results

The titer of the BWYV antiserum was 512 before the final booster, 1024 two weeks after the booster, and then it fell to 512 on the next bleeding. Antiserum from the third bleeding was used in all the experiments.

The TAS-ELISA could reliably detect PLRV and BWYV at 1 ng/ml or 100 pg/well (Fig. 2). The mean A_{405} readings for 1 ng/ml of PLRV and BWYV were 0.111 and 0.125 respectively, more than fifteen times the mean readings for the virus-free controls.

The ELISA results are presented as histograms (Sutula *et al.*, 1986) and each histogram represents a data set for each location and year in which samples were collected (Figs 3A-8B). An interval of 0.025 OD is used for all of the histograms on the x axis.

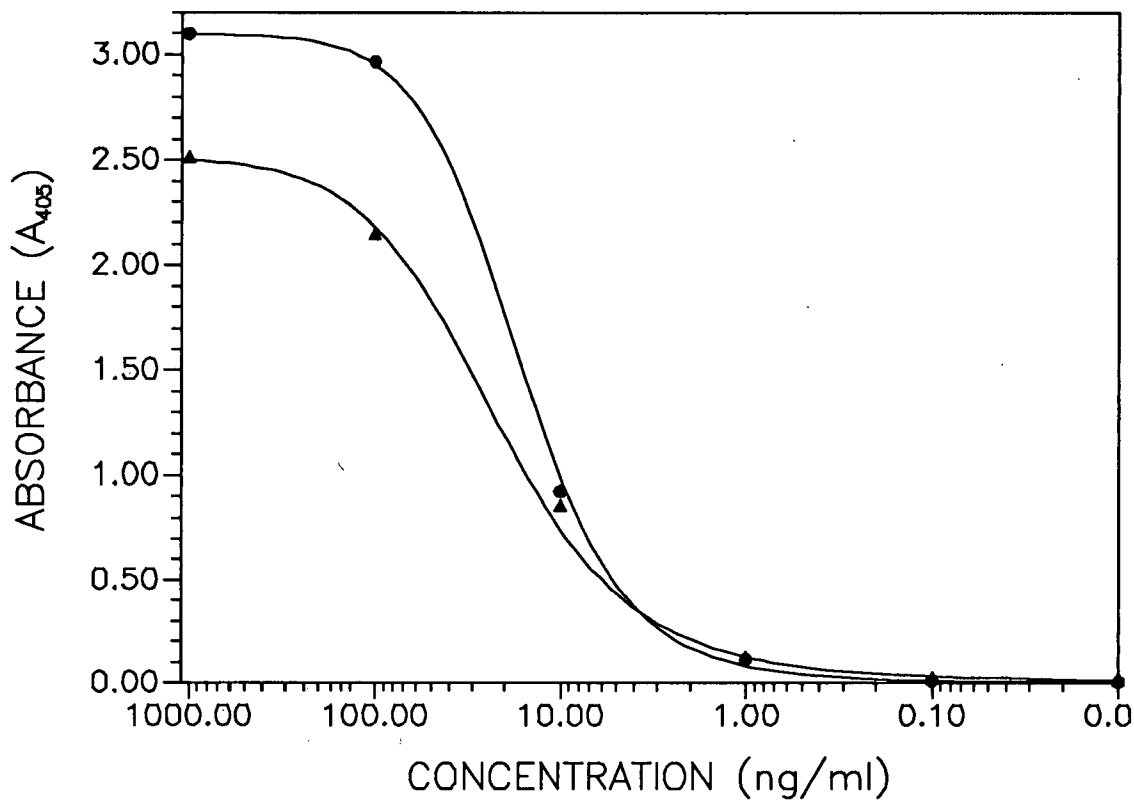


Fig. 2 TAS-ELISA analysis of purified potato leafroll virus (PLRV •) and beet western yellows virus (BWYV ▲) serially diluted in virus-free potato leaf sap diluted 1:9 with grinding buffer. Each point is the mean of two tests. The mean of the ELISA readings (A_{405}) of five virus-free control plants was 0.007 for the PLRV test and 0.008 for the BWYV test.

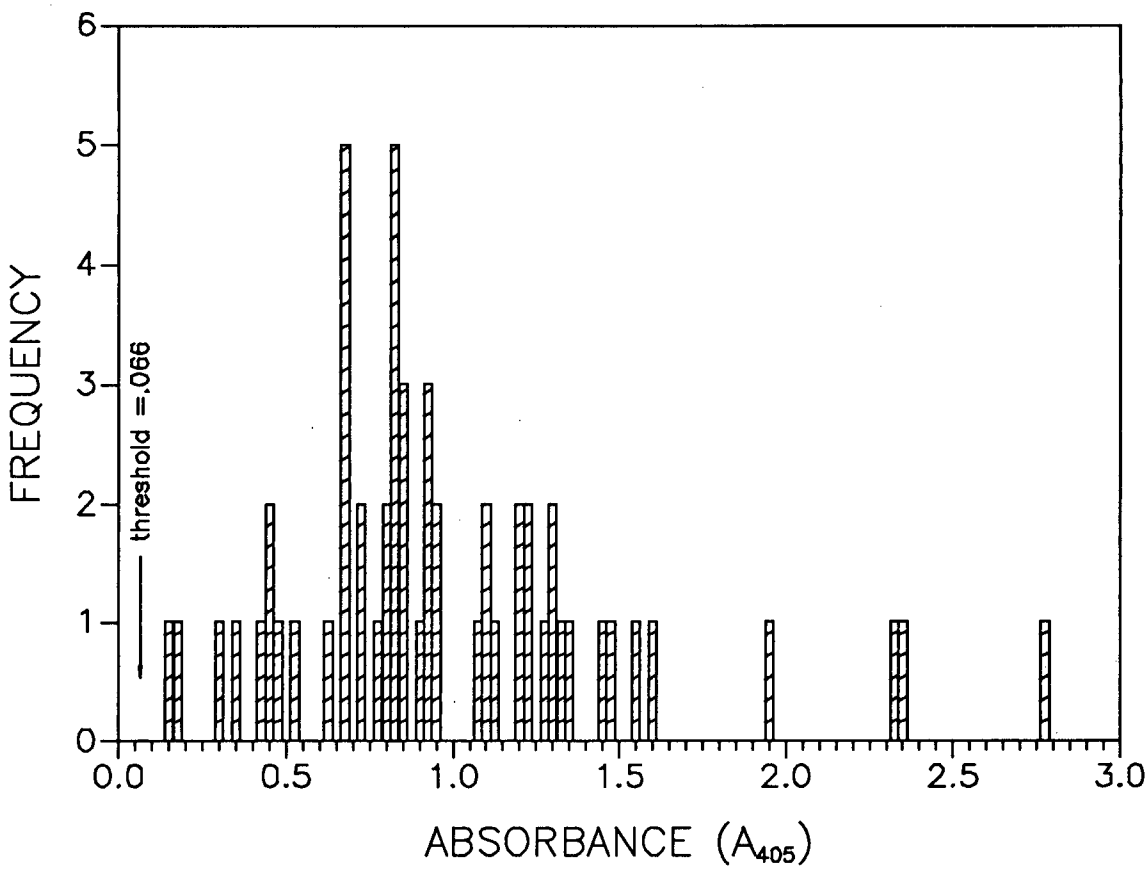


Fig. 3A. Histogram of 1986 Florida winter test ELISA results (mean of two tests) for potato leafroll virus in 55 potato leafroll disease samples.

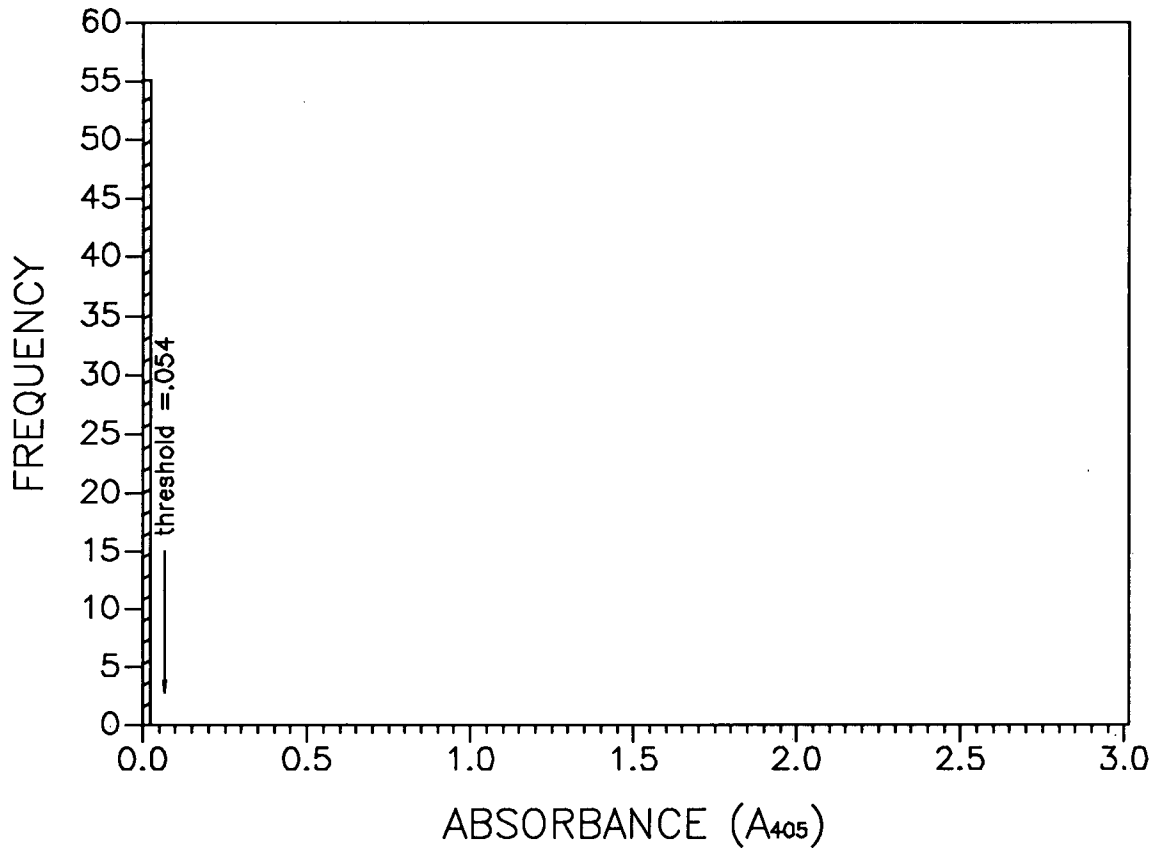


Fig. 3B. Histogram of 1986 Florida winter test ELISA results (mean of two tests) for beet western yellows virus in 55 potato leafroll disease samples.

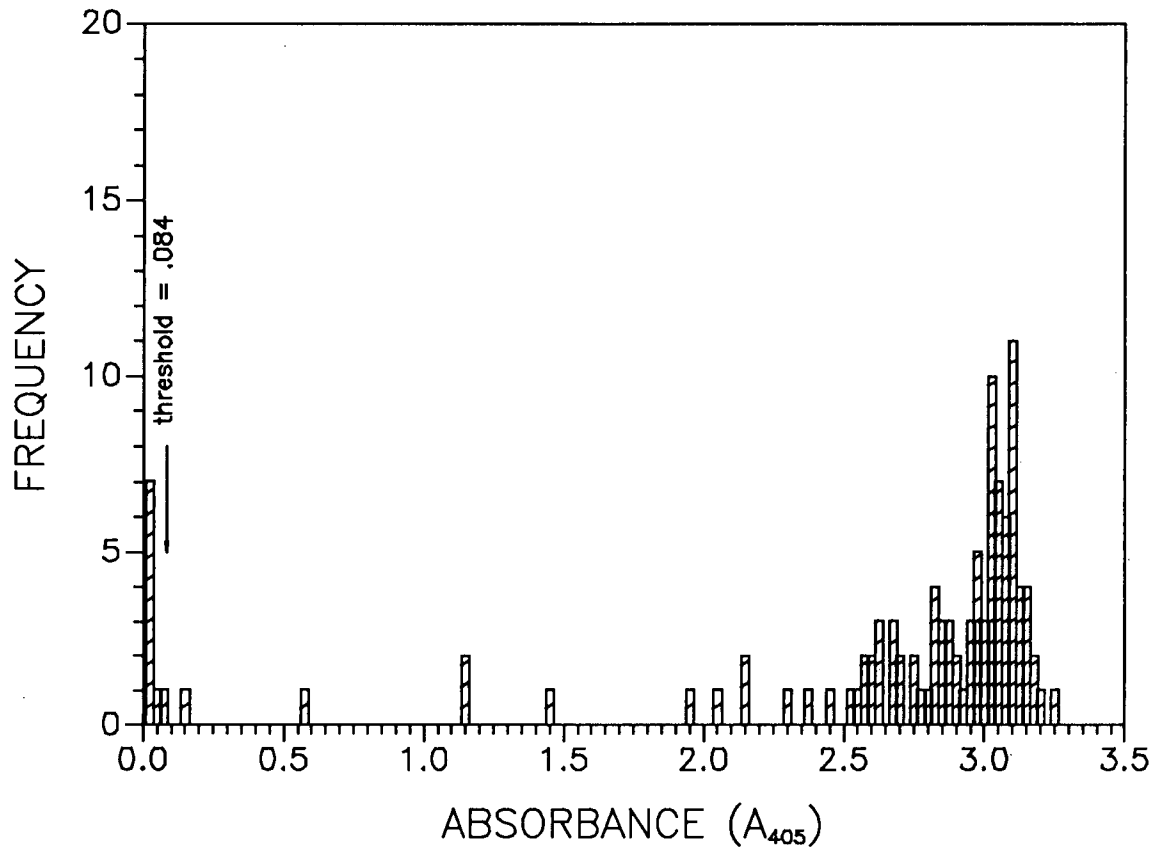


Fig. 4A. Histogram of 1987 California winter test ELISA results (mean of two tests) for potato leafroll virus in 257 potato leafroll disease samples.

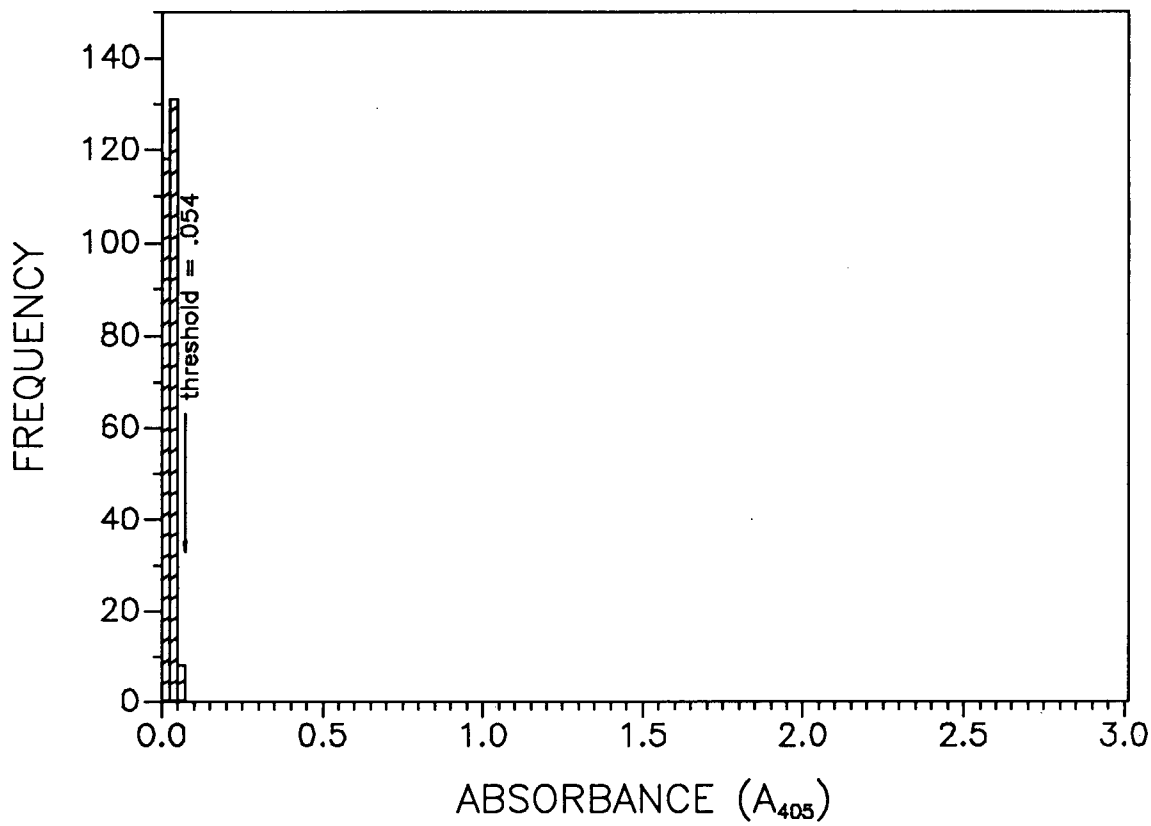


Fig. 4B. Histogram of 1987 California winter test ELISA results (mean of two tests) for beet western yellows virus in 257 potato leafroll disease samples.

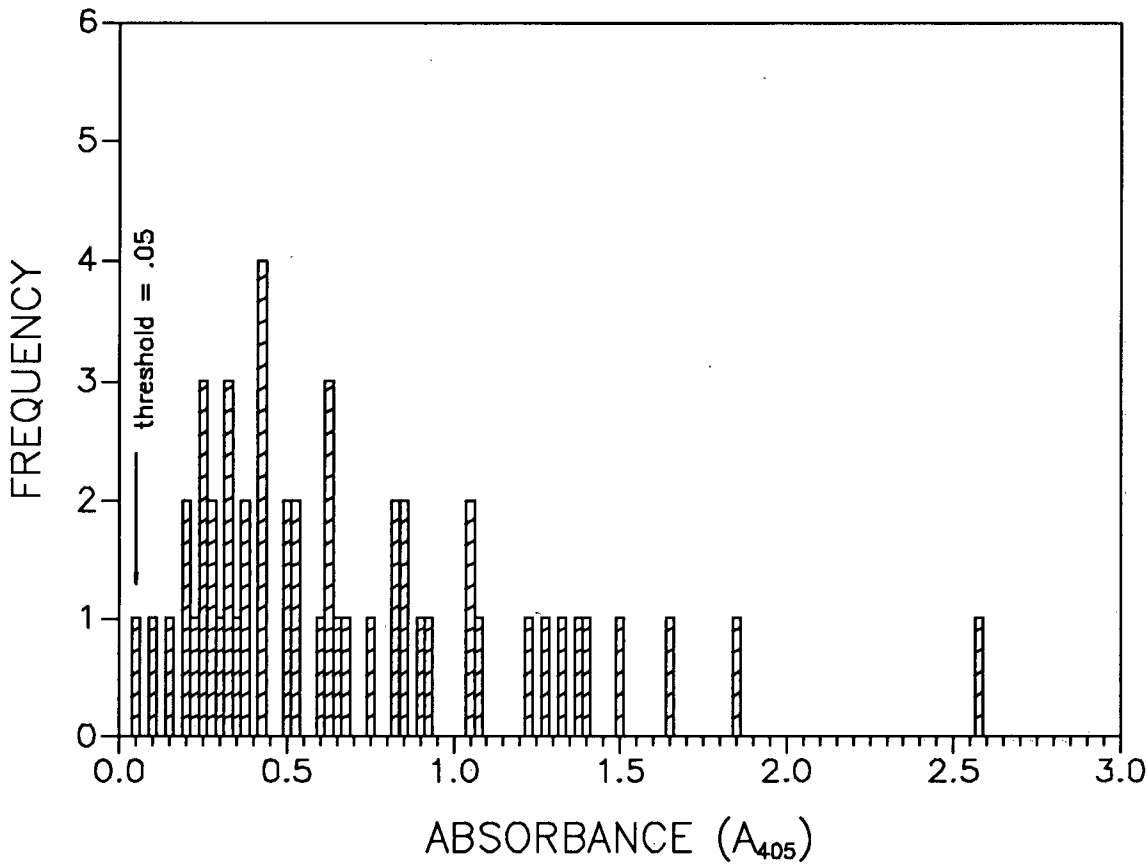


Fig. 5A. Histogram of 1987 Florida winter test ELISA results (mean of two tests) for potato leafroll virus in 51 potato leafroll disease samples.

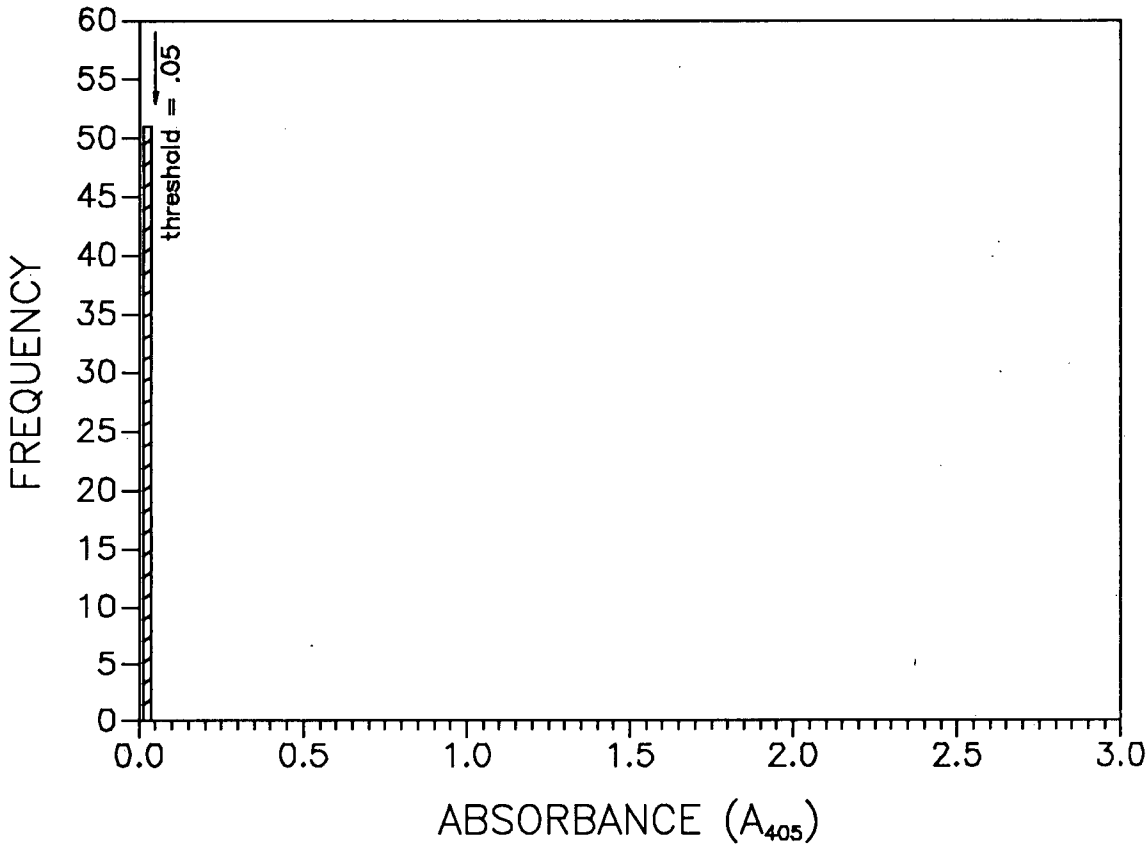


Fig. 5B. Histogram of 1987 Florida winter test ELISA results (mean of two tests) for beet western yellows virus in 51 potato leafroll disease samples.

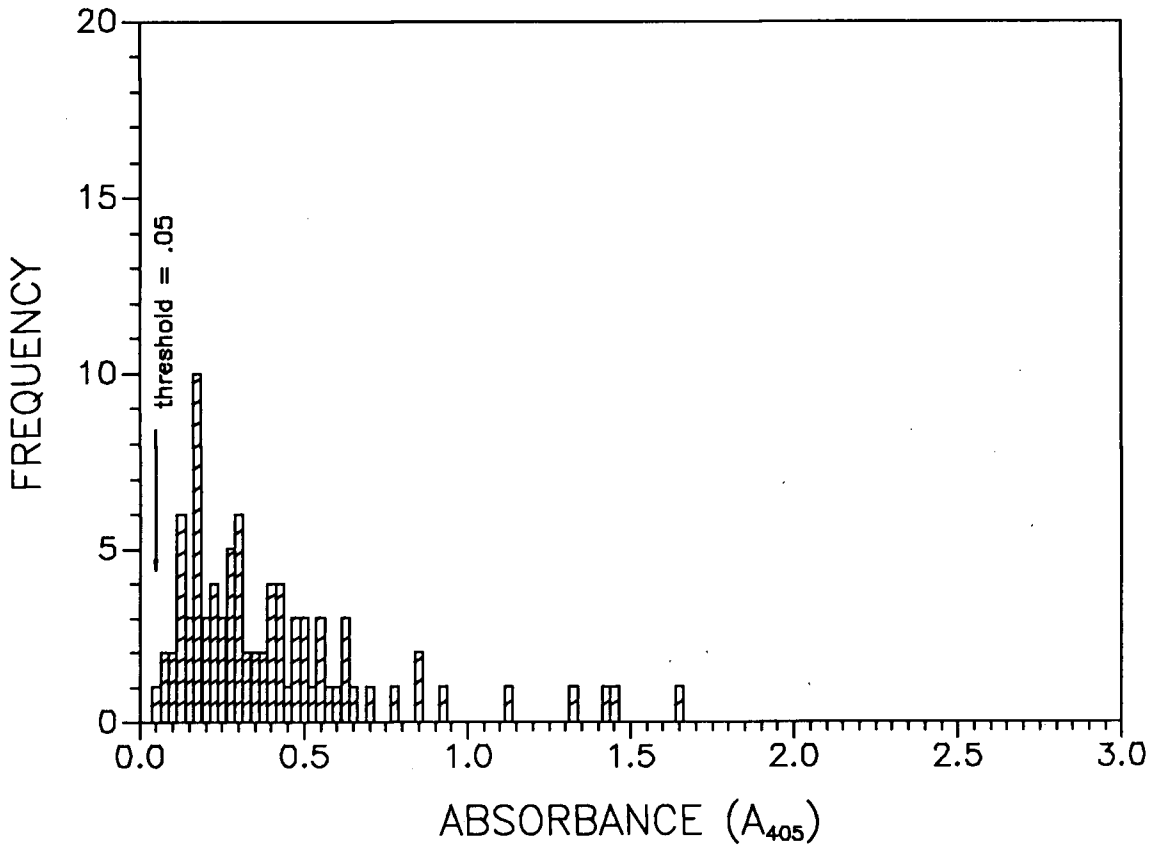


Fig. 6A. Histogram of 1987 Oregon winter test ELISA results (mean of two tests) for potato leafroll virus in 86 potato leafroll disease samples.

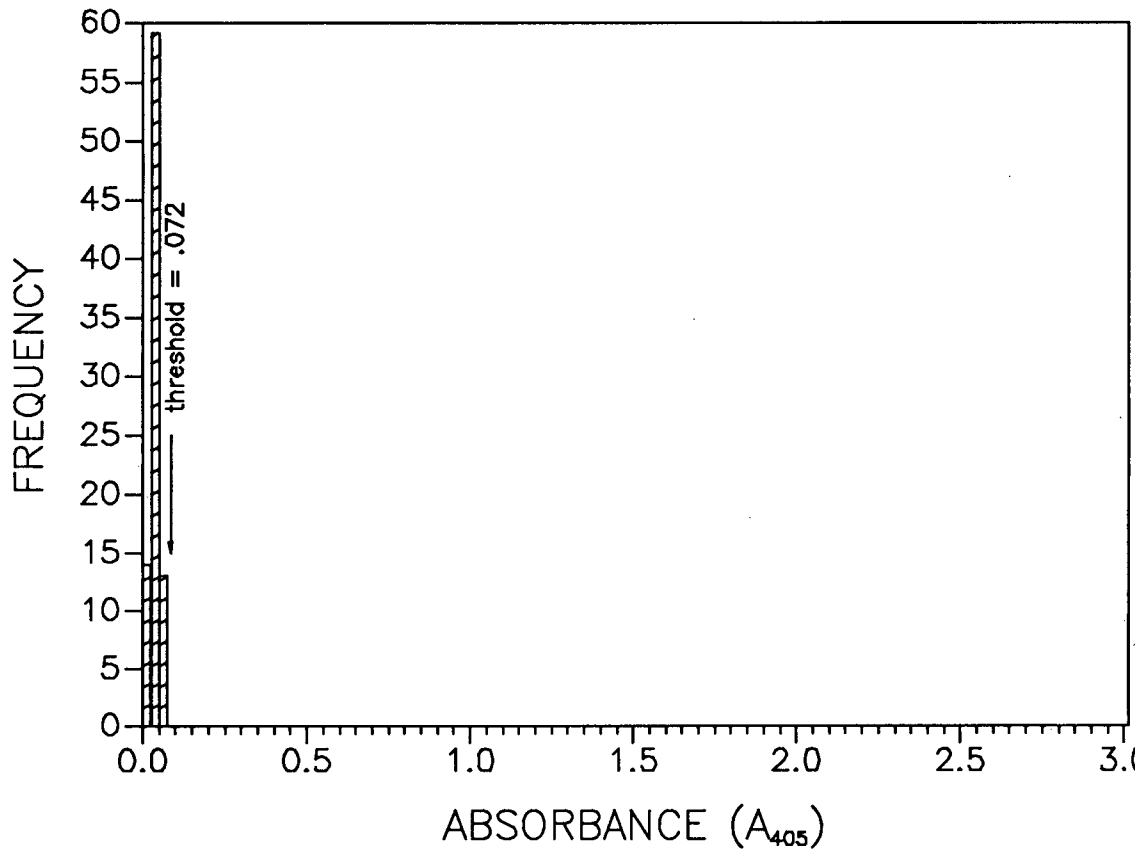


Fig. 6B. Histogram of 1987 Oregon winter test ELISA results (mean of two tests) for beet western yellows virus in 86 potato leafroll disease samples.

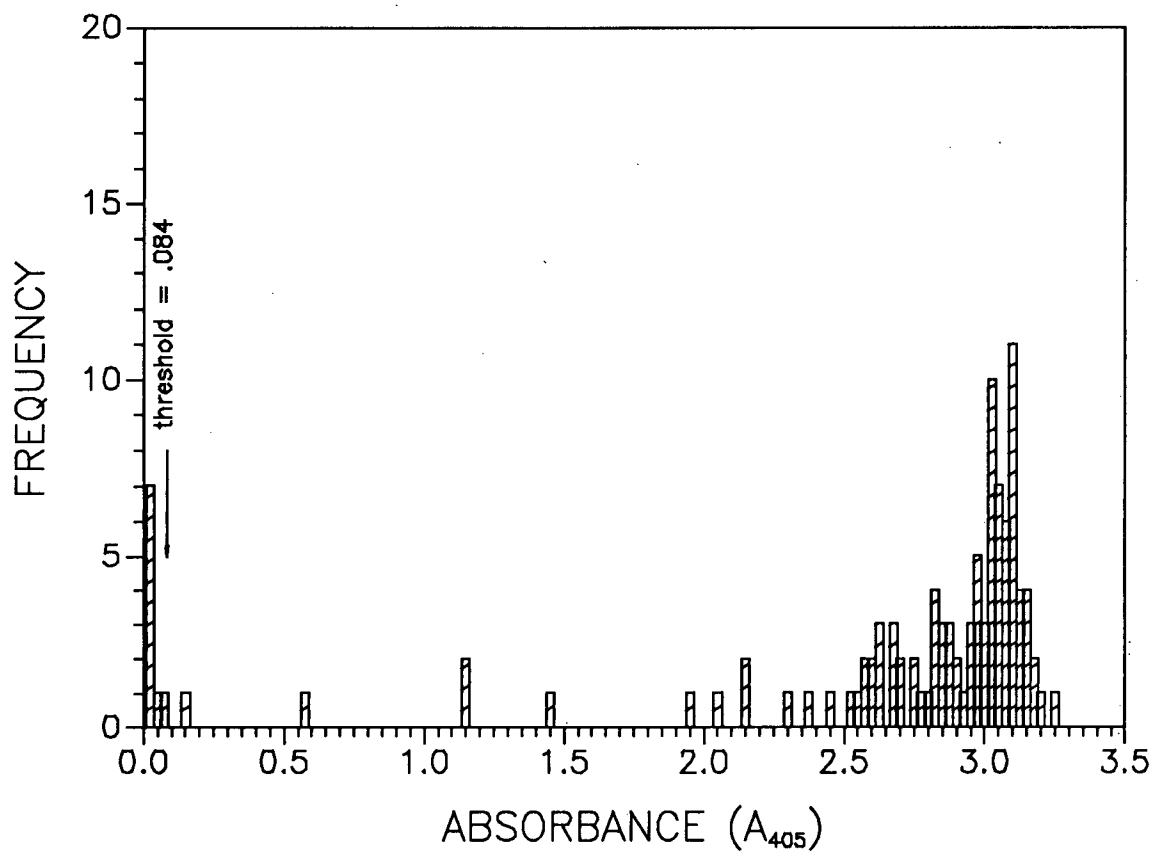


Fig. 7A. Histogram of 1988 California winter test ELISA results (mean of two tests) for potato leafroll virus in 109 potato leafroll disease samples.

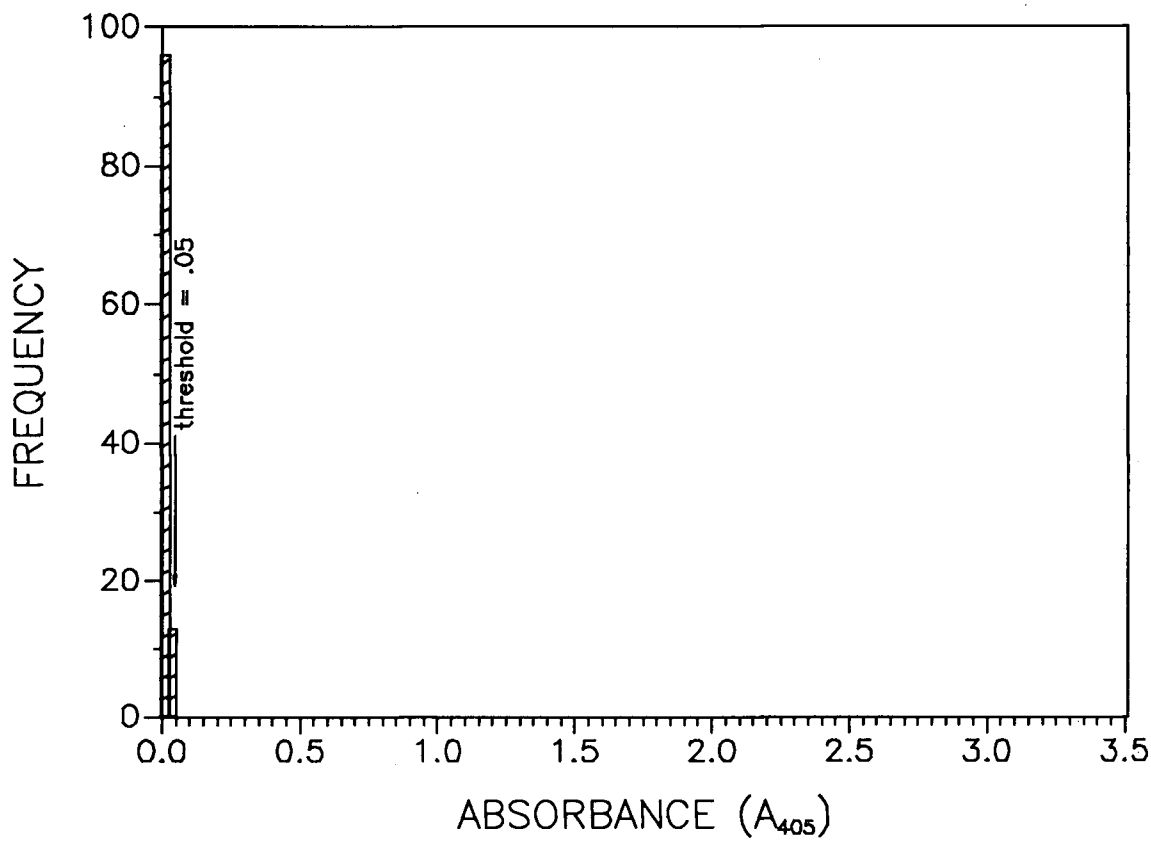


Fig. 7B. Histogram of 1988 California winter test ELISA results (mean of two tests) for beet western yellows virus in 109 potato leafroll disease samples.

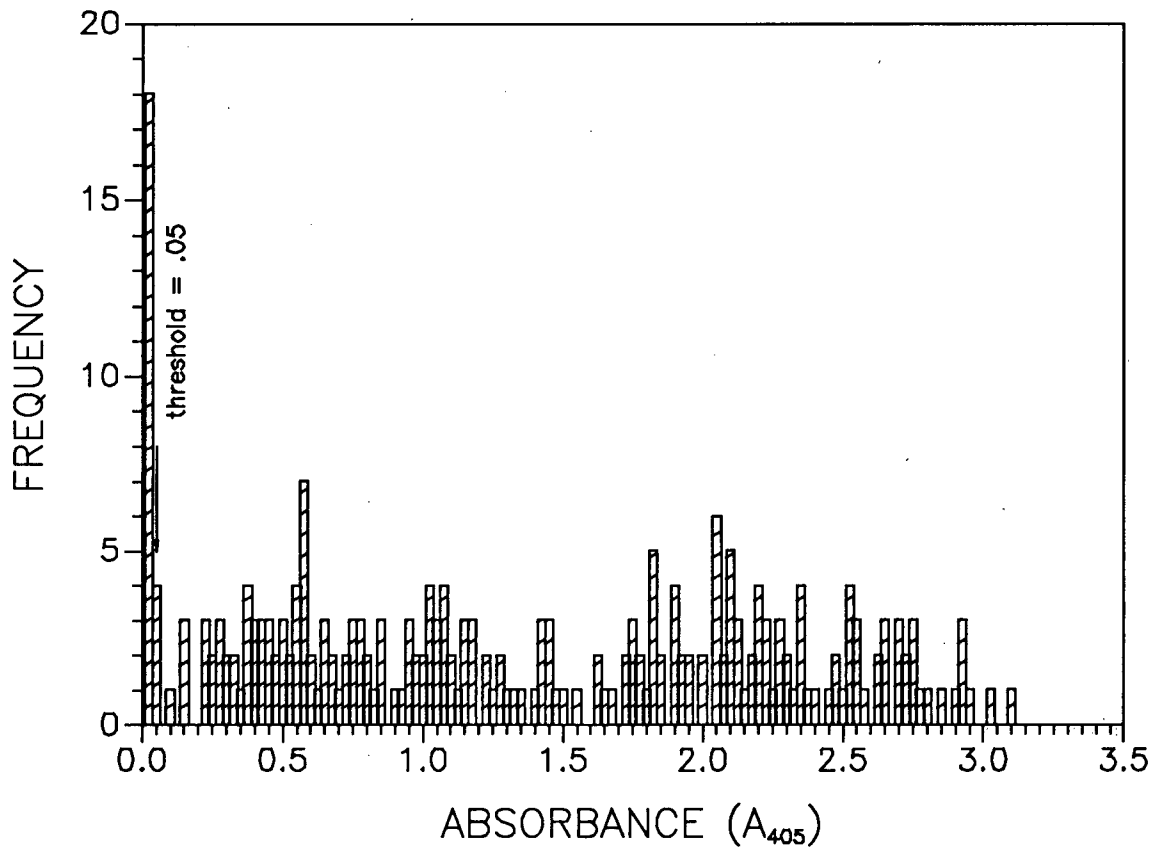


Fig. 8A. Histogram of 1988 Florida winter test ELISA results (mean of two tests) for potato leafroll virus in 243 potato leafroll disease samples.

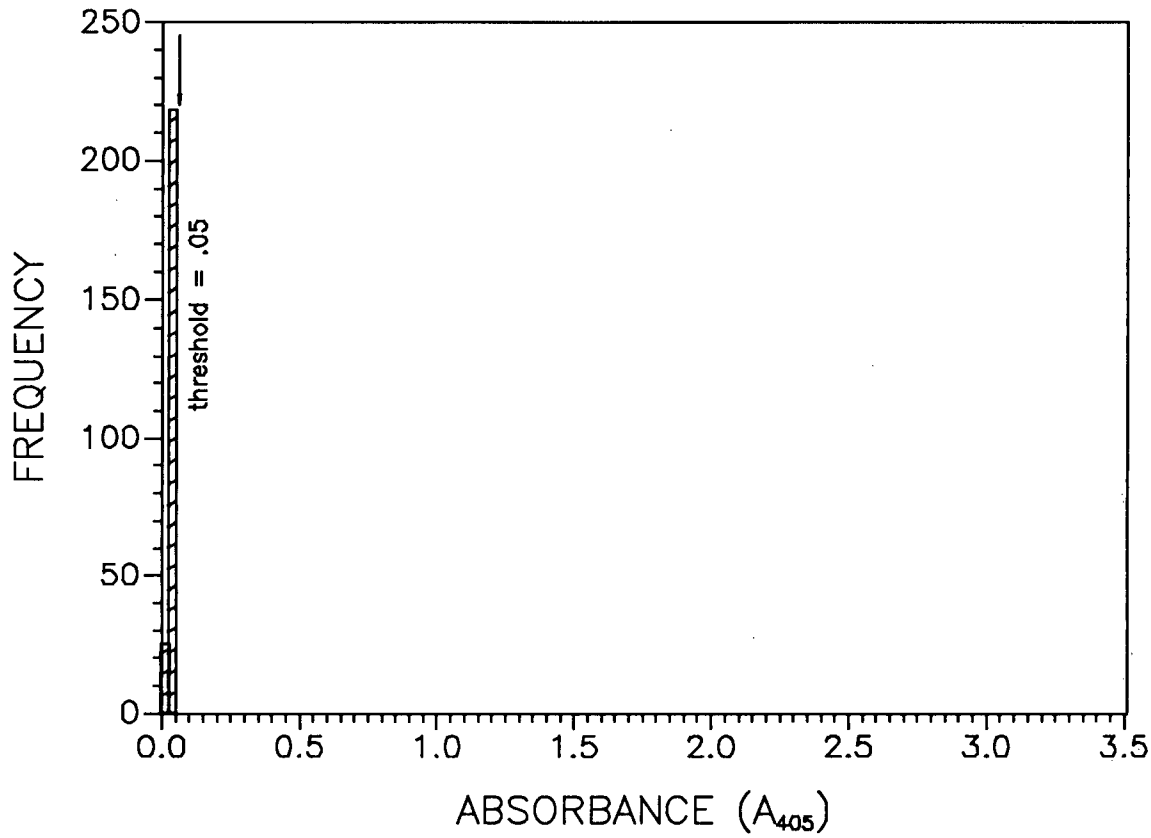


Fig. 8B. Histogram of 1988 Florida winter test ELISA results (mean of two tests) for beet western yellows virus in 243 potato leafroll disease samples.

None of the potato samples tested positive for BWYV; in all cases the data were clustered near zero. BWYV-infected *P. pubescens*, used as a positive control, produced a strong signal ($A_{405} \geq 1.0$) in all of the BWYV ELISA tests.

Most of the samples, 772/801 (96.4%), tested positive for PLRV. Neither PLRV nor BWYV could be recovered, by aphid transfers to indicator hosts, from 28 of the samples that tested negative for both viruses. One sample that was scored negative for both viruses by TAS-ELISA tested positive in the aphid transmission test to *P. pubescens*. The indicator plant tested positive for PLRV by TAS-ELISA.

3.4 Discussion

The results indicate that BWYV is neither a common nor important component of a complex that causes potato leafroll disease. The small number of samples (28/801 or 3.5%) that were identified in the field as having leafroll symptoms, but that tested negative for PLRV and BWYV, were probably affected by a physiological leafrolling. Nonvirus leafroll (LeClerg, 1944) is a symptom caused by the impairment of carbohydrate translocation from the foliage. When starch accumulates in the leaves they become leathery and roll upwards and the symptoms are easily mistaken for potato leafroll virus disease. PLRV-like symptoms

may be caused by Rhizoctonia stem canker and other diseases, mechanical injury to the stems, and soil nutritional conditions such as nitrogen toxicity or boron deficiency (Hooker, 1981).

The samples tested in this study represented a wide range of cultivars and geographical origins. The results are distinctly different from those reported by Sibara and Slack (1985b) and Gallenberg *et al.* (1987) ; both groups found BWYV to be common in potato leafroll disease samples from Canada and the United States. This is particularly interesting because they also collected many samples from the Florida winter test plots that I had sampled. The questions arise; did BWYV, common in many of their samples collected in 1983 to 1986, suddenly disappear in the years following, or did these investigators detect something other than BWYV with their BWYV polyclonal antiserum, or did the MAb that I used in my study fail to detect BWYV isolates occuring in the potatoes? It is very unlikely that BWYV was common in potato up until 1986 and then suddenly disappeared in the following years because both PLRV and BWYV are efficiently vectored by the GPA and BWYV has a much wider host range than PLRV. The BWYV MAb used here detected all isolates of BWYV against which it was screened including many strains (BMYV, TuYV, and the RPV isolate of BYDV) from several locations in six countries. Moreover, tests using BWYV polyclonal antiserum prepared against a British Columbia isolate of BWYV gave identical results (section 4.3); no potato leafroll

disease samples tested positive for BWYV. Some of the samples collected from the Florida winter test plots in 1988 were also tested independently by a commercial laboratory (Agdia Inc., Mishawaka, Indiana) for PLRV and BWYV. The results were the same; *viz.* no positive assays for BWYV (Dr. C. Sutula, personel communication).

Reconciliation of the differences between my results and those of Sibara and Slack (1985b), and of Gallenberg *et al.* (1987) requires some speculation. Because the actual ELISA readings from the experiments of Sibara and Slack (1985b) were not presented in their abstract and the paper has not yet been published, I will comment only on the results of Gallenberg *et al.* (1987).

Gallenberg *et al.* (1987) noted that the BWYV antiserum they used had a higher level of background interference and an overall lower level of reaction with infected samples than the PLRV antisera they used. However, they did not attempt to cross-absorb the globulin to reduce non-specific reactions and possibly increase the signal-to-noise ratio. Moreover, they prepared the gamma globulin by ammonium sulfate precipitation only and further purification by filtration through DEAE cellulose was not attempted. Although they rated many samples as weak positives, they did not apparently confirm their ELISA results with aphid transmission tests. Because *P. pubescens* is

a host for both PLRV and BWYV, it could have been used as a transmission host and the plants could have been retested later serologically after BWYV had developed a reasonable titer. Without an independent test, their weak ELISA results are not unequivocal proof that BWYV was present in the samples they tested. To obtain these ELISA results they had to use high reagents concentrations. The coating antibody and conjugate were used at 4 µg/ml and sap was diluted 1:5 with sample buffer. Recent work by Gunn and Pares (1988) demonstrated the presence of a stress-induced antigen which co-purified with PLRV. The antigen also appeared to be produced in uninfected but physiologically stressed potato plants, and reacted with PLRV antisera in ELISA tests, resulting in false-positive results. It is possible that antibodies to a stress-induced protein may be present in the BWYV polyclonal antiserum which would be one explanation for the low signal-to-noise ratio in their ELISA results. This may also explain why some of their samples tested positive for BWYV. The samples that were tested represented many different cultivars, and cultivars may respond differently to physiological stress. In Florida, where their samples were collected, many of the plots were on sites with less than ideal soils. In fact, it was not uncommon to find locations with less than one foot of soil over the coral parent material. At one site I saw a gravel ridge running through the test plots; potatoes planted here would be growing under conditions of stress.

The results presented here indicate that, in Canada and the United States, potato leafroll disease is caused by one virus, PLRV. I could not find any evidence to support the earlier findings that BWYV is a common component of potato leafroll disease. My results support the work of Tamada *et al.* (1984); they found no evidence of antigenic variation among potato isolates of PLRV. From a practical viewpoint it appears that BWYV does not present a risk to potato cultivation at the present time.

Chapter 4

Comparison of ELISA and nucleic acid spot hybridization for detection and identification of PLRV and BWYV

4.1 Introduction

The introduction of ELISA to plant virology by Voller *et al.* (1976) and Clark and Adams (1977) was a technological breakthrough (Clark *et al.*, 1986). ELISA has become widely accepted and used as a rapid and sensitive method of immunodetection, to study relationships among plant viruses, as a diagnostic tool, and as a quantitative assay to measure the concentration of virus in different plant tissues (Eweida, Oxelfelt, and Tomenius, 1988). The application of hybridoma technology to plant virology has further enhanced the performance of ELISA as a diagnostic tool by eliminating or reducing the background problems often associated with polyclonal antiserum.

Rapid nucleic acid hybridization techniques using complementary DNA (cDNA) were first developed for the detection of Epstein-Barr virus in animal cells (Brandsma and Miller, 1980) and shortly afterward for the detection of potato spindle tuber viroid (Owens and Diener, 1981; Salazar *et al.*, 1983). This technique became known as dot-blot or nucleic acid spot

hybridization (NASH) and has become a useful alternative to ELISA as a highly specific and sensitive method of identifying and comparing plant viruses (Maule, Hull, and Danson, 1983; Baulcombe, Flavell and Jellis, 1984a,b). NASH is particularly suited to the study of luteoviruses because of their low concentrations in plant tissues and because of the possibility of genomic masking (or transcapsidation). When two viruses replicate simultaneously in the same plant cell some of the virus particles may contain the genome of one virus completely encapsidated by the coat protein of the other virus. This phenomenon is called genomic masking or transcapsidation and has been demonstrated to occur with some luteoviruses (Creamer and Falk, 1990). It is obvious that ELISA would only detect the antigenic properties of the coat protein whereas NASH can identify the viral nucleic acid. Genomic masking of heterologous viral RNA by PLRV protein has never been reported to occur in nature despite the common occurrence of PLRV in mixed infections with other potato viruses. However, Waterhouse and Murant (1983) have presented experimental evidence that PLRV can substitute for carrot red leaf virus (CRLV) in packaging carrot mottle virus nucleic acid (CMotV). Although *Myzus persicae* does not normally transmit CMotV, it can from plants infected with both PLRV and CMotV.

The objectives of this study were to produce cDNA clones of BWYV RNA which can be used in NASH assays to detect viral

nucleic acid; and to compare NASH with ELISA and aphid transmission tests for the detection of PLRV and BWYV in potato leafroll disease samples from Canada and the United States.

4.2 Materials and methods

4.2.1 Virus isolates, purification, and RNA extraction

A sugar beet isolate of BWYV (MacCarthy, 1969) was propagated and purified as described in section 2.2.1. RNA was extracted by vigorously mixing (Maxi Mix II, Thermolyne Corporation, Dubuque, Iowa) purified virus in alkaline-SDS (0.25 M Tris-HCl, pH 8.9, 1.0 mM EDTA, 2% (w/v) SDS) with an equal vol of phenol:chloroform:isoamyl alcohol (25:24:1). The emulsion was broken by centrifugation at 10,000 x *g* for 2 min in an Eppendorf microcentrifuge (Brinkman Instruments, Ltd., Rexdale Ontario). The aqueous phase was pipetted into a sterile microcentrifuge tube and set on ice. The organic phase was re-extracted with an equal vol of sterile, deionized water and the aqueous phases were combined. An equal vol of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the combined aqueous phase, the phases were mixed briefly and centrifuged as above. The aqueous phase was pipetted into another sterile microcentrifuge tube and the RNA was precipitated by the addition of 0.1 vol of 2 M sodium acetate,

pH 5.8 and 2 vol of absolute ethanol. After mixing well and incubating overnight at -20 C, the RNA was pelletted by centrifugation at 10,000 x *g* for 15 min. The pellet was washed with 70% ethanol and recovered by centrifugation, dried under vacuum, and resuspended in sterile, deionized water. The concentration of RNA was estimated spectrophotometrically using an extinction coefficient of 25 $(mg/ml)^{-1}$ cm^{-1} at 260 nm. The purity and integrity of the RNA was determined by electrophoresis in denaturing agarose gels containing methylmercuric hydroxide (Bailey and Davidson, 1976). The RNA's were precipitated with ethanol, resuspended in deionized sterile water, and stored at -70 C until used.

Five BWYV isolates: BWYV-BC, BWYV-Sal(9), BWYV RY-1-R, BWYV RY-7, BWYV-NETH; and four PLRV isolates: PLRV-BC, PLRV-ST4, PLRV-ORE, and PLRV-ID (Table III) were used to determine the specificity of cloned BWYV cDNA.

4.2.2 Synthesis and cloning of cDNA

BWYV RNA was polyadenylated *in vitro* by the method of Sippel (1973). The poly(A)-tailed RNA, heat-denatured at 68 C for 5 min and immediately quenched on ice, served as a template for oligo(dT)-primed, first-strand cDNA synthesis using cloned Moloney murine leukemia virus (M-MLV) reverse transcriptase

(Bethesda Research Laboratories, Gaithersburg, Maryland). The second strand reaction was essentially as described by Gubler and Hoffman (1983), using *Escherichia coli* DNA polymerase I and RNase H (Bethesda Research Laboratories). Double-stranded cDNA was dC-tailed at the 3′ termini using terminal transferase (Bethesda Research Laboratories) and annealed to *PstI*-digested pUC9, dG-tailed by the same method. The recombinant DNA was used to transform competent *E. coli* DH5α cells which were then plated on Luria-Bertani agar containing ampicillin and X-gal. Ampicillin-resistant recombinants were screened by *in situ* colony hybridization (Gergen, Stern, and Wensink, 1979) using random primed, ^{32}P-labelled cDNA synthesized using BWYV RNA as a template (Taylor, Illemensee, and Summers, 1976). Fifty colonies showing the strongest signals were selected and the plasmids were isolated using the alkaline lysis method (Maniatis, Fritsch, and Sambrook, 1982). Plasmids were digested with *PstI* to release the DNA inserts and the mixture was electrophoresed in agarose gels to determine the size of the inserts.

4.2.3 Northern blots

Approximately 100 ng of PLRV and BWYV RNA, and 1 µg of total RNA from healthy potato and *P. pubescens*, prepared by the method of Siegel *et al.* (1976), were analyzed by electrophoresis

in denaturing methylmercury gels (Bailey and Davidson, 1976) and transferred to charge-modified nylon membranes of Nytran (Schleicher and Schuell Inc., Keene, New Hampshire) as described by Vrati, Mann, and Reed (1987).

4.2.4 Collection of samples

With the assistance of seed potato officials from twenty states or provinces (Table VI), 127 tuber samples were collected from plants showing symptoms of potato leafroll disease at the California and Florida winter test plots. In addition, 38 tuber samples were obtained from the winter test program at Oregon State University. The samples, representing 8 cultivars, originated in four provinces and seven states. The tubers were placed in labelled paper bags, packed in coolers, and shipped by air to Vancouver. The tuber samples were stored for 4 months at 4 C, planted in 6-inch plastic pots and the resulting plants were grown in an aphid-free greenhouse. Leaf samples were collected after all of the plants were at least 15 cm high.

Healthy potato plants were grown from virus-free tubers in a separate aphid-free greenhouse. Healthy seedlings of ground cherry (*Physalis pubescens*) and shepherd's purse (*Capsella bursa-pastoris*) were grown from seed and kept in the same greenhouse as the virus-free potatoes.

4.2.5 Preparation of plant extracts

Total nucleic acid extracts from both healthy and virus-infected potato, and BWYV-infected *P. pubescens* leaves were prepared by a modification of the method of Habili, McInnes and Symons (1987). Intact leaf tissue (0.25 g) of each sample was crushed between the rollers of a sap extractor (Erich Pollähne, F.G.R.) during the dropwise addition of 0.5 ml TS buffer (TS buffer is 50 mM Tris-HCl, pH 7.4, 2.0% SDS). Each slurry sample was collected in a 1.5 ml microfuge tube, mixed with an equal vol of water-saturated phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), mixed for 15 sec, and centrifuged at 10,000 x *g* at room temperature for 5 min. The aqueous phase (400 µl) was pipetted into a clean, sterile 1.5 ml microcentrifuge tube and the nucleic acids were precipitated by adding 2.5 vol chilled absolute ethanol, mixing briefly, and incubating at -20 C overnight. After centrifugation at 10,000 x *g* for 15 min at room temperature, the nucleic acid pellet was washed with cold 70% ethanol, dried under vacuum, resuspended in 40 µl of 0.1 mM EDTA, and stored at -70 C.

4.2.6 Preparation of nucleic acid probes

Random primed, ^{32}P-labelled cDNA probes were synthesized using PLRV and BWYV RNAs as templates by the method described by

Taylor *et al.* (1976). A recombinant DNA clone of PLRV in the plasmid vector Bluescript (Stratagene, San Diego, California) was obtained from L. Kawchuk and R. Martin, Agriculture Canada, Vancouver. The clone, designated pLP79, contained an insert of approximately 3.5 kb (Kawchuk *et al.*, 1989). A recombinant DNA clone of BWYV, pBW79 described in section 4.3, in the plasmid vector pUC9 contained an insert of approximately 0.7 kb. Recombinant plasmid PLRV and BWYV DNA were prepared according to the modified alkaline lysis protocol (Maniatis *et al.*, 1982) and labelled with [α-^{32}P] dATP using the oligo-labelling procedure of Feinberg and Vogelstein (1983,1984). Unincorporated ^{32}P-labelled nucleotides were removed by precipitating the probes twice with ammonium acetate and ethanol. The specific activities of the probes were greater than 1 x 10^9 cpm/μg DNA.

4.2.7 NASH procedures

Nitrocellulose filters (Schleicher and Schuell, Inc.) were cut to 12 x 15 cm, marked lightly at 96 equally spaced points with a blunt lead pencil (HB), soaked in glass distilled water for 5 min and then in 20x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 15 min and air dried. Charge-modified nylon filters of Nytran (Schleicher and Schuell), were cut and marked with pencil as above and soaked in glass-distilled water before air drying.

Each sample of total nucleic acid was thawed, mixed briefly, and 2 µl was spotted on the premarked filters. Each sample was spotted twice on both nitrocelluose and Nytran filters. After spotting, the membranes were dried under a lamp for 5 min and then baked under vacuum at 80 C for 2 hr.

The filters were then transferred to plastic, snap-seal containers and prehybridized at 50 C for 4 hr in prehybridization buffer consisting of 50% (v/v) deionized formamide, 1.5x SSPE (SSPE is 0.18 M NaCl, 0.01 M sodium phosphate, pH 7.0, 0.01 M EDTA), 0.5% BLOTTO, 1% (w/v) SDS, and 0.5 mg/ml sheared, denatured salmon sperm DNA (Reed, 1986).

The hybridizations themselves were carried out at 50 C [Tm = -15] (Martin and D'Arcy, 1990) for 16 hr in fresh buffer containing 50% deionized formamide, 10% dextran sulfate, 1.5x SSPE, 0.5% BLOTTO, and 0.5 mg/ml sheared, denatured salmon sperm DNA (Reed, 1986).

At the completion of hybridization, the membranes were removed from the hybridization solution and washed briefly in 2x SSC, then successively by vigorous agitation for 15 min at room temperature in the following solutions (using approximately 50 ml/membrane): (1) 2.0x SSC/0.1% SDS, (2) 0.5x SSC/0.1% SDS, and (3) 0.1x SSC/0.1% SDS. The membranes were given a final wash at 50 C for 20 min. in prewarmed 0.1x SSC/1% SDS, rinsed in 0.1x

SSC, blotted lightly to remove excess moisture, and immediately wrapped in plastic film. The membranes were exposed to X-OMAT film (Kodak) for 24 to 72 hr at -70 C in an X-ray cassette containing Lightning Plus (Dupont) intensifying screens.

4.2.8 Antisera and monoclonal antibodies

As in section 3.2.2.

4.2.9 Double antibody sandwich ELISA

A modification of the double antibody sandwich ELISA (DAS-ELISA) of Clark and Adams (1977) was used. All reagents were used at 100 µl/well except for blocking steps which were 300 µl/well. Linbro flat-bottomed microtiter plates (Flow Laboratories Inc.) were coated with purified immunoglobulin diluted to 1 µg/ml in PBS overnight at 4 C. The plates were then blocked with 2% BLOTTO in PBS for 30 min at room temperature. The blocking agent was shaken out and plant extract added and incubated overnight at 4 C. Each plant sample (0.1 g) was extracted with a sap press (Erich Pollähne, F.G.R.) while adding 900 µl grinding buffer (PBS: 0.5% Tween 20: 0.2% BLOTTO: 2% PVP, v:v:v:w) dropwise on the rollers. Following the incubation step, the plant extract was washed out of the wells

with PBS-T, three times for 1 min each. The plates were blocked again, as before. Blocking agent was shaken out of the wells and alkaline phosphatase conjugates (cross-absorbed for 1 hr at room temperature with sap from 1 g healthy potato leaf tissue in 10 ml grinding buffer) diluted to 1 µg/ml in PBS-T were added and incubated for 3 hr at room temperature. The wells were washed again as above except that the final wash was with tap water to remove any remaining phosphate. Substrate (ρ-nitrophenyl phosphate, Sigma 104-105, 0.5 mg/ml in 10% diethanolamine, pH 9.8) was added and incubated for 1 hr at room temperature. The absorbance at 405 nm (A_{405}) of each well was determined in a Titertek Multiscan MCC plate reader (Flow Laboratories Inc.). Reactions were considered to be positive when the A_{405} readings were greater than three times the mean of the readings for five healthy control samples (Voller *et al.*, 1977).

4.2.10 Triple antibody sandwich ELISA

Each sample was tested twice for PLRV and BWYV using virus-specific MAbs and TAS-ELISA, exactly as described in section 2.2.4/3.2.4.

4.2.11 Aphid transmission tests

As in section 3.2.5

4.3 Results

Approximately 200 transformants, containing cDNA sequences homologous to BWYV genomic RNA, were selected by colony hybridization. The majority of the inserts were less than 1000 bp as determined by agarose gel electrophoresis. The origin of one insert of approximately 0.7 kb in plasmid pBW79 was confirmed by northern blot analysis. A ^{32}P-labelled probe synthesized from pBW79 was hybridized specifically to BWYV RNA but did not hybridize with PLRV RNA or with total RNA purified from healthy potato or ground cherry.

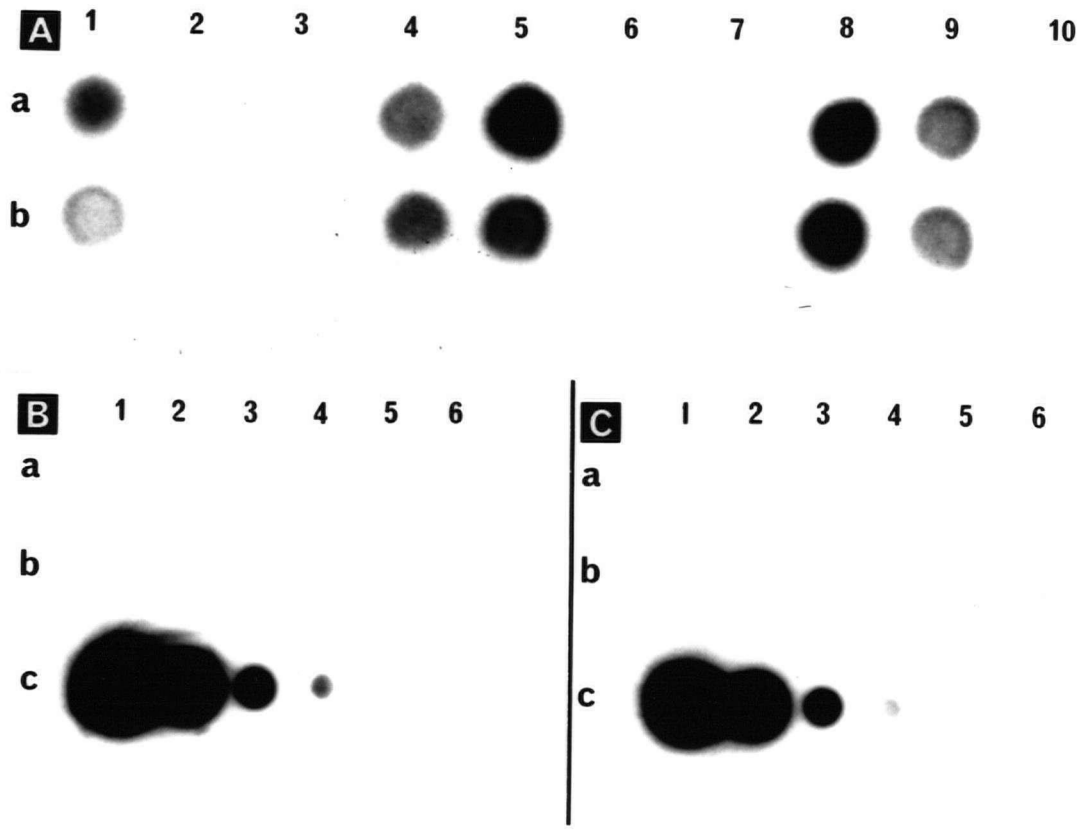
The specificity of pBW79 was demonstrated by its hybridization to RNA of five BWYV isolates from Canada, USA, Germany, and the Netherlands; and by its failure to hybridize to RNA of four PLRV isolates and RNA from virus-free potato plants (Fig. 9A).

The limit of detection of BWYV RNA by a ^{32}P-oligolabelled probe from pBW79 and random primed cDNA prepared from BWYV RNA was 100 pg in a tenfold dilution series (Fig. 9B and 9C).

Fig. 9. (A) Detection of beet western yellows virus (BWYV) isolates by nucleic acid spot hybridization using ^{32}P-oligolabelled pBW79. Each spot represents nucleic acid extracted from 10 mg of leaf tissue. The samples are: 1a,b BWYV-BC; 2a,b PLRV-BC; 3a,b PLRV-ORE; 4a,b BWYV Sal(9); 5a,b BWYV RY-7; 6a,b PLRV ST4; 7a,b PLRV-ID; 8a,b BWYV RY-1-R; 9a,b BWYV-NETH; virus-free potato. The autoradiograph was exposed for 72 hr.

(B) Detection of BWYV-BC RNA in a tenfold dilution series from 100 ng (1c) to 1 pg (6c) using ^{32}P-labelled, random primed cDNA prepared from BWYV RNA. Equivalent amounts of RNA from virus-free potato and potato leafroll virus (PLRV) were spotted at 1a to 6a and 1b to 6b, respectively. Samples of viral RNA were mixed with RNA from 10 mg of virus-free potato leaves before spotting. The autoradiograph was exposed for 24 hr.

(C) The same as (B) except the probe was ^{32}P-oligolabelled pBW79.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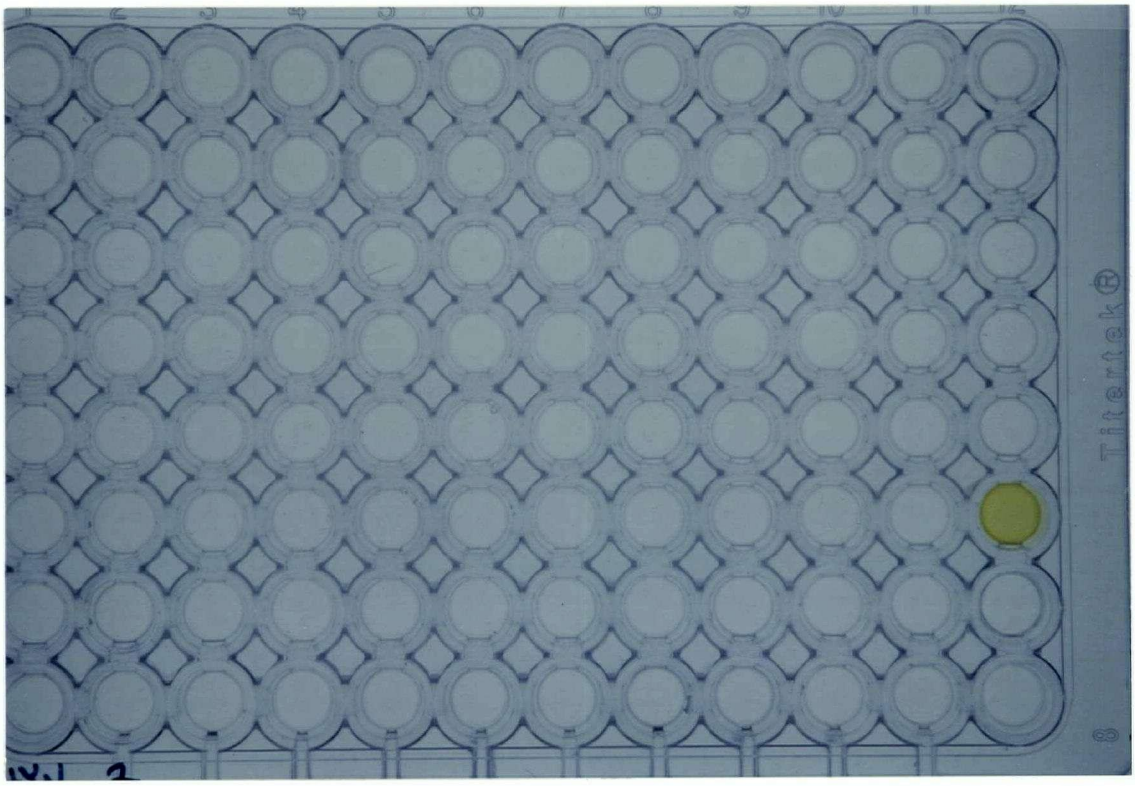


Fig. 10. Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for beet western yellows virus (BWYV) in potato leafroll disease samples. Virus-free control samples were tested in wells 12a-e, BWYV-infected *Physalis pubescens*, the positive control, was in well 12f. No BWYV was detected and there was no cross-reaction with the potato leafroll virus positive control in well 12g. The rows are identified as a-h from top to bottom and the columns are numbered 1-12 from left to right.

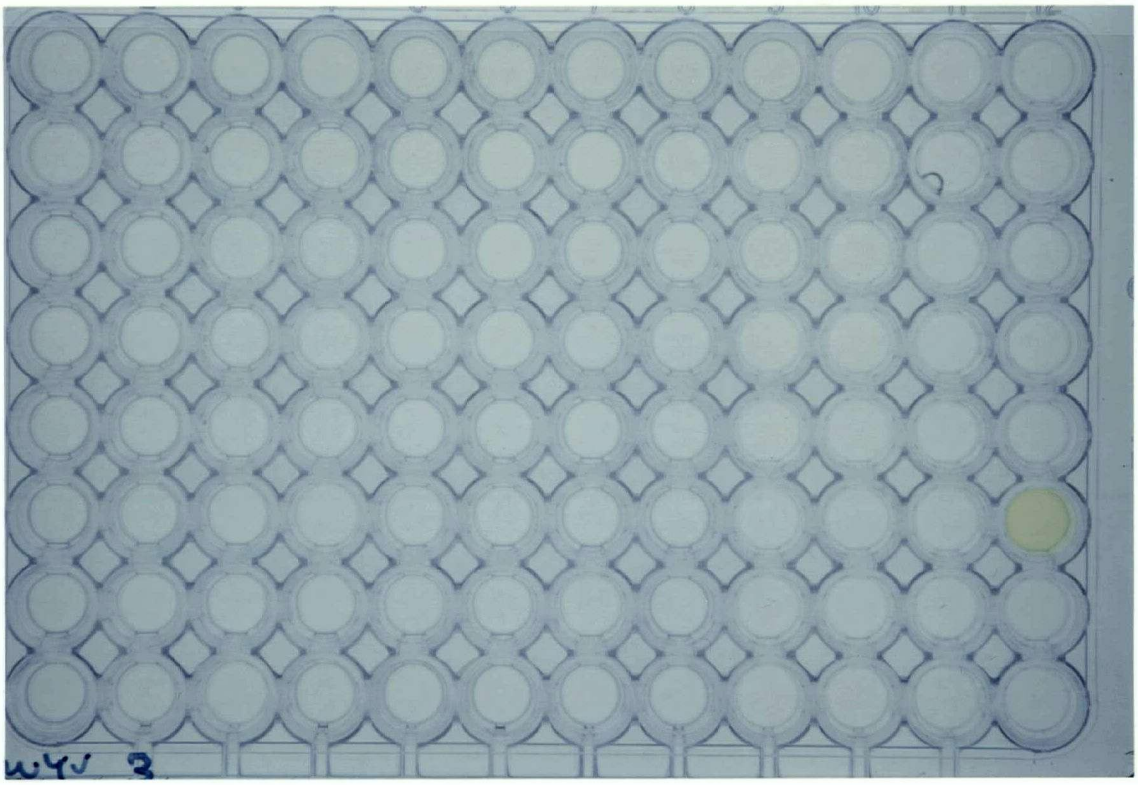


Fig. 11. Triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) for beet western yellows virus (BWYV) in potato leafroll disease samples. Virus-free control samples were tested in wells 12a-e. The positive control, BWYV-infected *Physalis pubescens*, was in well 12f. No BWYV was detected and there was no cross-reaction with the potato leafroll virus positive control in well 12g. The rows are identified as a-h from top to bottom and the columns are numbered from 1-12 from left to right.

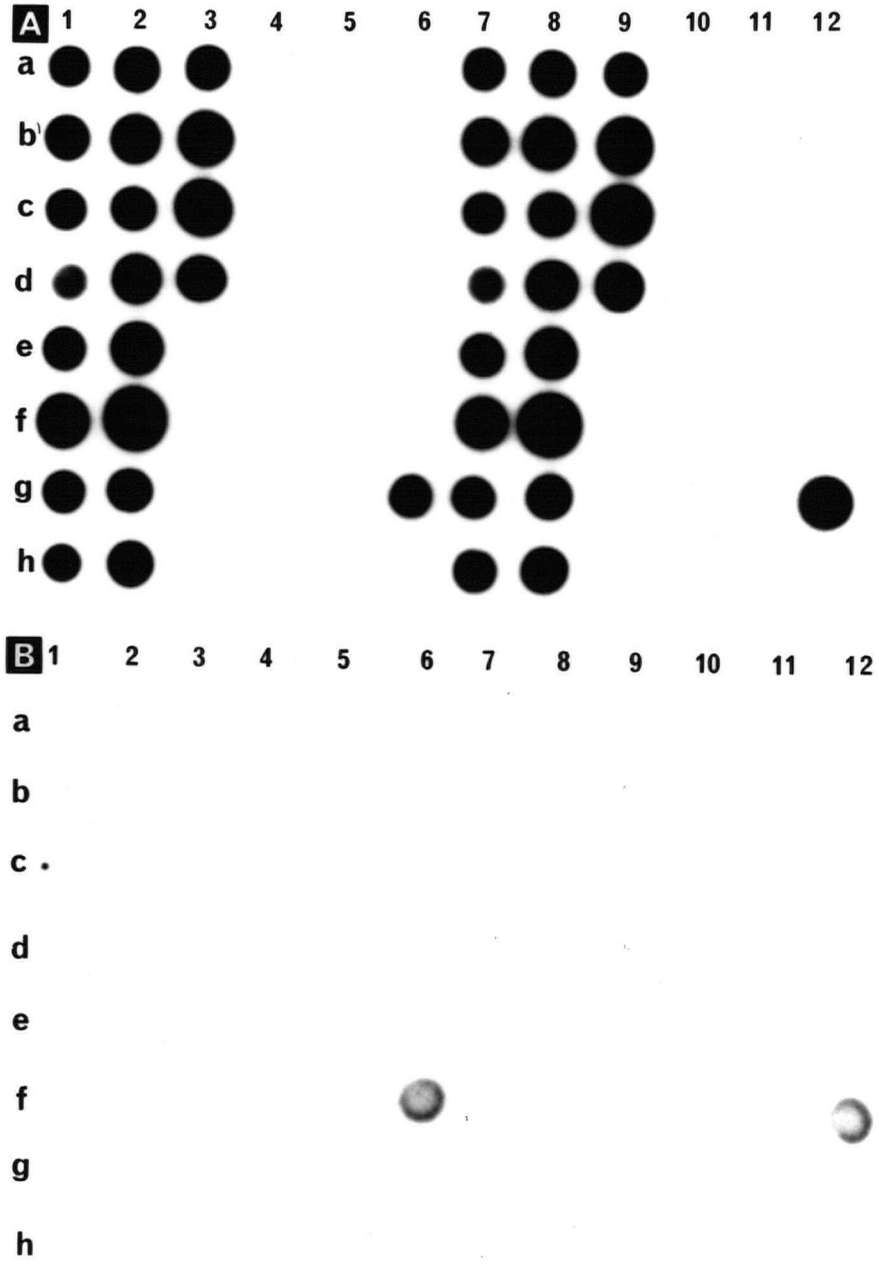
The probes did not cross-hybridize with PLRV RNA or RNA purified from virus-free potato leaves. None of the potato leafroll disease samples tested positive for BWYV by either DAS- or TAS-ELISA (Figs. 10 and 11) , or by NASH using either cloned (Fig. 12) or random primed cDNA probes (Fig. 13). BWYV was not transmitted, using aphids, from any of the potato leafroll samples to ground cherry or shepherd's purse indicator plants as determined by TAS-ELISA. In contrast, all of the potato leafroll disease samples tested positive for PLRV by TAS-ELISA and NASH using a cloned cDNA probe from pLP79. One false-negative result was obtained by both DAS-ELISA and NASH using random primed cDNA prepared from PLRV RNA. PLRV was recovered from the potato leafroll disease samples that tested negative, using aphid transmission to ground cherry. All of the PLRV isolates infected ground cherry and most (119/165) infected shepherd's purse (Fig. 14).

Equivalent results (Fig. 15) were obtained by NASH using either Nytran or nitrocellulose membranes except that the signals from Nytran were more diffuse than those from nitrocellulose.

Fig. 12. Nucleic acid spot hybridization (NASH) of 20 potato leafroll disease samples. Each spot is duplicated on both membranes and represents nucleic acid extracts from 10 mg of leaf tissue. Virus-free control samples are located at positions 6- and 12a-f, and symptomless-potato control samples collected at the winter test plots are spotted at positions 3- and 9e-h. Potato leafroll virus (PLRV) positive controls are at positions 6- and 12g, and beet western yellows virus (BWYV) positive controls are at positions 6 and 12f. Positions 4-, 5-, 10- and 11a-h are blank.

(A) Detection of PLRV with ^{32}P-oligolabelled pLP79.

(B) Detection of BWYV with ^{32}P-oligolabelled pBW79.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1 2 3 4 5 6 7 8 9 10 11 12
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Fig. 13. Nucleic acid spot hybridization (NASH) of potato leafroll disease samples. Each sample is spotted on both membranes and represents nucleic acid extracts from 10 mg of leaf tissue. Virus-free control samples are located at positions 12a-f, and symptomless-potato control samples collected at the winter test plots are spotted at the following positions: 1a; 2f,g; 3c,f,g; 5g; 7g; 8d; and 10d. The potato leafroll virus (PLRV) positive control is at position 12g, and the beet western yellows virus (BWYV) control is at position 12f. Positions 10g,h; and 11a-h are blank.

(A) Detection of PLRV with ^{32}P-labelled random primed cDNA prepared from PLRV RNA.

(B) Detection of BWYV with ^{32}P-labelled random primed cDNA prepared from BWYV RNA.

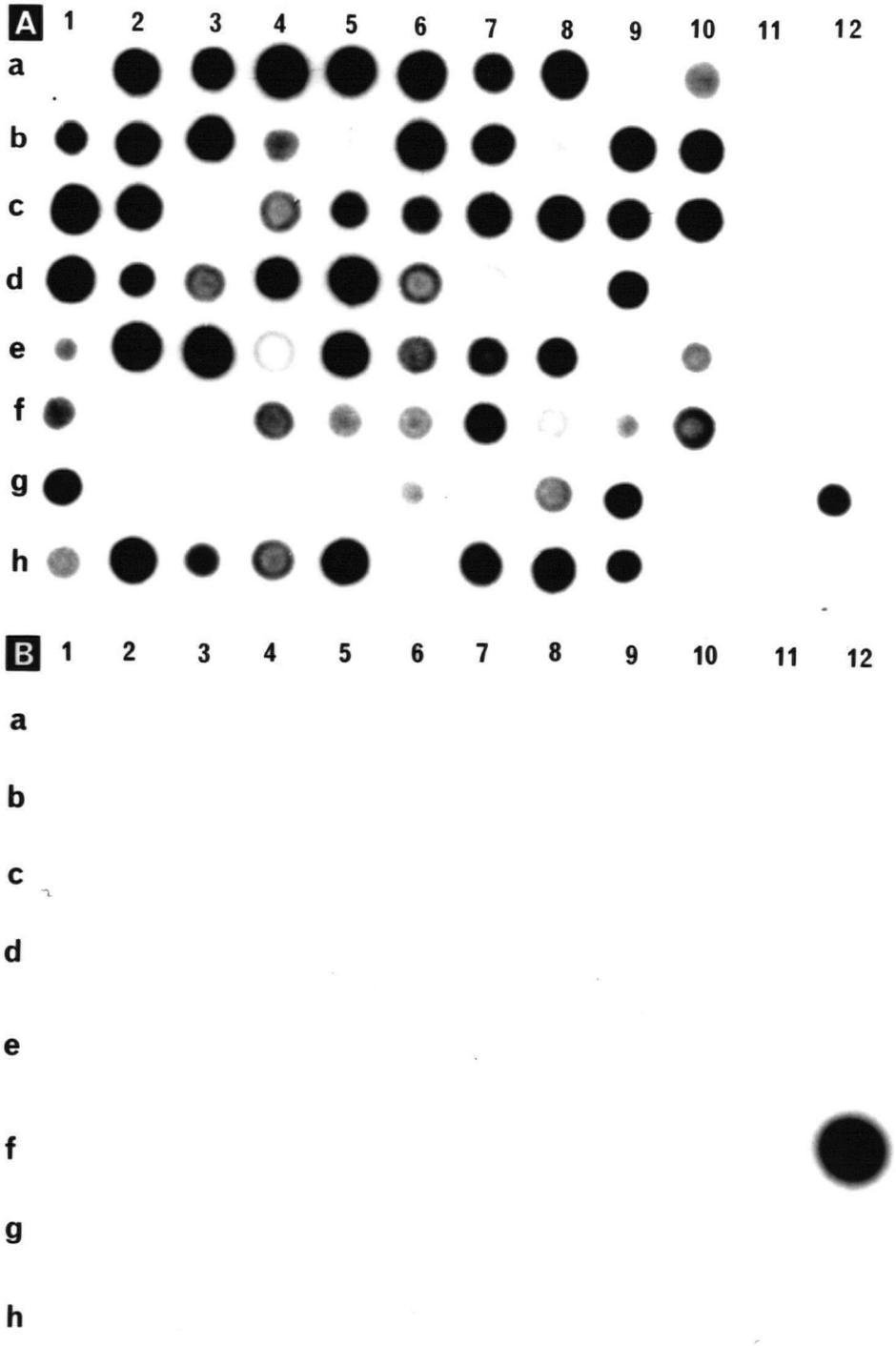
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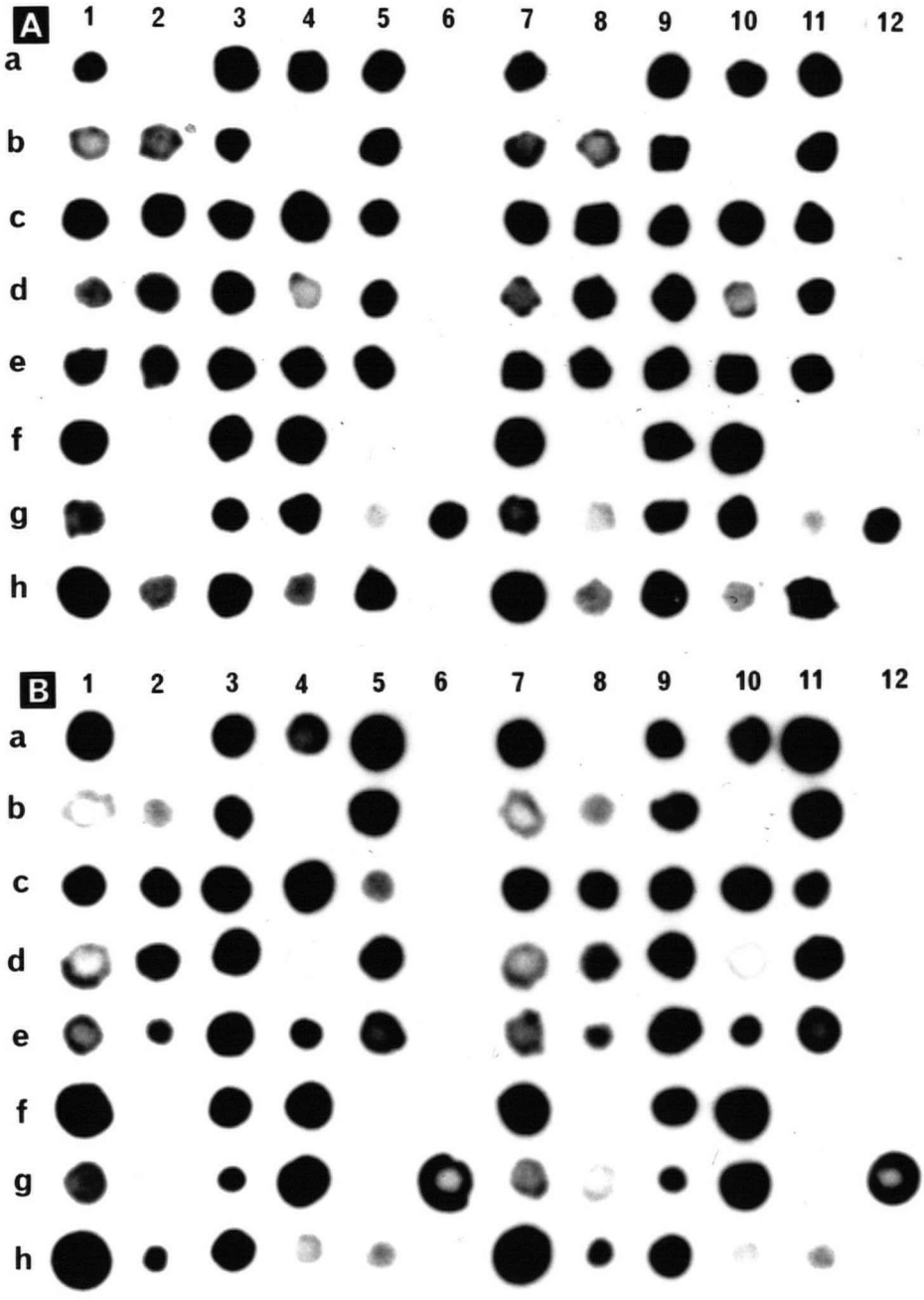
Fig. 14. *Capsella bursa-pastoris* infected with potato leafroll virus (right), and healthy plant (left).

Fig. 15. Nucleic acid spot hybridization (NASH) of potato leafroll disease samples with ^{32}P-oligolabelled pLP79. Each sample represents 10 mg of leaf tissue and is duplicated on both the (A) nitrocellulose membrane and (B) Nytran membrane. Virus-free control samples are located at positions 6- and 12a-f, and symptomless-potato control samples collected at the winter test plots are located at the following positions: 2- and 8a; 2- and 8f; 4- and 10b; and 5- and 11f. Potato leafroll virus positive controls are at positions 6- and 12g, and beet western yellows virus positive controls are at positions 6- and 12f.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4.4 Discussion

The results show that BWYV was not detected in potato leafroll disease samples from Canada and the United States by ELISA or NASH, whereas all of the samples tested positive for PLRV. Most of the PLRV isolates infected shepherd's purse and it is now clear that this plant is not a reliable diagnostic host for BWYV as was previously reported (Duffus, 1981a,b). Several research reports confirm that shepherd's purse is a host for some strains of PLRV (Fox *et al.*, 1990; Syller, 1985; Thomas, 1984) and it is being investigated as a potential overwintering host for PLRV in some potato-producing areas in Canada and the United States (Fox *et al.*, 1990).

In this study, equivalent results were obtained by ELISA and NASH, although based on the detection of different macromolecules. The results presented here show that both ELISA and NASH using either cloned or random primed cDNA, are useful and sensitive methods for screening plants for the presence of PLRV and BWYV. Under optimal conditions, approximately 1 ng/ml (100 pg/well) of antigen can be detected by ELISA (van Regenmortel, 1982). NASH is at least as sensitive. With random primed cDNA, 100 pg of purified BWYV RNA/spot was easily detected and 10 pg/spot was barely detectible after a 48 hr exposure. Cloned cDNA from pBW79 (approximately 0.7 kb) was less sensitive but 100 pg of BWYV RNA could be easily detected.

The sensitivity of NASH could be increased by using a mixture of cloned probes, complementary to the entire RNA genome, or by using RNA probes. Salazar, Balbo, and Owens (1988) have demonstrated that as little as 0.33 pg of PSTV RNA could be detected using RNA probes prepared by transcription of plasmid DNA templates containing a promotor for bacteriophage SP6 polymerase.

When extreme sensitivity is required, the polymerase chain reaction (PCR) provides an unusually simple method for making virtually an unlimited number of copies of cDNA from viral RNA, before detection with nucleic acid probes (Kawasaki, 1990). Byrne *et al.*, (1988) used this technique for the detection of retroviral RNA after first producing cDNA, using M-MLV reverse transcriptase, and then allowing the standard *Taq*-polymerase-based system to amplify the cDNA.

Although both ELISA and NASH allow sensitive and specific detection of PLRV and BWYV, at the present time ELISA is the method of choice for routine testing of plant samples, especially if the laboratory is not set up for handling radioactive isotopes. Storing and handling the reagents for ELISA are not technically demanding; MAbs can be stored as ascites fluid diluted 1:1 with saturated ammonium sulfate at 4 C for several years without loss of titer. NASH requires considerable handling; using a sap press for total nucleic acid

preparation, several hundred samples can be processed in a day compared to about one thousand samples for ELISA. Furthermore, autoradiography requires a dark room and is more time-consuming than reading ELISA plates. The specificity and sensitivity of molecular hybridization makes the method a valuable adjunct to ELISA. If sensitive discrimination between viral strains is required, the flexibility of the hybridization step in NASH allows control of the stringency of hybridization. Another major advantage of NASH is that the method is not restricted to one region of the genome; for plant-pathogenic viruses, only about 2-5% of the nucleic acid of the viral genome is represented in the antigenic determinants of the coat protein (Miller and Martin, 1988).

A major advantage of cloned probes is their ease of preparation in contrast to purifying virus and extracting RNA to make random primed cDNA probes. Furthermore, viral RNA may not be 100% pure. Jayasena, Randles, and Barnett (1984) and Smith *et al.*, (1988) have shown that host DNA may co-purify with luteovirus RNA either as a result of attachment to, or encapsidation in, luteovirus particles. Contaminating DNA may also become labelled during the preparation of random primed cDNA (Taylor *et al.*, 1976) and may lead to false-positive results.

Nonradioactive probes are comparable to ^{32}P-labelled probes in sensitivity and have a distinct advantage in terms of safety, cost, and stability. Habili *et al.* (1987) have shown that photobiotin-labelled probes are stable for at least 8 months when stored at -20 C. This technique shows considerable promise for the detection and diagnosis of the luteoviruses. Recent developments in nonradioactive nucleic acid detection methods, resulting in sensitivity in the subpicogram range (Carlson *et al.*, 1990), may lead the way for nucleic acid hybidization techniques to replace ELISA as the standard method for routine virus detection and diagnosis.

The results of ELISA, NASH, and aphid transmission tests reported here refute earlier reports that BWYV is a common and important component of a complex causing potato leafroll disease. To the contrary, the results support the hypothesis that potato leafroll disease is caused by one virus or a group of closely related viruses properly called PLRV. Keese *et al.* (1990) have recently reported that there is little genetic diversity among the viruses named PLRV.

Chapter 5

Susceptibility of potato to BWYV isolates from Canada and the United States

5.1 Introduction

There is an obvious discrepancy between research that suggests that BWYV occurs widely in North America and is an important component of a potato leafroll disease complex (Duffus, 1981a,b; Sibara and Slack, 1985a,b; and Gallenberg *et al.*, 1987); and experimental results that find BWYV to be absent or undetectable in potato leafroll-affected plants (Clarke, Powelson, and Beraha, 1983; Ellis and Wieczorek, 1988; Ellis, 1989). Tamada *et al.* (1984) failed to find virus isolates resembling BWYV in potato in Britain; Barker (1986) failed to infect potato with British isolates of BWYV; Marco (1984) could not infect potato with BWYV isolates from Israel, and Casper (1983) failed to transmit virus isolates from leafroll infected potatoes to a host range typical of BWYV. Despite these results, the possibility exists that some isolates of BWYV in North America differ enough from the European isolates that they may infect potato. The objective of this experiment was to determine the susceptibility of potato to seven isolates of BWYV from Canada and the United States.

5.2 Materials and methods

5.2.1 Virus isolates and transmission tests

Three isolates of BWYV from Canada and four from the United States were used in this study. Two of these isolates, IPTT752 and BW/PL-5.2, were in plants with mixed infections of BWYV and PLRV. Two isolates of PLRV, one from British Columbia and one from Washington State were included as controls. The isolates of BWYV and PLRV used, the original host from which they were isolated, when known, and the donor of the isolate are listed in Table VII. All of the isolates were maintained in ground cherry (*Physalis pubescens*).

Table VII. Virus isolates used in aphid transmission tests

Virus isolate	Origin	Host	Donor*
BWYV-BC	B.C., Canada	sugar beet	1
BWYV-12H	B.C., Canada	common groundsel	2
BWYV-210E	B.C., Canada	common groundsel	2
BWYV-CA	California, USA	common mallow	2
BWYV-D3	Washington, USA	ground cherry	3
IPTT752	Washington, USA	turnip	3
BW/PL-5.2	Washington, USA	ground cherry	3
PLRV-LR7	Washington, USA	potato	3
PLRV-BC	B.C. Canada	potato	4

* 1. H.R. MacCarthy, Agriculture Canada, Vancouver Research Station, Vancouver, B.C.
2. P.J. Ellis, Agriculture Canada, as above.
3. P.E. Thomas, USDA, Prosser, Washington.
4. N.S. Wright, Agriculture Canada, Vancouver Research Station, Vancouver, B.C.

Virus-free GPA were reared on Chinese cabbage. Apterous aphids were allowed an acquisition access feed of 72 hr or longer on ground cherry infected with each virus isolate. About 20 of these now viruliferous aphids were transferred with camel hair brushes to each test plant and allowed an inoculation access feed of 72 hr, after which they were killed by spraying the plants with pirimicarb at 0.25 g ai/l. Two test plants each of shepherd's purse and ground cherry at the four true-leaf stage, and 10 Russet Burbank potato plants about 4-5 cm high were inoculated with each virus isolate. About six weeks after inoculation, virus-free GPA were allowed a 72 hr access feed on leaves of the potato test plants and then they were transferred to shepherd's purse and ground cherry indicator plants for inoculation as above.

5.2.2 ELISA

Potato and the other indicator plants were assayed for PLRV and BWYV using TAS-ELISA as described in section 3.2.4. Each plant was tested at least twice at six weeks following inoculation.

5.3 Results

The results of the transmission tests are presented in Table VIII.

Table VIII. Mean virus-specific TAS-ELISA (A_{405}) results of attempts to transmit beet western yellows virus (BWYV) and potato leafroll virus (PLRV) isolates to potato.

Virus isolate	Shepherd's purse		Ground cherry		Potato	
	BWYV[1]	PLRV[2]	BWYV	PLRV	BWYV	PLRV
BWYV-BC	0.375 (2/2)[3]	0.027 (0/2)	1.203 (2/2)	0.017 (0/2)	0.005 (0/10)	0.008 (0/10)
BWYV-12H	1.978 (0/2)	0.015 (0/2)	0.751 (2/2)	0.014 (0/2)	0.000 (0/10)	0.002 (0/10)
BWYV-210E	1.303 (2/2)	0.008 (0/2)	1.325 (2/2)	0.013 (0/2)	0.000 (0/10)	0.000 (0/10)
BWYV-CA	0.187 (2/2)	0.016 (0/2)	0.239 (2/2)	0.017 (0/2)	0.004 (0/10)	0.006 (0/10)
BWYV-D3	0.782 (2/2)	0.016 (0/2)	1.120 (2/2)	0.007 (0/2)	0.007 (0/10)	0.006 (0/10)
IPTT-752	1.250 (2/2)	0.141 (2/2)	0.273 (2/2)	2.640 (2/2)	0.000 (0/10)	0.431[4] (6/10)
BW/PL-5.2	0.702 (2/2)	0.026 (0/2)	0.949 (2/2)	2.870 (2/2)	0.000 (0/10)	0.364[5] (5/10)
PLRV-BC	0.000 (0/2)	2.887 (2/2)	0.000 (0/2)	3.047 (2/2)	0.001 (0/10)	1.934 (10/10)
PLRV-LR7	0.010 (0/2)	1.212 (2/2)	0.009 (0/2)	3.084 (10/10)	0.005 (0/10)	2.473 (10/10)
Cont.[6] VF-GPA	0.000 (0/2)	0.022 (0/2)	0.000 (0/2)	0.031 (0/2)	0.000 (0/10)	0.007 (0/10)
Cont.[7] No GPA	0.007 (0/4)	0.021 (0/4)	0.001 (0/4)	0.007 (0/4)	0.008 (0/10)	0.008 (0/10)

[1]Mean BWYV TAS-ELISA (A_{405}) reading
[2]Mean PLRV TAS-ELISA (A_{405}) reading
[3]Numbers in brackets indicate # positive by TAS-ELISA/# tested
[4]Mean PLRV TAS-ELISA (A_{405}) reading for 6 of 10 potato plants rated positive by TAS-ELISA
[5]Mean PLRV TAS-ELISA (A_{405}) reading for 5 of 10 potato plants rated positive by TAS-ELISA
[6]Virus-free control plants in which 20 nonviruliferous GPA (VF-GPA) were allowed to feed for 72 hr
[7]Virus-free control plants maintained free of aphids

5.4 Discussion

None of the potato plants tested positive by ELISA for BWYV, either when inoculated with a single BWYV isolate or when inoculated with a mixed infection of PLRV and BWYV from ground cherry. The presence of virus in the inoculating aphids was confirmed by inoculation and infection of the susceptible indicator plants, shepherd's purse and ground cherry. The TAS-ELISA results were confirmed by the back testing of inoculated potatoes with aphid transmission to the indicator plants. BWYV could not be recovered by aphids from any (0/90) of the inoculated potatoes. In contrast, PLRV was readily recovered from many (31/40) of the plants inoculated with this virus. The aphid transmission results support the results of serological and nucleic acid hybridization tests that could not confirm the presence of BWYV in leafroll-affected potatoes in Canada and the United States (Ellis, 1989; Ellis, 1990; Ellis and Wieczorek, 1988). Therefore, like Casper (1983), Marco (1985), and Barker (1986) I have not found any evidence that potato is a host for BWYV. Although there is a possibility that some BWYV isolates can infect potato, there may be an alternative explanation for the earlier reports that BWYV is a widespread and important component of the potato leafroll disease in North America. The evidence is now overwhelming that PLRV does indeed infect shepherd's purse (Fox *et al.*, 1990; Syller, 1985; and Thomas, 1984), and the use of this plant as a differential host for BWYV

and PLRV is an error. Because BWYV and PLRV are serologically related luteoviruses and because they have at least one epitope in common (section 2.3.2), polyclonal antiserum may have led to questionable results and conclusions. The application of sensitive and specific serological tests (section 3.3) and nucleic acid hybridization tests (section 4.3) combined with the biological data presented here strongly suggest that BWYV is not a threat to table stock or seed-potato production at the present time.

Chapter 6

A survey of weeds as reservoirs for BWYV and PLRV

6.1 Introduction

BWYV occurs in a wide range of crop and weed hosts in the United States (Duffus, 1977; Timmerman, D'Arcy, and Splittstoesser, 1985) and has recently been implicated as an important component of potato leafroll disease (Duffus, 1981a,b). In Canada, the importance of weeds as reservoirs of potato viruses has not been closely studied (Singh, 1987). Because of the known wide host range of BWYV and its potential threat to both seed and table potato crops (Duffus, 1981a,b), a survey was conducted to evaluate common weeds, wild species and some volunteer crop plants as reservoirs of viruses inducing potato leafroll symptoms. An additional objective was to determine the occurrence of BWYV and PLRV in weeds in the potato-producing areas of British Columbia. In this paper, native and naturalized flora, and volunteer or abandoned cultivated crop plants are considered to be weeds. A preliminary report has been published (Ellis, 1988).

6.2 Materials and methods

6.2.1 Collection of samples

Foliage samples of common weeds found in British Columbia, and some volunteer crop plants, were collected in the main potato-producing areas of the province. The samples were collected from fence rows, irrigation ditches, and within both seed and table potato fields. The plants were collected at many different growth stages - from four expanded true leaves to the senescent growth stage (Lutman and Tucker, 1987). Symptoms were not used as a criterion for selection of samples because luteovirus symptoms may be easily confused with those of drought, senescence, waterlogging, nutritional imbalance, or herbicide injury (Duffus, 1977). Each sample consisted of a stem and leaves, in good condition, from the middle of the plant canopy. Samples were kept in plastic bags at 4 C until used. Collection sites were selected at random from a list of potato producers provided by staff of Agriculture Canada and the British Columbia Ministry of Agriculture and Fisheries.

6.2.2 Antisera and monoclonal antibodies

As in section 3.2.3.

6.2.3 ELISA

Each sample was tested twice using the TAS-ELISA procedure described in section 3.2.4. Positive and negative controls consisted of BWYV-infected ground cherry (*Physalis pubescens*), PLRV infected potato, and healthy greenhouse plants of the species being tested grown from seed. Infection with either BWYV or PLRV was indicated when absorbance readings (A_{405}) of sample wells were greater than twice the mean absorbance reading of five healthy controls (Timmerman *et al.*, 1985) or 0.05, whichever was the greater. Plant samples rated positive by TAS-ELISA were further tested, by aphid transmission to indicator plants, to check the validity of ELISA results.

6.2.4 Aphid transmissions

For aphid transmission tests, about 20 virus-free GPA were allowed a 48 hr access to leaf samples in sealed petri dishes. After acquisition, the aphids were placed on healthy indicator plants (*P. pubescens*) for an inoculation access period of 72 hr. The aphids were then killed with pirimicarb at 0.25 g ai/l. The plants were kept in an aphid-free greenhouse for six weeks and checked for symptoms. Leaf samples were taken from the indicator plants at that time and assayed for BWYV and PLRV by TAS-ELISA.

6.3 Results

A total of 10,182 weed samples, representing 98 species in 22 families, were tested by ELISA for virus infection (Table IX). BWYV was found in 101 (1.0%) of the samples and PLRV in six samples (0.06%). The species testing positive are listed in Table X. Aphid transmission tests were attempted on all of the samples that tested positive by ELISA except for a few samples that had become desiccated. Aphid transmission experiments confirmed the TAS-ELISA results in all cases, except for the transmission of BWYV from 2 of 3 samples of prickly lettuce and 5 of 7 samples of scentless chamomile.

Table IX. Plants surveyed for beet western yellows virus and potato leafroll virus in the potato-producing areas of British Columbia

Family	Botanical name*	Common name*	#
Amaranthaceae	*Amaranthus retroflexus* L.	redroot pigweed	290
Boraginaceae	*Lappula echinata* Gilib.	bluebur	2
Caryophyllaceae	*Spergula arvensis* L.	corn spurry	364
	Stellaria media (L.) Vill.	chickweed	626
Chenopodiaceae	*Beta vulgaris* L.	table beet	7
	Chenopodium album L.	lamb's-quarters	26
	Spinacia oleracea L.	garden spinach	3
Compositae	*Achillea millefolium* L.	yarrow	12
	Arctium minus (Hill) Bernh.	common burdock	6
	Aster sp.	wild aster	5
	Bellis perennis L.	English daisy	6
	Chrysanthemum leucanthemum L.	ox-eye daisy	9
	Cirsium arvense (L.) Scop.	Canada thistle	260
	C. vulgare (Savi) Tenore	bull thistle	3
	Erigeron canadensis L.	Canada fleabane	5

Table IX. Continued

Compositae	*Gnaphalium uliginosum* L.	low cudweed	1
	Hypochoeris radicata L.	spotted cat's-ear	230
	Lactuca muralis (L.) Gaertn.	wall lettuce	6
	L. sativa L.	garden lettuce	3
	L. scariola L.	prickly lettuce	297
	Matricaria maritima L.	scentless chamomile	209
	M. matricarioides (Less.) Porter	pineappleweed	65
	Senecio jacobaea L.	tansy ragwort	9
	S. vulgaris L.	common groundsel	459
	Solidago canadensis L.	Canada goldenrod	8
	Sonchus arvensis L.	smooth perennial sow-thistle	3
	S. asper (L.) Hill	spiny annual sow-thistle	13
	S. oleraceus L.	annual sow-thistle	22
	Tanacetum vulgare L.	tansy	7
	Taraxacum officinale Weber	dandelion	456
Convolvulaceae	*Convolvulus arvensis* L.	field bindweed	10
Cruciferae	*Alyssum alyssoides* L.	small alyssum	1
	Armoracia rusticana P. Goertn., B. Mey & Scherb.	horseradish	1

Table IX. Continued

Cruciferae	*B. napus var. napobrassica* L.	rutabaga	49
	B. oleracea var. botrytis L.	ball cabbage	1
	B. oleracea var. capitata L.	cauliflower	8
	B. pekinesis Rupr.	Chinese cabbage	1
	Camelina microcarpa Andrz.	small-seeded false flax	64
	Capsella bursa-pastoris (L.) Medic.	shepherd's purse	1309
	Cardamine oligosperma Nutt.	little western bittercress	100
	Cardaria draba (L.) Desv.	heart-podded hoary cress	31
	Descurainia pinnata (Walt.) Britt.	green tansy mustard	125
	Lipidium perfoliatum L.	clasping-leaved pepper-grass	1
	Nasturtium officinale R. Br.	water cress	177
	Nestlia paniculata (L.) Desv.	ball mustard	2
	Raphanus raphanistrum L.	wild radish	35
	Rorippa sylvestris (L.) Bess.	creeping yellow cress	190
	Sinapis arvensis L.	wild mustard	255
	Sisymbrium altissimum L.	tumble mustard	9
	S. officinale (L.) Scop.	hedge mustard	233
	Thlaspi arvense L.	stinkweed	653

Table IX. Continued

Geraniaceae	*Erodium cicutarium* (L.) L'Her.	stork's-bill	174
	Geranium molle L.	dovesfoot geranium	2
Labiatae	*Galeopsis tetrahit* L.	hemp-nettle	194
	Lamium amplexicaule L.	henbit	7
	Melissa officinalis L.	lemon-balm	6
	Mentha arvensis L.	field mint	9
	Prunella vulgaris L.	heal-all	3
Leguminosae	*Medicago lupulina* L.	black medic	10
	M. sativa L.	alfalfa	23
	Pisum sativum L.	garden pea	5
	Trifolium pratense L.	red clover	213
	T. repens L.	white clover	125
	Vicia sp.	vetch	2
Malvaceae	*Malva neglecta* Wallr.	common mallow	102
	M. parviflora L.	small-flowered mallow	3

Table IX. Continued

Onagraceae	*Epilobium angustifolium* L.	fireweed	25
	E. minutum Lindl.	small-flowered willow-herb	15
	Oenothera erythrosepala Borb.	red-sepaled evening-primrose	1
Papaveraceae	*Papaver nudicaule* L.	Iceland poppy	1
	P. somniferum L.	opium poppy	8
Plantaginaceae	*Plantago lanceolata* L.	narrow-leaved plantain	158
	P. major L.	broad-leaved plantain	63
Polygonaceae	*Polygonum aviculare* L.	prostrate knotweed	12
	P. convolvulus L.	black bindweed	106
	P. lapathifolium L.	pale smartweed	264
	P. persicaria L.	lady's-thumb	221
	P. scabrum Moench	green smartweed	8
	Rumex acetosella L.	sheep sorrel	190
	R. crispus L.	curled dock	335

Table IX. Continued

Portulacaceae	*Montia perfoliata* (Donn) Howell	miner's lettuce	8
	Portulaca oleracea L.	purslane	101
Ranunculaceae	*Ranunculus repens* L.	creeping buttercup	199
Rosaceae	*Fragaria vesca* L.	wild strawberry	73
	Geum macrophyllum Willd.	large-leaved avens	40
	Rosa sp.	wild rose	2
	Rubus hispidus L.	trailing blackberry	2
Rubiaceae	*Gallium aparine* L.	cleavers	56
	G. boreale L.	northern bedstraw	16
Scrophulariaceae	*Digitalis purpurea* L.	foxglove	4
	Verbascum thapsus L.	common mullein	2
Solanaceae	*Lycopersicon esculentum* Mill.	garden tomato	4
	Solanum nigrum L.	black nightshade	393
	S. tuberosum L.	table potato	212

Table IX. Continued

Umbelliferae	*Coriandrum sativum* L.	coriander	2
	Daucus carota L.	wild carrot	1

*Alex, Cayoutte, and Mulligan (1980) and Hitchcock and Cronquist (1973)

Table X. Reservoir hosts of beet western yellows virus (BWYV) and potato leafroll virus (PLRV) in southern British Columbia

Test	Host plant	Infected /sample	ELISA (A_{405}) positive samples	ELISA (A_{405}) negative controls	*Location
BWYV	*Senecio vulgaris*	75/459	0.051-1.570	0.004	A,D,W
	Matricaria maritima	7/209	0.111-0.538	0.010	V
	Capsella bursa-pastoris	5/1309	0.096-0.272	0.008	A,B
	Lactuca scariola	3/297	0.114-0.528	0.034	B
	Cardamine oligosperma	2/100	0.082-0.812	0.022	B
	Erodium cicutarium	2/174	0.285-0.436	0.010	A
	Polygonum lapathifolium	2/264	0.610-1.187	0.030	P
	Stellaria media	1/626	0.213	0.005	W
	Sisymbrium officinale	1/233	0.218	0.008	B
	Cardaria draba	1/31	0.353	0.014	W
	Brassica napus	1/49	0.149	0.015	P
	Raphanus raphanistrum	1/35	0.264	0.022	P
PLRV	*Solanum tuberosum*	3/212	1.996-2.478	0.001	L
	Capsella bursa-pastoris	2/1309	0.244-0.513	0.015	P
	Solanum nigrum	1/393	0.205	0.013	P

*Location A - Abbotsford
B - Burnaby
D - Delta
L - Lumby
P - Pemberton
V - Vancouver Island
W - Westham Island

6.4 Discussion

TAS-ELISA was used to identify BWYV and PLRV reservoir hosts in the potato-production areas of British Columbia. Confirmation of the ELISA results by aphid transmission tests validated TAS-ELISA as a rapid way of detecting luteoviruses in plants. Failure to transmit virus from all ELISA-positive test samples may have resulted from poor acceptance of some hosts by laboratory aphids reared on Chinese cabbage (*Brassica pekinensis*) or to the poor condition of some samples that were stored for up to two weeks before the transmission tests.

Several reports of non-specific reactions in ELISA (Gugerli, 1979; Nolan and Campbell, 1984; Timmerman *et al.*, 1985; Gunn and Pares, 1988) emphasize the need to use high quality antiserum and suitable controls (healthy plant sap for each species tested) and to verify positive results by another method. The two blocking steps, before and after the addition of plant sample, and the use of monoclonal antibodies in the TAS-ELISA protocol effectively reduced non-specific reactions (Table X).

Weeds are important reservoirs of both vectors and viruses for several important crops (Duffus, 1971), especially BWYV, in California and the Pacific Northwest (Wallis, 1967a,b). The results show that both BWYV and PLRV are present in the potato-

producing areas of British Columbia but they do not indicate that weed reservoirs are important sources of potato leafroll-inducing viruses. Although BWYV was found in about 1% of the samples tested, it does not appear to present any threat to the potato crop in British Columbia. I have not been successful in many attempts to transmit BWYV to potato (section 5.3) and these results are in agreement with those of MacCarthy (1969), Marco (1984), and Barker (1986). Weed reservoirs of BWYV are likely to be important sources of inoculum for the many crops that are susceptible to this virus and many of the virus reservoirs are also good hosts for the most efficient vector, GPA. BWYV was first recorded in British Columbia by MacCarthy (1969) when it was recovered from sugar beet. Although sugar beets are no longer grown commercially in British Columbia, BWYV has survived in the weed population on Westham Island, near the location where it was originally found. BWYV was also found in weeds in agricultural fields in the Fraser Valley and near Pemberton.

PLRV was found in only six samples and three of these were volunteer potatoes. Infected volunteer potatoes have long been considered the most important source of inoculum (Banttari *et al.*, 1990). Finding a few black nightshade and shepherd's purse plants naturally infected with PLRV (Table X) suggests that weeds may occasionally play some part in the epidemiology of potato leafroll disease. Shepherd's purse has been reported as an experimental host of PLRV but not to my knowledge as a

natural host. In the lower mainland of British Columbia and other areas of the Pacific Northwest, shepherd's purse is a winter annual that could quite possibly act as an overwintering host of PLRV. Thomas and Kaniewski (1986) reported that BWYV and PLRV could overwinter in cruciferous weeds. Fox *et al.* (1990) have recently reported that PLRV was transmitted to and recovered from two crucifers, tumble mustard and shepherd's purse by GPA under laboratory and greenhouse conditions. Although the authors suggested that these common weeds may be important sources of PLRV infection of commercial potatoes, they also gave evidence that the weeds are relatively poor sources of inoculum.

Black nightshade is an annual and, although not an overwintering host for either virus or vector, it is a preferred host of GPA (Tamaki, 1975) and may be a reservoir of virus and vectors for the spread of current season leafroll. Klein (1985) also reported black nightshade as a natural host for PLRV in a weed survey of the San Luis Valley of Colorado.

Reports of PLRV transmission to Cruciferae by Salaman and Wortly (1939) were not confirmed by the experiments of Helson and Norris (1943). Even as recently as 1981, shepherd's purse was thought to be a host for BWYV but not for PLRV (Duffus, 1981a,b). In section 4.3, I have shown that PLRV isolates from many locations in Canada and the United States are transmissible

to shepherd's purse. Thomas (1981) and Hassan, Thomas, and Mink (1984) have also demonstrated that PLRV and the tomato strain of PLRV (TYTV) cause symptomless infection of shepherd's purse. Nevertheless, in much of Canada and the United States, most authorities would agree that PLRV-infected volunteer potato plants are the most important sources of primary inoculum of PLRV (Banttari *et al.*, 1990; Thomas,1983). Another source is low grade potatoes for planting material. A recent study by Vernon (1988) indicates that in the densely settled Fraser Valley of British Columbia, backyard gardens are almost certainly the most important reservoir of PLRV-infected potato. Many of these home gardeners plant ordinary commercial table potatoes, often from California, do not apply insecticides, and are not aware that their potatoes are infected with a virus.

The results presented here represent a conservative estimate of virus infection because ELISA is not sensitive enough to detect virus at concentrations below 1 ng/ml (section 3.3). If the virus concentration in some of the samples was below this limit, due to recent infection or to restricted multiplication of the viruses in some hosts, the sample would have been scored falsely negative. Fox *et al.* (1990) showed that the concentration of PLRV may be higher in the root tissue than in the foliage of tumble mustard and shepherd's purse, but another study (Eweida *et al.*, 1988), showed that the distribution of luteoviruses in root and shoot tissue may be

dependent on the growth stage at which the plants became infected.

The identification of weed hosts of plant viruses can help in the understanding of the ecological relationships that contribute to disease outbreaks. In Canada, more than a thousand different plants can be regarded as weeds but only about 230 species are considered economically important (Frankton and Mulligan, 1970). Until this study, no report appears to have been published linking potato virus disease spread with weed hosts in Canada.

Most of the weeds infected with BWYV are in the Cruciferae or Compositae and are common in British Columbia. Those that are winter annuals (fall-sprouted annuals) or perennials may serve as overwintering sources of BWYV or PLRV. When these plants grow in protected areas, near the Pacific Coast, they also serve as overwintering plants for the summer form of vector aphids, particularly GPA.

BWYV is of considerable economic importance (Ashby, Bos, and Huijberts, 1979; Duffus, 1977; Walkey and Pink, 1990). Its presence in weeds in agricultural areas of British Columbia indicates that a source of inoculum is usually present. In years when GPA are abundant, BWYV probably is a threat to many susceptible vegetable crops. MacCarthy (1969) has shown that

garden beet, spinach, lettuce, pea, broccoli, cauliflower, turnip, and Chinese cabbage are all susceptible to a local isolate. The presence of BWYV in weeds is not surprising considering the wide host range of this virus and its occurrence in the province for at least 20 years.

Chapter 7

Summary and conclusions

7.1 Summary of results

The objectives outlined in section 1.4 were achieved:

1. Several MAbs were produced that discriminate between PLRV and BWYV. Two MAbs, 510H and 112E, detected all of the strains of BWYV tested including the RPV isolate of BYDV now recognized as a strain of BWYV. MAb 510H was selected for large scale testing because of its broad specificity to BWYV, detecting 16 isolates from six countries, and because it was produced in high titer in ascites fluid. MAbs 26BE and 41BC detected all of the PLRV isolates tested including the solanum yellows virus strain. One MAb detected an epitope common to both PLRV and BWYV. None of the MAbs reacted with other luteoviruses nor with healthy plant sap.

2. In total, 801 samples of potato leafroll disease were collected and tested for PLRV and BWYV in 1986, 1987, and 1988 using TAS-ELISA and virus-specific MAbs. The samples represented 32 cultivars, originating in 8 Canadian provinces and 12 American states. None of the samples tested positive for

BWYV whereas 772 (96.4%) tested positive for PLRV. Neither PLRV nor BWYV could be recovered, by aphid transfers to indicator hosts, from 28 of the samples that tested negative for both viruses. One sample that was scored negative for both viruses by ELISA, tested positive with an aphid transmission test to *P. pubescens;* the indicator plant tested positive for PLRV by TAS-ELISA.

3. NASH was compared with ELISA and aphid transmission tests for detection of PLRV and BWYV in 165 potato plants showing symptoms of potato leafroll disease. None of the plants tested positive for BWYV by DAS-ELISA, TAS-ELISA, or NASH using either a cloned BWYV probe or random primed cDNA prepared from BWYV RNA. The indicator plants all tested negative for BWYV by TAS-ELISA. All of the 165 potato leafroll disease samples tested positive for PLRV using TAS-ELISA and NASH with a cloned PLRV cDNA probe. TAS-ELISA, using MAb 371A, generally produced a stronger signal for PLRV than DAS-ELISA using PLRV polyclonal antiserum. DAS-ELISA produced one false-negative; the same sample tested positive by TAS-ELISA, NASH, and aphid transmission to *P. pubescens*. NASH, using random primed cDNA prepared from PLRV RNA, also produced one false-negative. None of the five virus-free negative control samples or 18 symptomless field samples tested positive for either virus. Seventy-two percent (119/165) of the PLRV isolates infected shepherd's purse.

4. The susceptibility of potato to BWYV was tested by aphid inoculation of Russet Burbank plants with three isolates of BWYV from Canada and four from the United States. Two of the isolates were in a mixed infection with PLRV. The potato plants were assayed for PLRV and BWYV by TAS-ELISA, and by aphid transmission to indicator plants six weeks after inoculation. None of the plants tested positive for BWYV by ELISA. The ELISA results were confirmed by the aphid transmission tests. BWYV could not be recovered, by aphid transfers to indicator plants, from any of the inoculated potato plants. PLRV was detected in and recovered from plants inoculated with both PLRV and BWYV.

5. Common weeds were surveyed in the potato-producing areas of British Columbia for PLRV and BWYV. In total, 10,098 weed samples, representing 98 species in 22 plant families, were collected and tested by TAS-ELISA from 1986 to 1989. BWYV was detected in 1% of the plants tested; the hosts were: chickweed, common groundsel, heart-podded hoary cress, hedge mustard, little western bittercress, prickly lettuce, shepherd's purse, pale smartweed, rutabaga, scentless chamomile, stork's-bill, and wild radish. PLRV was detected in three volunteer potato plants, two samples of shepherd's purse, and one black nightshade plant. The low incidence of PLRV in hosts other than potato suggests that weeds are of minor importance in the epidemiology of potato leafroll disease in British Columbia.

7.2 Conclusions

The results presented in this thesis provide an unambiguous picture of the insignificance of BWYV in potato leafroll disease. BWYV does not present a threat to either seed or table potato production. Potato leafroll disease is caused by a single virus, PLRV. The virus exists as a number of biological variants that are antigenically identical or closely related (Stace-Smith, 1987). The natural host range of PLRV is limited primarily to the Solanaceae but some species in other plant families may become infected. At the present time there is no convincing evidence that hosts other than potato are important reservoirs for PLRV in British Columbia. However, the occurrence of PLRV in a very few naturally infected winter annuals suggests that in areas where there is a significant early flight of GPA, weeds may play a role in the epidemiology of potato leafroll disease. With the development of ELISA as a routine test for detecting plant viruses and the availability of specific monoclonal antibodies to PLRV, we can now reliably detect PLRV in potato and other hosts. TAS-ELISA is sensitive enough to detect PLRV in single aphid vectors (Martin and Ellis, 1987) and can be used to monitor aphid populations for the virus, to allow assessment of risks and more precise timing of potato top-kill dates.

Recently Kawchuck, Martin, and McPherson (1990) have succeeded in producing transgenic potato plants, cultivars Desiree and Russet Burbank, that express the coat protein gene of PLRV. This same strategy has been used to provide cross-protection from several plant viruses such as tobacco mosaic virus (Abel *et al.*, 1986), alfalfa mosaic virus (Loesch-Fries *et al.*, 1987), potato virus X (Hemenway *et al.*, 1988), and cucumber mosaic virus (Cuozzo *et al.*, 1988). However, by reducing viral replication, this method may result in plants that become infected with virus but at levels below the detection limit of ELISA.

Another new technology that will have a significant impact on detection and diagnosis of PLRV is the polymerase chain reaction (PCR). The PCR technique allows virtually unlimited amplification of cDNA, from viral RNA, before detection with nucleic acid probes (Kawasaki, 1990; Rotbart, 1990). As newer cultivars of potato are released with better resistance to PLRV (Barker and Harrison, 1985) it will become increasingly difficult to detect the virus. PCR and nucleic acid probes will allow us to separate cultivars which are carriers of the virus from those which do not allow the virus to replicate. The same technique will have a significant application in the detection of other potato pathogens, such as potato spindle tuber viroid and bacterial ringrot.

The recent advancements in biotechnology combined with traditional plant breeding techniques will undoubtly result in the release of excellent potato cultivars with improved disease resistance in the next decade.

Bibliography

Abel, P. P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.

Alex, J. F., Cayouette, R., and Mulligan, G. A. 1980. Common and botanical names of weeds in Canada. Publ. 1397. Agriculture Canada, Ottawa. 132 pp.

Ashby, J. W. 1984. Bean leaf roll virus. No. 286 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, England.

Ashby, J. W., Bos, L., and Huijberts, N. 1979. Yellows of lettuce and some other vegetable crops in the Netherlands caused by beet western yellows virus. Neth. J. Plant Pathol. 85:99-111.

Ashby, J. W., and Johnstone, G. R. 1985. Legume luteovirus taxonomy and current research. Australas. Plant Path. 14:2-7.

Bailey, J. M., and Davidson, N. 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. 70:75-85.

Banttari, E. E., Ellis, P. J., and S. M. P. Khurana. 1990. Integrated Pest Management for Viruses, Viroid and Mycoplasma-like Organisms of Potato. APS Press (in press).

Banttari, E. E., and Goodwin, P. H. 1985. Detection of potato viruses S, X, and Y by enzyme-linked immunosorbent assay on nitrocellulose membranes (Dot-ELISA). Plant Dis. 69:202-205.

Barker, H. 1986. Failure of British isolates of beet western yellows to infect potato. Ann. Appl. Biol. 109:445-447.

Barker, H., and Harrison, B. D. 1985. Restricted multiplication of potato leafroll virus in resistant potato genotypes. Ann. Appl. Biol. 107:205-212.

Barker, H., Mayo, M. A., and Robinson, D. J. 1984. Polygenic resistance to potato leafroll virus (PLRV). Rep. Scott. Crop Res. Inst. 1983:194.

Baulcombe, D., Flavell, R. B., Boulton, R. E., and Jellis, G. J. 1984. The sensitivity and specificity of a rapid nucleic acid hybridization method for the detection of potato virus X in crude sap samples. Plant Pathol. 33:361-370.

Bishop, G. W. 1967. A leaf roll virus control program in Idaho's seed potato areas. Am. Potato J. 44:305-308.

Bishop, G. W., and Guthrie, J. W. 1964. Home gardens as a source of green peach aphid and virus diseases in Idaho. Am. Potato J. 41:28-34.

Brandsma, J., and Miller, G. 1980. Nucleic acid spot hybridization: rapid quantitative screening of lymphoid cell lines for Epstein-Barr viral DNA. Proc. Natl. Acad. Sci. USA 77:6851-6856.

Byrne, B. C., Li, J. J., Sninsky, J., and Poiesz, B. J. 1988. Detection of HIV-1 RNA sequences by an *in vitro* DNA amplification. Nucleic Acids Res. 16:4165.

Byrne, D. N., and Bishop, G. W. 1979. Relationship of green peach aphid numbers to spread of potato leafroll virus in southern Idaho. J. Econ. Ent. 72:809-811.

Carlson, D. P., Superko, C., Mackey, J., Gaskill, M. E., and Hansen, P. 1990. Chemiluminescent detection of nucleic acid hybridization. Focus 12:9-12.

Casper, R. 1983. Beet western yellows virus in potatoes in Germany? EARP Conf. Sept. 5-9, 1983, Braunschweig.

Casper, R., Meyer, S., Lesemann, D. -E., Reddy, D. V. D., Rajeshwari, R., Misari, S. M., and Subbarayudu, S. S. 1983. Detection of a luteovirus in groundnut rosette diseased groundnuts (*Arachis hypogaea*) by enzyme-linked immunosorbent assay and immunoelectron microscopy. Phytopath. Z. 108:12-17.

Casper, R. 1988. Luteoviruses. Pages 235-258 in: The Plant Viruses: Polyhedral Virions with Monopartitite RNA Genomes. R. Koenig, ed. Plenum Press, New York.

Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.

Clark, M. F., Lister, R. M., and Bar-Joseph, M. 1986. ELISA techniques. Methods Enzymol. 118:742-766.

Clarke, R. G., Converse, R. H., and Kojima, M. 1980. Enzyme-linked immunosorbent assay to detect potato leafroll virus in potato tubers and viruliferous aphids. Plant Dis. 64:43-45.

Clarke, R. G., Powelson, M. L., and Beraha, L. 1983. Association of viral, bacterial and fungal pathogens with vascular discoloration of Russet Burbank potato tubers. Am. Potato J. 60:235-243.

Creamer, R., and Falk, B. W. 1990. Direct detection of transcapsidated barley yellow dwarf luteoviruses in doubly infected plants. J. Gen. Virol. 71:211-217.

Cuozzo, M., O'Connell, K. M., Kaniewski, W., Fang, R., Chua, N., and Tumer, N. E. 1988. Viral protection in transgenic tobacco plants expressing the cucumber mosaic coat protein or its antisense RNA. Bio/technology 6:549-557.

D'Arcy, C. J., Hewings, A. D., Burnett, P. A., and Jedlinski, H. 1983. Comparative purification of two luteoviruses. Phytopathology 73:755-759.

D'Arcy, C. J., Torrance, L., Martin, R. R. 1989. Discrimination among luteoviruses and their strains by monoclonal antibodies and identification of common epitopes. Phytopathology 79:869-873.

Davis, P. B., and Pearson, C. K. 1978. Characterization of density gradients prepared by freezing and thawing a sucrose solution. Anal. Biochem. 91:343-349.

Dodds, J. A., and Hamilton, R. I. 1976. Structural interactions between viruses as a consequence of mixed infections. Adv. Virus Res. 20:33-86.

Duffus, J. E. 1960. Radish yellows, a disease of radish, sugar beet and other crops. Phytopathology 50:389-394.

Duffus, J. E. 1964. Host relationships of beet western yellows virus strains. Phytopathology 54:736-738.

Duffus, J. E. 1971. Role of weeds in the incidence of virus diseases. Annu. Rev. Phytopathol. 9:319-340.

Duffus, J. E. 1972. Beet western yellows virus. No. 89 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, England. 4pp.

Duffus, J. E. 1977. Aphids, viruses and the yellow plague. Pages 361-383 in: Aphids as Virus Vectors. K. F. Harris and K. Maramorosch, eds. Acedemic Press, New York.

Duffus, J. E. 1979. Legume yellows virus, a new persistent aphid-transmitted virus of legumes in California. Phytopathology 69:217-221.

Duffus, J. E. 1981a. Beet western yellows virus - a major component of some potato leaf roll-affected plants. Phytopathology 71:193-196.

Duffus, J. E. 1981b. Distribution of beet western yellows virus in potatoes affected by potato leaf roll. Plant Dis. 65:819-820.

Duffus, J. E., and Gold, A. H. 1969. Membrane feeding and infectivity neutralization used in a serological comparison of potato leaf roll and beet western yellows viruses. Virology 37:150-153.

Duffus, J. E., and Johnstone, G. R. 1982a. The probable long association of beet western yellows virus with the potato leaf roll syndrome in Tasmania. Aust. J. Exp. Agric. Anim. Husb. 22:253-356.

Duffus, J. E., and Johnstone, G. R. 1982b. Some luteovirus diseases in Tasmania caused by beet western yellows and subterranean clover red leaf virus. Aust. J. Agric. Res. 35:821-830.

Duffus, J. E., and Rochow, W. F. 1978. Neutralization of beet western yellows virus by antisera against barley yellow dwarf virus. Phytopathology 68:45.

Dykstra, T. P., 1933. Weeds as possible carriers of leaf roll and rugose mosaic of potato. J. Agric. Res. 47:17-33.

Ellis, P. J. 1988. A survey of common weeds of British Columbia for beet western yellows virus and potato leafroll virus using monoclonal antibodies. (Abstr.) Can. J. Plant Pathol. 10:363-364.

Ellis, P. J. 1989. Failure to detect beet western yellows virus in potato leafroll disease samples from Canada and the United States. (Abstr.) Phytopathology 79:908.

Ellis, P. J. 1990. Potato leafroll disease. Pages 44-53 in: Proceedings of the Eighth Annual North American Seed Potato Seminar. Nov. 30 - Dec. 2, 1989. Idaho Falls, Idaho.

Ellis, P. J., and Wieczorek, A. 1988. Serological testing of potato leafroll disease samples using monoclonal antibodies specific for beet western yellows virus (BWYV) and potato leafroll virus (PLRV). (Abstr.) Phytopathology 78:1583.

Eskandari, F., Sylvester, E.S., and Richardson, J. 1979. Evidence for lack of propagation of potato leaf roll virus in its aphid vector, *Myzus persicae*. Phytopathology 69:45-47.

Eweida, M., Oxelfelt, P., and Tomenius, K. 1988. Concentration of virus and ultrastructural changes in oats at various stages of barley yellow dwarf virus infection. Ann. Appl. Biol. 112:313-321.

Falk, B. W., and Duffus, J. E. 1984. Identification of small single and double-stranded RNA's associated with severe symptoms in beet western yellows virus-infected *Capsella bursa-pastoris*. Phytopathology 74:1224-1229.

Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

Feinberg, A. P., and Vogelstein, B. 1984. Addendum: a technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137:266-269.

Fox, L., Biever, K. D., Duffus, J. E., and Thomas, P. E. 1990. Transmission of potato leafroll virus (PLRV) to wild Crucifers by *Myzus persicae* (Sulzer) and detection by enzyme-linked immunosorbent assay. Am. Pot. J. (in press).

Francki, R. I. B., Milne, R. G., and Hatta, T. 1985. Luteovirus group. Pages 137-152 in: Atlas of Plant Viruses. Volume 1. CRC Press, Inc. Boca Raton.

Frankton, C., and Mulligan, G. A. 1970. Weeds of Canada. Publication 948. Canada Department of Agriculture. 217pp.

Gallenberg, D. J. Zitter, T. A., and Jones, E. D. 1987. Comparison of three potato leafroll virus antisera and a single beet western yellows virus antiserum for luteovirus detection in potato. Am. Potato J. 64:97-108.

Gergen, J. P., Stern, R. H., and Wensink, P. C. 1979. Filter replicas and permanent collections of recombinant DNA plasmids. Nucleic Acids Research 7:2115-2136.

Gerlach, W. L., Miller, W. A., and Waterhouse, P. M. 1987. Barley Yellow Dwarf Newsl. 1:17.

Gildow, F. E. 1985. Transcellular transport of barley yellow dwarf virus into the hemocoel of the aphid vector, *Rhopalosiphum padi*. Phytopathology 75:292-297.

Gildow, F. E., Ballinger, M. E., and Rochow, W. F. 1983. Identification of double-stranded RNA's associated with barley yellow dwarf virus infection of oats. Phytopathology 73:1570-1572.

Gill, C. C., and Chong, J. 1979a. Cytopathological evidence for the division of barley yellow dwarf virus isolates into two subgroups. Virology 95:59-69.

Gill, C. C., and Chong, J. 1979b. Cytological alterations in cells infected with corn leaf aphid-specific isolates of barley yellow dwarf virus. Phytopathology 69:363-368.

Goding, J. W. 1986. Monoclonal Antibodies: Principles and Practice. Second Edition. Academic Press, New York. 315 pp.

Goldbach, R., and Wellink, J. 1988. Evolution of plus-strand RNA viruses. Intervirology 29:260-267.

Govier, D. A. 1985. Purification and partial characterization of beet mild yellowing virus and its serological detection in plants and aphids. Ann. Appl. Biol. 107:439-447.

Gubler, U., and Hoffman, B. J. 1983. A simple and very efficient method for generating cDNA libraries. Gene 25: 263-269.

Gunn, L. V., and Pares, R. D. 1988. Effect of potato physiology on the intepretation of ELISA results for potato leafroll virus. Plant Pathol. 37:516-521.

Habili, N., McInnes, J. L., and Symons, R. H. 1987. Nonradioactive, photobiotin-labelled DNA probes for the routine diagnosis of barley yellow dwarf virus. J. Virol. Methods 16:225-237.

Halk, E. L. 1986. Serotyping plant viruses with monoclonal antibodies. Methods Enzymol. 118:766-780.

Halk, E. L., and De Boer, S. H. 1985. Monoclonal antibodies in plant-disease research. Ann. Rev. Phytopathol. 23:321-350.

Harrison, B. D. 1984. Potato leafroll virus. No. 291 in: Commonw. Mycol. Inst./Assoc. Appl. Biol. Descriptions of Plant Viruses. Kew, Surrey, England. 5 pp.

Hassan, S., and Thomas, P. E. 1984. Etiological distinctions between tomato yellow top virus and potato leafroll and beet western yellows viruses. Plant Dis. 68:684-685.

Hassan, S., Thomas, P. E., and Mink, G. I. 1985. Tomato yellow top virus host range, symptomatology, transmission properties, and isolate variability. Phytopathology 75:287-291.

Helson, G. A. H., and Norris, D. O. 1943. Transmission of potato virus diseases. III. Susceptibility of Cruciferae to potato leaf roll virus. Jour. Counc. Sci. Indust. Res. (Australia) 16:261-262.

Hemenway, C., Fang, R., Kaniewski, W. K., Chua, N., and Tumer, N. E. 1988. Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. The EMBO Journal 7:1273-1280.

Hewings, A. D., and D'Arcy, C. J. 1984. Maximizing the detection capability of a beet western yellows virus ELISA system. J. Virol. Methods 9:131-142.

Hitchcock, L. C., and Cronquist, A. 1973. Flora of the Pacific Northwest. University of Washington Press. Seattle. 730 pp.

Hooker, W. J. 1981. Nonvirus leafroll. Pages 18-19 in: Compendium of Potato Diseases. W. J. Hooker ed. APS Press. St. Paul. 125 pp.

Jayasena, K. W., Randles, J. W., and Barnett, O. W. 1984. Synthesis of a complementary DNA probe specific for detecting subterranean clover red leaf virus in plants and aphids. J. Gen. Virol. 65:109-117.

Johnson, D. A., Gautsch, J. W., Sportsman, J. R., and Elder, J. H. 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Techn. 1:3-8.

Kannangara, T., Wieczorek, A., and Laverder, D. P. 1989. Immunoaffinity columns for isolation of abscisic acid in conifer seedlings. Physiologia Plantarum 75:369-373.

Kawasaki, E. S. 1990. Amplification of RNA. Pages 21-27 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelford, J. I. Sninsky, and T. W. White eds. Acedemic Press, Inc. New York. 482pp.

Kawchuk, L. M., Martin, R. R., Rochon, D. M., and McPherson, J. 1989. Identification and characterization of the putative potato leafroll virus coat protein gene. J. Gen. Virol. 70:783-788.

Keese, P., Martin, R. R., Kawchuk, L. M., Waterhouse, P. M., and Gerlach, W. L. 1990. Nucleotide sequences of an Australian and a Canadian isolate of potato leafroll luteovirus and their relationship with two European isolates. J. Gen. Virol. 71:719-724.

Kirkpatrick, H. C. 1948. Indicator plants for studies with the leafroll virus of potatoes. Amer. Potato J. 25:283-290.

Kirkpatrick, H. C., and Ross, A. F. 1952. Aphid transmission of potato leafroll virus to solanaceous species. Phytopathology 42:540-546.

Klein, R. E. 1985. Factors in the epidemiology of potato leafroll in the San Luis Valley of Colorado. Ph.D. thesis, Colorado State University, Colorado. 90 pp.

Koenig, R. 1978. ELISA in the study of homologous and heterologous reactions of plant viruses. J. Gen. Virol. 40:309-318.

Koenig, R. 1981. Indirect ELISA methods for the broad specificity detection of plant viruses. J. Gen. Virol. 55:53-62.

Köhler, G., and Milstein, C. 1975. Continuous culture of fused cell secreting antibodies of predefined specificity. Nature 256:495-497.

Kozak, M. 1986. Regulation of protein synthesis in virus-infected animal cells. Adv. Virus Res. 31:229-292.

Kubo, S. 1981. Toabacco necrotic dwarf virus. No. 234 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./ Assoc. Appl. Biol., Kew, Surrey, England.

Kyriakou, A., Close, R. C., and Ashby, J. W. 1983. A strain of beet western yellows virus in Canterbury, New Zealand. N.Z. J. Agric. Res. 26:271-277.

LeClerg, E. L. 1944. Non-virus leafroll of Irish potatoes. Am. Potato J. 21:5-13.

Leonard, S. H., and Holbrook, F. R. 1978. Minimum aquisition and transmission times for potato leaf roll virus by the green peach aphid. Ann. Entomol. Soc. Am. 71:493-495.

Loesch-Fries, L. S., Merlo, D., Zinner, T., Burhop, L., Hill, K., Krahn, K., Jarvis, N., Nelson, S., and Halk, E. 1987. Expression of alfalfa mosaic virus RNA4 in transgenic plants confers virus resistance. The EMBO Journal 6:1845-1851.

Lutman, P. J. W., and Tucker, G. G. 1987. Standard descriptions of growth stages of annual dicotyledonous weeds. Ann. Appl. Biol. 110:683-687.

MacCarthy, H. R. 1969. A newly recorded virus disease of sugar beet in British Columbia. Can. Plant Dis. Survey 49:135-138.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 545pp.

Marco, S. 1984. Beet western yellows virus in Israel. Plant Dis. 68:162-163.

Marco, S. 1985. Serological reaction of beet western yellows and potato leafroll viruses in ELISA. Phytoparasitica 13:201-207.

Martin, R. R. 1987. The use of monoclonal antibodies for virus detection. Can. J. Plant Pathol. 9:177-181.

Martin, R. R., and Converse, R. H. 1985. Purification, properties and serology of strawberry mild yellow-edge virus. Phytopath. Z. 114:21-30.

Martin, R. R., and D'Arcy, C. J. 1990. Relationships among luteoviruses based on nucleic acid hybridization and serological studies. Intervirology 31:23-30.

Martin, R. R., and Ellis, P. 1986. Detection of potato leafroll and beet western yellows viruses in aphids. (Abstr.) in: Proceedings of Workshop on Epidemiology of Plant Virus Diseases, Orlando, Florida, August 6-8, 1986.

Martin, R. R., Keese, P. K., Young, M. T., Waterhouse, P. M., and Gerlach, W. L. 1990. Evolution and molecular biology of luteoviruses. Annu. Rev. Plant Pathol. (In press).

Martin, R. R., and Stace-Smith, R. 1984. Production and characterization of monoclonal antibodies specific to potato leafroll virus. Can. J. Plant Pathol. 6:206-210.

Matthews, R. E. F. 1982. Classification and nomenclature of viruses. Intervirology 17:1-199.

Maule, A. J., Hull, R., and Donson, J. 1983. The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. J. Virol. Methods 6:215-224.

Mayo, M. A., and Barker, H. 1984. Translation products and RNA species of potato leafroll virus (PLRV). Rep. Scott. Crop Res. Inst. 1983:187.

Mayo, M. A., Barker, H., Robinson, D. J., Tamada, T., and Harrison, B. D. 1982. Evidence that potato leafroll virus RNA is positive-stranded, is linked to a small protein and does not contain polyadenylate. J. Virol. 59:163-167.

Mayo, M. A., Robinson, D. J., Jolly, C. A., and Hyman, L. 1989. Nucleotide sequence of potato leafroll luteovirus RNA. J. Gen. Virol. 70:1037-1051.

Milbrath, G. M., and Duffus, J. E. 1978. Solanum yellows, an apparently distinct member of the luteovirus group. Phytopathol. News 12:170.

Miller, S. A., and Martin, R. R. 1988. Molecular diagnosis of plant disease. Annu. Rev. Phytopathol. 26:409-432.

Miller, W. A., Waterhouse, P. M., and Gerlach, W. L. 1988. Sequence and organization of barley yellow dwarf virus genomic RNA. Nucleic Acids Research 16:6097-6111.

Miller, W. A., Waterhouse, P. M., Kortt, A. A., and Gerlach, W. L. 1988. Sequence and identification of the barley yellow dwarf virus coat protein gene. Virology 165:306-309.

Murphy, J. F., D'Arcy, C. J., and Clark, J. M. 1989. Barley yellow dwarf virus RNA has a 5'-terminal genome-linked protein. J. Gen. Virol. 70:2253-2256.

Natti, J. J., Kirkpatrick, H. C., and Ross, A. F. 1953. Host range of potato leafroll virus. Am. Potato J. 30:55-64.

Nolan, P. A., and Campbell, R. N. 1984. Squash mosaic virus detection in individual seeds and seed lots of cucurbits by enzyme-linked immunosorbent assay. Plant Disease 68:971-975.

Osler, R. 1984. Rice giallume, a disease related to barley yellow dwarf in Italy. Barley Yellow Dwarf, A Proceedings of the Workshop, CIMMYT, Mexico, December 6-8, 1983. 204pp.

Owens, R. A., and Diener, T. O. 1981. Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridisation. Science 213:670-672.

Peters, D. 1987. Spread of viruses in potato crops. Pages 126-145 in: Viruses of Potatoes and Seed-potato Production. J. A. de Bokx and J. P. H. van der Want, eds. PUDOC, Wageningen.

Peters, D., and Jones, R. A. C. 1981. Potato leafroll virus. Pages 68-70 in: Compendium of Potato Diseases. W. J. Hooker ed. APS Press, St. Paul

Prill, B., Maiss, E., Chansilpa, N., and Casper, R. 1988. Molecular cloning of single-stranded RNAs of potato leafroll virus and beet western yellows virus. J. Gen. Virol. 69:2397-2402.

Rajeshwari, R., and Murant, A. F. 1988. Purification and particle properties of groundnut rosette assistor virus and production of a specific antiserum. Ann. Appl. Biol. 112:403-414.

Siegel, A., Hari, V., Montgomery, I., and Kolacz, K. 1976. A messenger RNA for capsid protein isolated from tobacco mosaic virus-infected tissue. Virology 73:363-371.

Reed, K. C. 1986. Nucleic acid hybridizations with DNA bound to Zeta-Probe membrane. Bio-Rad Laboratories, Bulletin 1234, Richmond, CA.

Richter, J., Stanarius, A., Kuhne, T., Proll, E., and Kleinhempel, H. 1983. Investigations on the serological relation between potato leaf roll virus and beet mild yellowing virus. Archiv fur Phytopathologie und Pflanzenschutz 19:419-422. in Potato Abstracts 10:18.

Roberts, I. M., Tamada, T., and Harrison, B. D. 1980. Relationship of potato leafroll virus to luteoviruses: evidence from electron microscope serological tests. J. Gen. Virol. 47:209-213.

Rochow, W. F. 1984. Appendix I, Barley Yellow Dwarf, A Proceedings of the Workshop, CIMMYT, Mexico, December 6-8, 1983. 204 pp.

Rochow, W. F., and Duffus, J. E. 1978. Relationships between barley yellow dwarf and beet western yellows viruses. Phytopathology 68:51-58.

Rochow, W. F., and Duffus, J. E. 1981. Luteoviruses and yellows diseases. Pages 147-170 in: Handbook of Plant Viruse Infections and Comparative Diagnoses. E. Kurstak, ed. Elserier/ North-Holland, Amsterdam.

Rotbart, H. A. 1990. PCR amplification of enteroviruses. Pages 372-377 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelford, J. J. Sninsky, and T. J. White eds. Acedemic Press. New York. 482pp.

Rowhani, A., and Stace-Smith, R. 1979. Purification and characterization of potato leafroll virus. Virology 98:45-54.

Salaman, R. N., and Wortley, A. R. S. 1939. Potential hosts of potato viruses in garden and field. Nature 144:1049-1050.

Salazar, L. F., Owens, R. A., Smith, D. R., and Diener, T. O. 1983. Detection of potato spindle tuber viroid by nucleic acid spot hybridization: evaluation with tuber sprouts and true potato seed. Am. Potato J. 60:587-597.

Salazar, L. F., Balbo, I., and Owens, R. A. 1988. Comparison of four radioactive probes for the diaagnosis of potato spindle tuber viroid by nucleic acid spot hybridization. Potato Research 31:431-442.

Sela, I., Reichman, M., and Weissbach, A. 1984. Comparison of dot-blot molecular hybridization and enzyme-linked immunosorbent assay for detecting tobacco mosaic virus in plant tissues and protoplasts. Phytopathology 74:385-389.

Sutula, C. L., Gillett, J. M., Morrissey, S. M., and Ramsdell, D. C. 1986. Interpreting ELISA data and establishing the positive-negative threshold. Plant Dis. 70:722-726.

Shepard, J. F., and Claflin, L. E. 1975. Critical analysis of the principles of seed potato certification. Annu. Rev. Phytopathol. 13:271-293.

Shepherd, R. J., Francki, R. I. B., Hirth, L., Hollings, M., Inouye, T., MacLeod, R., Purcifull, D. E., Sinha, R. C., Tremaine, J. H., Valenta, V., and Wetter, C. 1976. New groups of plant viruses approved by the International Commettee on Taxonomy of Viruses. Intervirology 6:181-199.

Sibara, M. M., and Slack, S. A. 1985a. Multiplication and translocation of potato leafroll virus and beet western yellows virus in selected potato cultivars. (Abstr.) Phytopathology. 75:1291.

Sibara, M. M., and Slack, S. A. 1985b. Incidence of beet western yellows virus and potato leafroll virus in potato leafroll- affected potato plants. (Abstr.) Phytopathology 75:1291.

Singh, R. 1987. Role of weeds in potato virus spread. Pages 355-362 in: Potato Pest Management in Canada. G. Boiteau, R. P. Singh, and R. H. Parry, eds. Fredericton, New Brunswick.

Sippel, A. E. 1973. Purification and characteriqation of adenosinetriphosphate : ribonucleic acid adenyl transferase from *Escherichia coli*. Eur. J. Biochem. 37:31-34.

Smith, O. P., Harris, K. F., Toler, R. W., and Summers, M. D. 1988. Molecular cloning of potato leaf roll virus complementary DNA. Phytopathology 78:1060-1066.

Stace-Smith, R. 1987. Control of aphid-borne persistant viruses. Pages 54-69 in: Potato Pest Management in Canada. G. Boiteau, R. P. Singh, and R. H. Parry, eds. Fredericton, New Brunswick.

Syller, J. 1985. Comparison of some isolates of potato leaf roll virus in Poland. Phytopath. Z. 113:17-23.

Swanstrom, R., and Shank, P. R. 1978. X-ray intensifying screens greatly enhance the detection by autoradiography of the isotopes ^{32}P and ^{125}I. Anal. Biochem. 86:184-192.

Taggart, T. R., and Samloff, M. I. 1983. Stable antibody-producing murine hybridomas. Science 219:1228-1230.

Takanami, Y., and Kubo, S. 1979. Enzyme-assisted purification of two phloem-limited plant viruses: tobacco necrotic dwarf and potato leafroll. J. Gen. Virol. 44:153-159.

Tamada, T., Harrison, B. D., and Roberts, I. M. 1984. Variation among British isolates of potato leafroll virus. Ann. Appl. Biol. 104:107-116.

Tamada, T., and Kojima, M. 1977. Soybean dwarf virus. No. 179 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./ Assoc. Appl. Biol., Kew, Surrey, England.

Tamaki, G. 1975. Weeds in orchards as important alternate sources of green peach aphids in late spring. Environ. Entomol. 4:958-960.

Tamaki, G., and Fox, L. 1982. Weed species hosting viruliferous green peach aphids, vector of beet western yellows virus. Environ. Entomol. 11:115-117.

Tamaki, G., Fox, L., and Butt, B. H. 1979. Ecology of the green peach aphid as a vector of beet western yellows virus of sugarbeets. U.S. Dep. Agric. Tech. Bull. 1599. 16 pp.

Taylor, J. M., Illemensee, R., and Summers, J. 1976. Effecient transcription to RNA into DNA by avian sarcoma virus polymerase. Biochimica et Biophysica Acta 442:324-330.

Thomas, P. E. 1983. Sources and dissemination of potato viruses in the Columbia Basin of the northwestern United States. Plant Dis. 67:744-747.

Thomas, P. E., and Kaniewski, W. K. 1986. Overwintering of potato leafroll and beet western yellows viruses in winter annual weeds. (Abstr.) Phytopathology 76:847.

Thomas, J. E. 1981. Tomato yellow top - a probable luteovirus from Australia. Aust. J. Pathol. 10:33-34.

Thomas, J. E. 1984. Characterization of an Australian isolate of tomato yellow top virus. Ann. Appl. Biol. 104:79-86.

Thottappilly, G., Kao, Y. J., Hooper, G. R., and Bath, J. E. 1977. Host range, symptomatology, and electron microscopy of a persistent, aphid transmitted virus from alfalfa in Michigan. Phytopathology 67:1451-1459.

Timmerman, E. L., D'Arcy, C. J., and Splittstoesser, W. E. 1985. Beet western yellows virus in Illinois vegetable crops and weeds. Plant Dis. 69:933-936.

Turl, L. A. D. 1983. The effect of winter weather on the survival of aphid populations on weeds in Scotland. EPPO Bull. 13:139-143.

Veidt, I., Lot, H., Leiser, M., Scheidecker, D., Guilley, H., Richards, K., and Jonard, G. 1988. Nucleotide sequence of beet western yellows virus RNA. Nucleic Acids Res. 16:9917-9932.

Vernon, R. S. 1988. Computerized integrated pest management. ARDSA Project-11001. Annual reports.

Voller, A., Barlett, A., Bidwell, D. E., Clark, M. F., and Adams, A. N. 1976. The detection of viruses by enzyme-linked immunosorbent assay. J. Gen. Virol. 33:165-167.

Voller, A., Bidwell, D. E., and Bartlett, A. 1977. The enzyme-linked immunosorbent assay (ELISA). Flowline Publications, Guernsey, United Kingdom. 48pp.

Vrati, S., Mann, D . A., and Reed, K. C. 1987. Aldaline northern blots: transfer of RNA from agarose gels to Zeta-Probe membrane in dilute NaOH. Molecular Biology Reports 1:1-4.

van Regenmortel, M. H. V. 1982. Serology and Immunochemistry of Plant Viruses. Academic Press, New York.

Walkey, D. G. A., and Pink, D. A. C. 1990. Studies on resistance to beet western yellows virus in lettuce (*Lactuca sativa*) and the occurrence of field sources of the virus. Plant Pathol. 39:141-155.

Wallis, R. L. 1967a. Green peach aphids and the spread of beet western yellows in the Northwest. J. Econ. Entomology 60:313-315.

Wallis, R. L. 1967b. Some host plants of the green peach aphid and beet western yellows virus in the Pacific Northwest. J. Econ. Entomology 60:904-907.

Waterhouse, P. M., Gerlach, W. L., and Miller, W. A. 1986. Serotype-specific and general luteovirus probes from cloned cDNA sequences of barley yellow dwarf virus. J. Gen. Virol. 67:1273-1281.

Waterhouse, P. M., Gildow, F. E., and Johnstone, G. R. 1988. Luteovirus group. No. 339 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.

Waterhouse, P. M., and Murant, A. F. 1982. Carrot red leaf virus. No. 249 in: Commonw. Mycol. Inst./ Assoc. Appl. Biol., Kew, Surrey, England.

Waterhouse, P. M., and Murant, A. F. 1983. Further evidence on the nature of the dependence of carrot mottle virus on carrot red leaf virus for transmission by aphids. Ann. Appl. Biol. 103:455-464.

Wright, N. S., and MacCarthy, H. R. 1963. Expression and detection of leafroll virus strains in potato. Am. Potato J. 40:154-162.

Wright, N. S., MacCarthy H. R., and Forbes A. R. 1970. Epidemiology of potato leaf roll virus in the Fraser River delta of British Columbia. Am. Potato J. 47:1-8.