Neth. J. Pl. Path. 84 (1978) 149–156

Potato leafroll virus: antiserum preparation and detection in potato leaves and sprouts with the enzyme-linked immunosorbent assay (ELISA)

D. Z. MAAT and J. A. DE BOKX

Research Institute for Plant Protection (IPO), Wageningen

Accepted 14 April 1978

Abstract

Potato leafroll virus (PLRV) was purified from *Physalis floridana*, applying freezing, low-speed centrifuging, ammonium sulphate precipitation, clarification with chloroform and butanol, ultracentrifuging and sucrose-gradient centrifuging. Three antisera with titers from 64 to 256 were prepared, one of them being sufficiently specific to be used in the enzyme-linked immunosorbent assay (ELISA).

With this test PLRV could be detected reliably in the foliage of secondarily infected, glasshouse-grown potato plants of six cultivars tested, and in sprouts of four or five of them. The results indicate that ELISA may be used successfully for routine testing of foliage of glasshouse-grown potato plants.

Introduction

Potato leafroll, the disease caused by potato leafroll virus (PLRV; cryptogram */*: */* : S/S : S,(I)/Ve/Ap), is known in Europe for more than a century and may still be the major virus disease of potato in many countries. Yields of infected plants may be reduced by as much as 50%. Many efforts to breed for resistance have not yet led to successful control. Lack of reliable detection methods greatly hampers such breeding programs as well as certification schemes.

In spite of many experiments to develop a suitable detection method (Bode, 1962), only the callose test was applicable routinely. This test (de Bokx, 1967) is now being used to a limited extent in some European countries for testing tubers, but it is not fully reliable. Therefore, potato virologists are searching for better detection techniques. The recently developed enzyme-linked immunosorbent assay (ELISA; Clark and Adams, 1977) seems promising. Casper (1977) was already able to detect PLRV with this test in different plant parts of potato and *Physalis floridana*. He suggested that routine indexing for PLRV with ELISA in phytosanitary programs might be possible.

PLRV has been purified by Peters (1967a and b), Kojima *et al.* (1969), Murayama and Kojima (1974), and Sarkar (1975, 1976). Successful antiserum preparation has been reported by Kojima and Murayama (1972) and Murayama and Kojima (1974). Their antiserum has been used by Caspar (1977) in his ELISA experiments with the virus. We also have now prepared antisera to PLRV and tested potato leaves and sprouts to develop ELISA into a routine method for potato seed certification.

Materials and methods

Virus propagation. From *Physalis floridana* inoculated by *Myzus persicae* with PLRV from 'Bintje' potato, the virus was further transmitted in the same way to a group of *P. floridana* plants. These were then propagated by cutting and rooting in plastic trays with potting soil and covered with a transparant lid. After about two weeks they were transplanted to 14 cm plastic pots with a standard potting soil and grown in a glasshouse at a temperature of about 20°C. From the resulting plants stem and leaf material was harvested three or more times. After each harvest NPK-fertilizer was supplied to ensure further growth.

Virus purification. The buffers used were those described by Sarkar (1975). For homogenizing and resuspending the first virus sediment, 0.1 M phosphate buffer, pH 7.3, containing 0.01 M 2-mercaptoethanol, and in all other cases 0.05 M phosphate buffer, pH 6.5, was used.

Unless otherwise stated, centrifuging at low speed was at 8000 rev/min for 10 min in a Sorvall RC2-B centrifuge, the rotor type depending on the quantity of material. Centrifuging at high speed was in a Beckman ultracentrifuge for 3 h at 27 000 rev/min, 3 h at 30 000 rev/min or 2 h at 40 000 rev/min using rotors 30, 35 and 50, respectively, or, in later experiments, 3.5 h at 31 500 rev/min or 2.5 h at 45 000 rev/min using rotors 35 and 50, respectively.

For the first sucrose-gradient centrifuging 0.5 ml of partially purified virus, obtained from 250 or 500 g of plant material was layered on top of a sucrose gradient from 10–40% and spun for 3 h at 24 000 rev/min in a Beckman SW 27 rotor. The gradients were prepared linear with volume, using an LKB-Ultrograd gradient mixer. Virus zones were isolated with an ISCO density-gradient fractionator. For the second sucrose-gradient centrifuging in the purification procedure each of the tubes was loaded with the virus obtained from 500 g or 1 kg of plant material.

In general, treatments were performed at 3°C.

Plant material (stems and leaves) was used chilled at 3°C, or frozen. Portions of 500 g were homogenized in 2000 ml of buffer, pH 7.3, in a one-gallon Waring blendor during 3–5 min. The homogenate was divided into portions of 400 ml, which were blended further in a high-speed 'Virtis' homogenizer during 2 min at 45 000 rev/min in an ice-bath. This homogenate was filtered through cheese-cloth after which the sap was centrifuged at low speed. Solid $(NH_4)_2SO_4$ was added to the supernatant, 38 g per 100 ml of juice and stirred for 15 min. The solution was centrifuged during 10 min at 10 000 rev/min and the sediment obtained resuspended in about 1/12 of the original sap volume of buffer, pH 7.3 and stirred for 1 h. Per 100 ml of this suspension 20 ml of a mixture of equal volumes of chloroform and n-butanol were added and mixed in a Waring blendor. The suspension was kept for 1 h and then centrifuged at low speed. The supernatant obtained was stored overnight at 3°C and centrifuged at low speed, or was kept frozen for 16 h to several days, thawed and centrifuged at low speed. Then the supernatant was centrifuged at high speed. The sediment of 500 g of leaf material was resuspended in 10 ml of buffer, pH 6.5, stirred for 1 h, centrifuged at low speed and the supernatant again centrifuged at high speed. Now the sediment was resuspended in 0.5 or 1 ml of buffer, stored overnight, and subjected to sucrose-gradient centrifuging, 0.5 ml per tube. The virus zone was isolated and injected into

a rabbit, or was reconcentrated to 0.5 ml by dialysis against a concentrated solution of polyethylene glycol or by high-speed centrifuging, and centrifuged on a second sucrose-gradient, 0.5 or 1 ml per tube. The virus zone was isolated and used for injection without further treatment.

Antiserum preparation. Rabbits were injected intravenously at varying intervals and with varying amounts of virus, depending on the availability and concentration of virus preparations.

One rabbit (A) was injected with virus from the first sucrose gradient. Two other rabbits (B and C) were injected with material from the second sucrose gradient. For C the virus was obtained from fresh plant material and no freezing was applied in the purification procedure. Titers were determined two weeks after the last injection. For more details see Results and Table 2.

Ouchterlony double-diffusion test. Titers of the antisera prepared were determined in the Ouchterlony double-diffusion test, using (partially) purified virus preparations. For identification some of these virus preparations were tested with an antiserum to PLRV prepared by Dr M. Kojima, Sapporo, Japan.

ELISA. The methods used to prepare γ-globulin fractions and enzyme conjugates differed only in some details from those described by Clark and Adams (1977). The optimal concentrations of these reagents in ELISA were determined by testing dilution series of both of them against dilution series of crude extracts from PLRV-diseased as well as from PLRV-free *Physalis floridana* plants.

Leaf extracts were prepared with a power-driven crusher and the sap was diluted 1:6 with buffer. Extracts from sprouts were prepared in the same way or with a hand-press. For more details see Results.

Tests were performed according to Clark and Adams (1977), but for practical reasons incubation periods and temperatures differed. Coating with γ-globulin was performed in a refrigerator, overnight or for a few days. Incubation with plant extracts was for 2 to 3 h at room temperature or overnight in a refrigerator, incubation with enzyme conjugate always for 2 to 3 h at room temperature, and with the enzyme substrate for 3/4 to 1 h at room temperature. Results were read with a photometer at 405 nm. Between two reaction stages the plates were thoroughly washed with a gentle jet of tap water followed by rinsing with a solution of 0.05% Tween 20 in distilled water. Like Clark and Adams (1977) we used alkaline phosphatase and p-nitrophenyl phosphate as enzyme and substrate, respectively.

Plant material tested in ELISA. Besides leaves of *P. floridana*, potato leaves and sprouts were tested. More information on the potato material used is given in Table 1. This material was from the IPO collection, grown under conditions preventing contamination. Moreover it was checked regularly for the presence of viruses. Potato tubers, harvested from healthy and virus-infected fieldgrown plants in July 1977, were stored at 3°C in a store-house until December. Then one eye from each tuber was taken and planted in a glasshouse at about 20°C and with supplementary illumination. The rest of the tuber was stored in the dark at 18–25°C to promote sprouting. From the plantlets grown from the eyes the youngest completely expanded leaf was

Table 1. Potato cultivars tested, numbers of tubers taken for sprouting and from each of which one eye was planted in the glasshouse, and the viruses with which they were infected.

Cultivar	Virus isolates[1]				
	PLRV-'Bintje'	PVA-'Lichte Industrie'	PVY^N-'Gineke'	PVY^O-'Paul Kruger'	Virus-free
Alpha		3		3	2
Bintje	3		3	3	3
Désirée	3	3		3	3
Doré		3	3		2
Eigenheimer		3	3	3	3
Element	3		3		2
Mirka		3	3	3	3
Ostara	3				1
Radosa	3				1
Resy	3	3	3	3	4
Total	18	18	18	18	24

[1]PVA = potato virus A; PVY^N = potato virus Y, tobacco veinal necrosis strain; PVY^O = potato virus Y, common strain.

Tabel 1. Getoetste aardappelrassen, de aantallen knollen, die ter kieming werden weggelegd en waarvan één oog werd uitgeplant in de kas, en de virussen waarmee ze geïnfecteerd waren.

tested about 5 weeks after planting. Sprouts were tested 7 and 11 weeks after the tubers were submitted to 18–25°C. The sprouts at 7 weeks were thick and woody, and extracts were prepared with a handpress. After 4 more weeks new sprouts had formed. These were thin and sappy and from them sap was prepared with a power-driven crusher.

Results

Virus purification and antiserum preparation. Virus yields, as indicated by the size of the virus peaks in the ISCO density-gradient fractionator, proved to be higher when freshly harvested plant material was used instead of material stored frozen. Freezing after chloroform-butanol clarification gave lower, but longer and faster high-speed centrifugings higher yields.

The antiserum titers are given in Table 2. Due to its relatively high antibody titer to normal plant material (4 and 64 to normal plant material and PLRV, respectively) rabbit A was omitted in further experiments. The antiserum from rabbit B reached a titer of 64 to PLRV after three and of 256 after seven injections. This serum was much more specific than that of rabbit A and samples of B (titer 64) were used in the ELISA experiments. For rabbit C an antibody titer of 256 was reached after five injections, but this serum slightly reacted with normal plant material. When no further injections were given, the titers rapidly decreased to 16.

Partially purified virus preparations reacted with the specific homologous antiserum and with Kojima's antiserum with one single precipitation line. There was no spur formation between the reaction lines indicating a very close relationship between our virus isolate and Kojima's.

Table 2. Details of antiserum preparation.

Rabbit	Total number of injections given	Total amount of plant material worked up (kg)	Number of weeks between first injection and determination of antiserum titer	Antiserum titer to	
				PLRV	normal plant material
A	5	3.5	6	64	4
B	3	9	9	64	
	5	14	12	64	–
	7	18	16	256	±
C	5	4	4	256	1 or 4

– no reaction; ± reaction questionable.

Tabel 2. Bijzonderheden betreffende de antiserumbereiding.

ELISA. For coating 1 µg/ml of γ-globulin proved to be the optimum in combination with a γ-globulin concentration of ca 3.5 µg/ml in the enzyme conjugate.

In preliminary experiments the use of phosphate-buffered saline, containing 0.05% Tween 20 (PBS-Tween; Clark and Adams, 1977) for preparation of sap samples from potato sprouts and for diluting the enzyme conjugate gave rise to strong non-specific reactions. Addition of 2% polyvinylpyrrolidon (PVP; MW 10 000) to the PBS-Tween for diluting the sap and PVP + 0.2% ovalbumin to the PBS-Tween for dilution of the

Table 3. Results of ELISA with an antiserum to PLRV and potato leaves and sprouts, virus-free or infected with PLRV, PVA, PVY^N or PVY^O. For potato cvs tested see Table 1.

Virus	Number of samples tested	Range of extinction values at 405 nm 0 0.1 0.2 0.4 0.8 1.6
PLRV	18	ooooooooooooooo
	18	++++++++++++++++++++
	18	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
PVA	18	ooooo
	18	++++
	18	xxxxxxxx
PVY^N	18	ooooooo
	18	xxxxxxx
PVY^O	15^1_2	oooooo
	17^2	xxxxxxx
Virus-free	24	ooooooooo
	12_2	+++++++++
	17^2	xxxxxxxxxxx

[1]Leaves of 'Désirée' with PVY^O could not be tested because of plant death.
[2]Not all tubers sprouted for the second time.
ooo potato leaf material; +++ extracts from thick woody sprouts (see Materials and methods); xxx extracts from thin sappy sprouts (see Materials and methods).

Tabel 3. Resultaten van ELISA met een antiserum tegen het aardappelbladrolvirus en aardappelbladeren en spruiten, virusvrij of geïnfecteerd met bladrolvirus of de aardappelvirussen A, Y^N of Y^O. Voor de getoetste cultivars zie Tabel 1.

Table 4. Results per cultivar of ELISA with an antiserum to PLRV and potato sprouts with and without PLRV.

Cultivar[1]		Numbers of samples and ranges of extinction values			
		PLRV-free		PLRV-infected	
		number	range	number	range
Bintje	a	1	0.07	3	0.21–0.35
	b	9	0.05–0.17	3	0.46–1.0
Désirée	a	5	0.06–0.09	3	0.10–0.14
	b	8	0.07–0.09	3	0.18–0.24
Element	a	1	0.14	3	0.20–0.33
	b	3	0.10–0.12	3	0.36–0.58
Ostara	a	1	0.08	3	0.23–0.35
	b			3	0.33–0.42
Radosa	a	1	0.10	3	0.13–0.25
	b	1	0.09	3	0.10–0.34
Resy	a	5	0.07–0.08	3	0.24–0.45
	b	9	0.07–0.12	2	0.38–0.46

[1]a, extracts from thick woody sprouts (see Materials and methods); b, extracts from thin sappy sprouts (see Materials and methods).

Tabel 4. Resultaten per cultivar van ELISA met een antiserum tegen het aardappelbladrolvirus en aardappelspruiten met en zonder bladrolvirus.

enzyme conjugate strongly reduced these non-specific reactions. Therefore these additions were used routinely in our tests with potato material. The optimum sap dilution varied somewhat between experiments and the average of 1 :6 was chosen for routine work.

When testing leaf material from *P. floridana* it was easy to distinguish PLRV-infected from virus-free samples, extinction values for diseased material being at least two times those for virus-free material.

The results of testing potato leaves and sprouts are summarized in Table 3. In tests with leaf material extinction values varied from 0.04–0.13 for PLRV-free and from 0.20–0.60 for PLRV-infected material. For sprouts the ranges were 0.05–0.17 and 0.10–1.0 for PLRV-free and -infected material, respectively, thus showing an overlap. Table 4 presents the results for each cultivar. It shows that, using the thick, woody sprouts, it was difficult to distinguish between PLRV-free and PLRV-diseased material from the cvs Désirée, Element, and Radosa, and also from Radosa using the newly formed sappy sprouts. With Bintje, Resy, and possibly also Ostara the differences were clear with both types of sprouts, as was the case with Désirée and Element using young sappy sprouts.

Discussion

Ultracentrifuging is not very effective to concentrate very dilute virus suspensions (McNaughton and Matthews, 1971). The rather crude material was therefore concentrated before clarification with chloroform-butanol. Yields were much higher when leaves were used fresh and no freezing was applied to clarify virus suspensions.

This may explain why only 4 kg of plant material was needed to reach an antiserum titer of 256 in rabbit C, whereas for rabbit B 18 kg was needed to reach the same titer. Rabbit A required only 3.5 kg to obtain a titer of 64 to PLRV, but this antiserum had a relatively high titer to normal plant material. In general the plant material used to immunize rabbit A was frozen for a shorter period than that for rabbit B and only one sucrose-gradient centrifuging was applied. As antiserum B was more specific than antiserum C, freezing seems to have a positive effect on antiserum specificity, as had the application of a second sucrose-gradient centrifuging, but a negative effect on virus yield.

If possible, sap samples were prepared with a power-driven crusher, collecting a predetermined number of drops in tubes prefilled with a certain volume of buffer. In this way much more samples could be prepared than when the material had to be homogenized. This is particularly important for routine testing. In preliminary experiments results did not differ much between the two methods of preparing extracts, provided the optimum dilutions were used.

Although a limited number of samples were tested, the results indicate that ELISA can be applied reliably to test foliage of secondarily infected, glasshouse-grown potato plants for the presence of PLRV. Whether the same holds for field-grown plants and for plants with primary infection has to be investigated.

The method is less reliable when infected sprouts are tested. However, ELISA gave satisfactory results with four or five of the six cultivars tested so far, when comparing tests with PLRV-free and -infected sprouts from tubers of a given cultivar.

For Dutch seed-potato production, ELISA would be most valuable if it could be applied in tuber indexing to test freshly harvested dormant tubers. Unfortunately, these were not available at the time of our tests with leaves and sprouts. However, Casper (1977) could detect PLRV in different parts of potato tubers.

Samenvatting

Aardappelbladrolvirus: antiserumbereiding en aantoonbaarheid in aardappelbladeren en -spruiten met de 'enzyme-linked immunosorbent assay' (ELISA)

Aardappelbladrol, veroorzaakt door het aardappelbladrolvirus (PLRV) is reeds meer dan een eeuw in Europa bekend en is in veel landen vermoedelijk nog steeds de ernstigste virusziekte van de aardappel. De bestrijding wordt ernstig bemoeilijkt door het ontbreken van een betrouwbare toetsmethode. De callosetoets heeft als routinetoets voor het aantonen van PLRV in knollen op beperkte schaal ingang gevonden, maar is niet 100% betrouwbaar.

Sinds kort is voor virussen een zeer gevoelige serologische methode beschikbaar gekomen, de 'enzyme-linked immunosorbent assay' (ELISA). Hierbij wordt het virus in plantemateriaal aangetoond door middel van een enzymreactie, waarvan de kleuromslag met het blote oog of (bij voorkeur) met een fotometer wordt afgelezen. De uitslag van de fotometer (extinctie) is een aanduiding voor de aanwezigheid van het virus. De methode lijkt bruikbaar voor routinematige toepassing. Om de bruikbaarheid voor het aardappelbladrolvirus te onderzoeken werd dit virus gezuiverd, werden antisera bereid en werden loof en spruiten van bladrolvirusvrije en -zieke aardappels onderzocht.

Bij de viruszuivering uit *Physalis floridana*, werd gebruik gemaakt van bevriezing,

precipitatie door middel van ammoniumsulfaat, klaring met chloroform en butanol, centrifugering bij laag en hoog toerental en suikergradiëntcentrifugering. Met de viruspreparaten werden drie konijnen geïnjiceerd. De verkregen antisera bereikten titers van 64 tot 256 in de micro-precipitatietoets (Tabel 2). Eén hiervan (B) was voldoende specifiek om in ELISA gebruikt te worden.

Toetsingen werden uitgevoerd met blad van in een kas opgekweekte bladrolvrije en secundair geïnfecteerde aardappelplanten en met in het donker gekweekte spruiten. Tabel 1 geeft een overzicht van de getoetste rassen, de aantallen knollen, die ter kieming waren weggelegd en waarvan telkens één oog in de kas werd uitgeplant, en van de virussen waarmee ze geïnfecteerd waren. De resultaten van de toetsingen, weergegeven in de Tabellen 3 en 4, laten zien, dat het bladrolvirus betrouwbaar kan worden aangetoond in het blad van alle zes cultivars, maar in de spruiten van slechts vier of vijf, nl. Bintje, Element, Ostara, Resy en mogelijk Désirée. ELISA kan derhalve goed worden gebruikt voor het aantonen van het bladrolvirus in blad van secundair geïnfecteerde kasplanten. Of dit ook geldt voor veldplanten moet nog worden onderzocht.

ELISA zal voor de Nederlandse pootgoedteelt het meest waardevol zijn wanneer met deze toets het virus in vers gerooide, slapende knollen kan worden aangetoond (nacontrole). Helaas waren zulke knollen niet beschikbaar toen we onze proeven met blad en spruiten uitvoerden.

Acknowledgments

Thanks are due to J. Vink for virus purification and to P. G. M. Piron, C. Cuperus, and H. Brinks for raising plant material and assistance in serological testing.

References

Bode, O., 1962. Die Blattrollkrankheit der Kartoffel. Die durch die Virose bei der Kartoffel hervorgerufenen Veränderungen und die Möglichkeiten des Nachweises. Angew. Bot. 36: 86–116.

Bokx, J. A. de, 1967. The callose test for the detection of leafroll virus in potato tubers. Eur. Potato J. 10: 221–234.

Casper, R., 1977. Detection of potato leafroll virus in potato and in *Physalis floridana* by enzyme-linked immunosorbent assay (ELISA). Phytopath. Z. 90: 364–368.

Clark, M. F. & 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.

Kojima, M., Shikata, E., Sugawara, M. & Murayama, D., 1969. Purification and electron microscopy of potato leafroll virus. Virology 39: 162–174.

Kojima, M. & Murayama, D., 1972. An attempt to prepare antiserum against potato leaf-roll virus. Ann. phytopath. Soc. Japan 38: 255–257.

McNaughton, P. & Matthews, R. E. F., 1971. Sedimentation of small viruses at very low concentrations. Virology 45: 1–9.

Murayama, D. & Kojima, M., 1974. Antigenicity of potato leafroll virus. Proc. Japan Acad. 50: 322–327.

Peters, D., 1967a. The purification of potato leafroll virus from its vector *Myzus persicae*. Virology 31: 46–54.

Peters, D., 1967b. Potato leafroll virus, its purification from its vector *Myzus persicae*. Meded. L. E. B. Fonds No. 45. H. Veenman & Zonen, Wageningen, the Netherlands.

Sarkar, S., 1975. A rapid electron microscopic diagnosis of potato leafroll virus. Potato Res. 18: 227–236.

Sarkar, S., 1976. Potato leafroll virus contains a double-stranded DNA. Virology 70: 265–273.

Address

Instituut voor Plantenziektenkundig Onderzoek (IPO), Binnenhaven 12, 6709 PD Wageningen, the Netherlands.