ACQUISITION AND TRANSMISSION OF POTATO LEAFROLL VIRUS BY *MYZUS PERSICAE*

quantitative aspects

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Proefschrift
ter verkrijging van de graad van doctor
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BIBLIOTHEEK
LANDBOUWUNIVERSITEIT
WAGENINGEN

aan mijn ouders

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STELLINGEN

1. In haar onderzoek naar de kwantitatieve resistentie tegen *Xanthomonas campestris* pv. *oryzae* in rijst is Koch onterecht voorbijgegaan aan de mogelijkheden van ELISA.

Koch, M.F. (1990). Aspects of quantitative resistance to *Xanthomonas campestris* pv. *oryzae* in rice. Proefschrift Landbouwuniversiteit Wageningen.

2. Implicatie van het Meerjarenplan Gewasbescherming ten aanzien van een reductie in het gebruik van chemische grondontsmettingsmiddelen kan leiden tot een verhoogd optreden van door nematoden overgedragen plantevirussen.

3. Composteren van groente, fruit en tuinafval leidt tot verspreiding van bodempathogenen.

4. De epidemiologische significantie van het recent door Holmes voorgestelde 'strategic monitoring' ter duiding van gebieden met een verhoogd risico voor het optreden van infecties van het gerstevergelingsvirus in wintergranen zal toenemen indien er tevens kwantitatieve gegevens omtrent het optreden van menginfecties van stammen van dit virus bij worden betrokken.

Holmes, S.J. Strategic monitoring: a new approach to identifying BYDV high risk. Abstracts Anniversary Meeting, British Society for Plant Pathology, Bath, December 1990.

5. De door Creamer en Falk ontwikkelde immunohybridisatietoets levert een belangrijke bijdrage tot het kwantificeren van het optreden van heterologe inkapseling tussen stammen van het gerstevergelingsvirus.

Creamer, R. en Falk, B.W. 1990. Direct detection of transcapidated barley yellow dwarf luteoviruses in doubly infected plants. Journal of General Virology **71**, 211-217.

6. De constatering dat Bruenn de door het 'International Committee on Taxonomy of Viruses' voorgestelde weergave van afkortingen van plantevirussen niet hanteert, Flavivirussen nog niet als een afzonderlijke familie beschouwd en tabaksratelvirus een enkelvoudig genoom toedeelt, versterkt zijn afwijkend taxonomische voorstel met betrekking tot de phylogenetische verwantschap tussen virussen op basis van homologieën tussen RNA-afhankelijke RNA polymerasen niet.

Bruenn, J.A. (1991). Relationships among the positive strand and double-strand RNA viruses as viewed through their RNA-dependent RNA polymerases. Nucleic Acids Research **19**, 217-226.

7. Anticiperend op de commercialisering van genetisch gemodificeerde aardappelvariëteiten die het manteleiwit van bepaalde virussen tot expressie brengen, dienen verantwoordelijke keuringsdiensten het onderzoek naar niet-serologische detectiemethodieken te stimuleren.

8. Een methodiek die in de nacontrole van pootaardappelen de aanwezigheid van virussen rechtstreeks in de knol detecteert levert, vooral in zuid-Europese landen met een jaarrond teelt van aardappelen, een belangrijke bijdrage in het beteugelen van de virusverspreiding.

9. De door Van Beek, Wood & Hughes gerapporteerde lagere mortaliteit van *Trichoplusia ni* larven geïnoculeerd met AcMNPV-HOB virusdeeltjes die elk zeven tot negen nucleocapsiden bevatten in vergelijking tot inoculatie met deeltjes die slechts één nucleocapside bevatten, strookt niet met hun uitspraak dat de eerste groep virusdeeltjes effectief zeven tot negen maal meer infectieus is en hun eerdere resultaten met betrekking tot virusdosis en mortaliteit.

Van Beek, N.A.M., Wood, H.A. en Hughes, P.R. (1988). The number of nucleocapsids of enveloped *Autographa californica* nuclear polyhydrosis virus particles affects the survival time of neonate *Trichoplusia ni* larvae. Journal of Invertebrate Pathology **52**, 185-186.

Van Beek, N.A.M., Wood, H.A. en Hughes, P.R. (1988). Quantitative aspects of nuclear polyhedrosis virus infections in lepidopterous larvae: the dose-survival time relationship. Journal of Invertebrate Pathology **51**, 58-63.

10. De benaming 'groene virologie' dient vermeden te worden daar waar het de ecologisch geörienteerde plantevirologie betreft.

J.F.J.M. van den Heuvel
Acquisition and transmission of potato leafroll virus by *Myzus persicae*; quantitative aspects.
Wageningen, 22 mei 1991

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INTRODUCTION

Potato leafroll virus (PLRV) is a member of the luteovirus group. Luteoviruses affect many economically important crops all over the world including temperate, sub-tropical and tropical regions. Most members, like PLRV and bean leaf roll virus (Ashby, 1984), have a limited host range, whereas beet western yellows virus (Rochow and Duffus, 1981) and its close serological relatives infect a very wide range of monocotyledonous and dicotyledonous plants. Luteoviruses are not transmitted by mechanical inoculation but are persistently transmitted by aphids in a circulative manner. Apparently they do not replicate in their vectors (Eskandri, Sylvester & Richardson, 1979). Most members show a high degree of vector specificity.

PLRV has a monopartite RNA genome (Rowhani & Stace-Smith, 1979) and an isometrical particle morphology. PLRV particles have a diameter of about 23 nm (Peters, 1967) and their protein shell comprises 180 copies of one polypeptide species with a molecular mass of about 24 kDa (Rowhani & Stace-Smith, 1979; Waterhouse, Gildow & Johnstone, 1988). The recent determination of the complete nucleotide sequence of the PLRV genome revealed that it consists of approximately 5900 nucleotides (Van der Wilk *et al*, 1989, Mayo *et al*, 1989; Keese *et al*, 1990). It is comprised of six major open reading frames (ORF) of which ORF 3 was identified as the coat protein gene. Interviral amino acid sequence comparison of the coat protein ORFs showed strong homologies among different members of the luteovirus group.

PLRV is mainly restricted to the phloem tissue of an infected plant. Virus particles have been observed in mature sieve elements, in companion cells and in plasmodesmata connecting these two types of cells (Shepardson, Esau & McCrum, 1980). Occasionally, virus can be found in mesophyll cells. It induces a necrosis of a type called necrotic obliteration (Esau, 1967) as a primary pathological change. Necrosis involves sieve tube elements and the associated companion cells. Phloem parenchyma cells undergo hypertrophy and crush the necrotic cells. A large amount of callose is deposited in sieve tube elements and obstructs the transport of assimilates. Consequently, external symptoms, viz. interveinal chlorosis, leaf rolling and purpling of leaf margins become apparent and plants display severe stunting. PLRV infection can cause up to 60% reduction in potato crop yield. Furthermore, seed crops with a low incidence of PLRV are already rejected from certification schedules.

Although PLRV is persistently transmitted by several aphids species, *Myzus persicae* Sulzer is considered to be the principal and most efficient vector (Sylvester, 1980). Virus ingestion occurs when the aphid feeds on infected phloem cells. Ingested virus moves through the food canal between the maxillary stylets to the hypopharynx in which the gustatory organs are located. Virus continues to move through the lumen of the stomach region of the midgut. Arrived at the hindgut, the virus may pass the gut wall into the hemocoel or pass through to the rectum and leave the aphid in the honeydew. To be transmitted by the aphid, the virus present in the hemolymph has to pass through the accessory salivary gland to be released into the salivary duct. Along with salivary gland secretory products it is excreted during aphid's feeding.

Based on morphological information provided by electron microscopical images, Gildow (1987) developed a model for the mechanism of transcellular transport of luteoviruses through the hindgut cells of an aphid. In this hypothetical model, the first step leading to luteovirus transport through the hindgut is the attachment of a virus particle to the apical plasmalemma of the epithelium. Attachment of the virus, which is mediated by luteovirus-recognizing receptor molecules embedded in the membrane, could induce endocytosis of the particle into a coated pit. Coated pits are then budded off into the cytoplasm to become a coated vesicle. Several of these vesicles could fuse together forming a tubular vesicle which are directed toward the basal end of the cell and eventually contact and fuse with the basal plasmalemma. This results in exposure of the vesicle lumen and the virus particles it contains to the aphid hemocoel. The virus would then be free to diffuse through the extracellular basal lamina which is suspended in the hemolymph. At salivary gland level the mechanism of transport may also be receptor-mediated (Gildow, 1982) like the transcellular virus transport across hindgut cells. However, the direction of transport is opposite. The current opinion is that initial recognition of luteoviruses occurs at the level of the aphid's hindgut and that vector specificity is determined at the level of the accessory salivary gland. The particle protein will play an important role in these vector-virus interactions (Gildow & Rochow, 1981; Massalski & Harrison, 1987; Harrison & Robinson, 1988).

The process of luteovirus acquisition and transmission by aphids can be experimentally splitted into two phases, viz. the acquisition access period (AAP) and the inoculation access period (IAP). Transmission of these circulative viruses is furthermore characterized by the existence of a period of latency (LP), which is the period between the acquisition of an infectious virus dose and the moment at which the aphid can infect a plant. After this period is completed, the vector is infectious

for its further life span. The concepts of AAP, LP and IAP allow to measure virus transmission by aphids quantitatively. Studies on acquisition and transmission of PLRV by *M. persicae* revealed that the ability of aphids to transmit the virus differed widely among individuals and strongly depended on the level of symptom expression of the *P. floridana* source plant (MacKinnon, 1962 and 1963; Peters & Elderson, 1984). In the present study three major components determining the acquisition and transmission of PLRV by *M. persicae* were analyzed, viz. the feeding behaviour of aphids, the amount of virus acquired by aphids, and the intrinsic properties of the virus. The honeydew excretion of single *M. persicae* nymphs, as a measure of their feeding activities, was investigated during the AAP on PLRV-infected *P. floridana* plants and related to the efficiency of virus transmission (Chapter 1). The analysis of the second component required the development of a sensitive serological assay to detect PLRV antigen in individual aphids (Chapter 2). By means of this assay, the uptake of PLRV by *M. persicae* was studied from artificial diets containing defined amounts of PLRV, from leaves of infected *P. floridana* plants differing in symptom severity (Chapter 3), and from secondarily-infected potato plants of various genotypes with different levels of field resistance (Chapter 4). During these studies it became apparent that changes of the intrinsic properties of the virus particle also attributed to the observed differences in virus transmission. Therefore, the relationship between the transmissibility of PLRV isolates and their surfaces structures was further investigated. To this end, monoclonal antibodies (MAbs) were generated to PLRV (Chapter 5) and their corresponding epitopes were characterized as to their function in regulating the passage of PLRV through the aphid's body (Chapter 6).

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CHAPTER 1

TRANSMISSION OF POTATO LEAFROLL VIRUS IN RELATION TO THE HONEYDEW EXCRETION OF *MYZUS PERSICAE*

SUMMARY

Honeydew excretion of single *Myzus persicae* nymphs on potato leafroll virus (PLRV)-infected *Physalis floridana* plants was studied during the acquisition access period (AAP) in relation to the efficiency of virus transmission.

With increasing length of the AAP, the percentage of nymphs that transmitted the virus increased. These nymphs produced significantly more honeydew droplets during the AAP on PLRV-infected *P. floridana* plants than nymphs which failed to transmit the virus. However, the number of honeydew droplets excreted during the AAP by transmitting nymphs did not affect the length of the latency period. Nymphs which infected the first test plant after a short latency period produced a similar amount of honeydew during the AAP to those with a longer latency period.

Honeydew excretion recorded on plants of different ages, showed that nymphs feeding on bottom leaves of infected plants produced more honeydew droplets than on comparable leaves of healthy plants. On infected plants, nymphs produced more honeydew droplets on bottom leaves with pronounced symptoms than on top leaves that hardly showed any symptom of PLRV infection.

The concentration of viral antigen measured by ELISA was lower in top leaves than in bottom leaves of infected plants. Nevertheless, nymphs feeding on top leaves transmitted the virus more efficiently than those which used bottom leaves as virus source. When bottom leaves were used as a virus source, the percentage of viruliferous nymphs decreased with plant age. These results indicate that the availability of virus for acquisition by aphids declines with increasing plant age and symptom severity.

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INTRODUCTION

Virus transmission by aphids involves interactions between viruses, vectors and plants. Each of these biological entities has its inherent variability, and mutual interactions are influenced by environmental conditions (Rochow, 1974). Even within a specific vector-virus-plant system, virus transmitting efficiency differs widely among individuals as shown in tests carried out under strictly controlled conditions (Fargette, Jenniskens & Peters, 1982). Differences in the amount of virus acquired by individual aphids will account for the observed variation to a great extent. *Myzus persicae* (Sulz.) which had fed on top leaves of PLRV-infected *Physalis floridana* Rydb. were better transmitters than those which had fed on middle or bottom ones (MacKinnon, 1962). Moreover, Peters & Elderson (1984) demonstrated while using *P. floridana* plants infected under different regimes, that plants slightly affected by PLRV-infection were much better sources for virus acquisition by *M. persicae* than plants with severe symptoms. Both MacKinnon (1963) and Peters & Elderson (1984) suggested that their observations were related to differences in the availability of PLRV for acquisition by *M. persicae*.

In this paper, some factors that play a role in the acquisition and subsequent transmission of PLRV by *M. persicae* were analysed by studying the honeydew excretion of single *M. persicae* nymphs on *P. floridana* plants. Auclair (1958) and Mittler (1958) indicated that honeydew excretion is an appropriate measure of the amount of ingested phloem sap and therefore a useful parameter to measure feeding activities (Sylvester, 1988). Sylvester (1967) showed that the efficiency by which pea enation mosaic was transmitted, was positively correlated with the amount of honeydew excreted by *Acyrthosiphon pisum* (Harris). As PLRV is a phloem-restricted virus and *M. persicae* feeds chiefly on phloem tissue, the amount of honeydew excreted may be an indicator of the amount of PLRV imbibed by the aphids.

To gain more insight into the quantitative aspects of virus acquisition and transmission, the honeydew excretion of single *M. persicae* nymphs was monitored on leaves of infected *P. floridana* plants of varying age. The leaves differed in symptom severity, and their concentration of viral antigen was estimated by ELISA. The number of honeydew droplets excreted by nymphs during the AAP was compared with their ability to transmit PLRV.

MATERIALS AND METHODS

Aphids

M. persicae biotype WMp1, WMp2, WMp3, and WMp4 were obtained from Mr F.L. Dieleman, Wageningen Agricultural University (Reinink *et al*, 1989). Virus-free stocks of these biotypes were reared, in cohorts differing by one day in age, on *Brassica napus* L. ssp. *oleifera* (oilseed rape) in a greenhouse compartment at a 16-h photoperiod (L/D:16/8) at 20±2°C as reported by Van den Heuvel & Peters (1989).

Only first-instar nymphs, upto 24-h old, were used for recording honeydew excretion. They do not wander off the test plants as frequently as older nymphs or adults do, and they will not moult before the end of the honeydew recording. In addition, these nymphs were chosen as they transmit the virus more efficiently than older nymphs (MacKinnon, 1962).

Virus

PLRV was maintained by repeated aphid transfers on *P. floridana* in a glasshouse (L/D:16/8) at 23±2°C. All PLRV-infected *P. floridana* plants used in the experiments were infected in the cotyledon stage by single viruliferous *M. persicae* nymphs.

Recording of honeydew excretion

During the acquisition access period (AAP), the honeydew excretion was monitored using a 'honeydew clock', similar to the one described and illustrated by Ajayi & Dewar (1982). A metal frame supported a motor-driven rod which rotated at constant velocity and wound up five parallel strips of indicator paper (50 mm wide) which were supported horizontally and had previously been soaked in a solution of bromocresol purple in ethanol (2 mg/ml). Plants were placed close to the apparatus and a Perspex plate (40 x 20 x 2 mm), with three holes of 10 mm diameter, was clipped to the underside of a leaf which was supported over one of the indicator strips. At the start, three nymphs were placed in each hole but after 1 h, two nymphs were removed and the honeydew excretion of the remaining nymph was recorded. Honeydew droplets formed a linear pattern of blue spots on the yellow indicator paper and the excretion of 15 nymphs could be recorded simultaneously.

The total number of honeydew droplets excreted by each nymph was recorded, as well as the period between the start of the AAP and the production of the first honeydew droplet. The numbers of honeydew droplets excreted (a) were $\log_{10}(a+1)$

transformed before factorial analysis using the SAS program.

Infectivity testing

Directly after the AAP in which honeydew excretion was recorded, the nymphs were transferred individually to *P. floridana* seedlings, to test their infectivity using an inoculation access period (IAP) of 5 or 6 days, or to estimate the median latency period (LP_{50}) in a series of short IAP's. The LP_{50} is defined as the time interval from the start of the AAP to the end of that IAP in which the first transmission occurred, and was estimated by log-probit analysis of the time-series of cumulative percentages of nymphs transmitting the virus for the first time (Sylvester, 1965). The standard error (S.E.) of the LP_{50} was calculated following Finney (1962).

After infectivity testing, plants were fumigated and held in a glasshouse at 23±2°C (L/D:16/8) for symptom development.

Detection of viral antigen

The cocktail method of the enzyme-linked immunosorbent assay (cocktail-ELISA) was performed to detect viral antigen in homogenized leaves of PLRV-infected *P. floridana* (Van den Heuvel & Peters, 1989). Wells of Nunc-Immunoplate IF plates (Nunc, Denmark) were coated with 2 μg/ml gamma-globulins in coating buffer (0.05 M sodium carbonate, pH 9.6; 250 μl per well). After 3-h incubation at 37°C, wells were rinsed with tap water and filled with 200 μl of a cocktail of alkaline phosphatase-conjugated gamma-globulins (1 μg/ml) and ground leaf material, 20-fold diluted in sample buffer (0.02 M sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, 2% polyvinylpyrrolidone (M.W. about 44,000), 0.05% Tween 20 and 0.2% ovalbumin). After overnight incubation at 4°C, wells were washed and 200 μl of 2.7 mM p-nitrophenyl phosphate disodium salt in 10% diethanolamine, pH 9.8 was added. Colour development was allowed to proceed at room temperature. Absorbance was measured on a Titertek Multiskan colorimeter (Flow Laboratories Ltd, Irvine, Scotland) at 405 nm.

RESULTS

Differences in transmission efficiency between aphid biotypes

To determine the most suitable biotype of *M. persicae* to work with, 30 nymphs of each biotype were given an AAP of 24-h on *P. floridana* showing severe symptoms of PLRV-infection. Afterwards they were individually transferred to *P.*

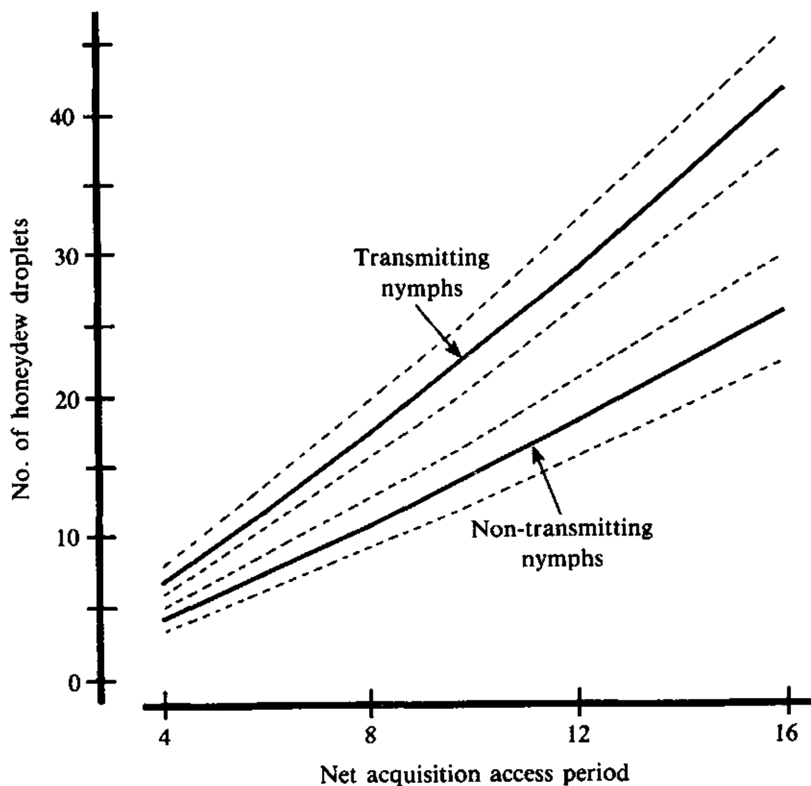

Fig. 1. The number of honeydew droplets excreted by transmitting and non-transmitting *M. persicae* nymphs during the net acquisition access period. Dashed lines represent the 95% fiducial limits.

floridana seedlings for an IAP of 72 h. Nymphs of biotype WMp1 were poor transmitters of the virus. Only one nymph succeeded in infecting a seedling. The nymphs of biotype WMp2 and WMp3 infected respectively 21 and 23 seedlings. Nymphs of biotype WMp4 were intermediate in transmission efficacy, 13 seedlings being infected. Differences in development of PLRV symptoms were not observed between seedlings infected by nymphs of the tested biotypes.

Since most experiments had to be carried out in autumn and winter, they were performed with the anholocyclic biotype WMp2.

The effect of the length of the AAP on virus transmission

The honeydew excretion of 49, 49, 51, 28 nymphs was recorded during an AAP

of 4, 6, 8 or 16 h on completely expanded leaves, showing clear symptoms of PLRV infection, of seven- to nine-week old *P. floridana* plants. The total number of honeydew droplets excreted and the period between the start of the AAP and the production of the first honeydew droplet was measured. Nymphs which had an AAP of 4, 6 or 16-h were placed on *P. floridana* seedlings for an IAP of 5-6 days. Nymphs which had an 8-h AAP were transferred eight times to fresh *P. floridana* seedlings at 12-h intervals to estimate the LP_{50}.

The percentages of nymphs that transmitted the virus during the IAP were 51, 55, 63 and 86% after the AAP's of 4, 6, 8 and 16 h respectively. The mean time intervals that elapsed before the first honeydew droplet was produced were 2.0, 2.8, 3.2 and 4.8 h during the AAP's of 4, 6, 8 and 16 h respectively, and did not differ between nymphs that eventually transmitted the virus and those that did not.

The net AAP (NAP) was determined for each nymph individually by subtracting the time interval between the start of the AAP and the production of the first honeydew droplet from the AAP. The NAP was $\log_{10}$ transformed for regression analysis. The equations of the transformed number of honeydew droplets excreted during the $\log_{10}$ (NAP) were $Y = (1.21 \pm 0.07)X + (0.17 \pm 0.05)$ ($\hat{\sigma}$, 0.22; r, 0.86; $P < 0.0001$) for transmitting nymphs and $Y = (1.18 \pm 0.11)X + (0.01 \pm 0.06)$ ($\hat{\sigma}$, 0.27; r, 0.79; $P < 0.0001$) for non-transmitting nymphs. The back-transformed values of these equations are graphically presented in Fig. 1. Transmitting nymphs produced 35 to 60% more honeydew droplets than non-transmitting nymphs.

The LP_{50} of the nymphs which had an AAP of 8 h was estimated to be 58 h. Transmitting nymphs which accomplished their first transmission after LP's shorter than 58 h, produced on average 15.2 (S.E. = 2.0; n = 17) honeydew droplets. Nymphs which did so after LP's longer than 58 h, produced 11.1 (S.E. = 2.2; n = 15) honeydew droplets in the AAP; the difference is only marginally significant ($P < 0.10$).

Leaf position and age of virus source plants

The honeydew excretion of single nymphs was recorded during a period of 8 h on top or bottom leaves of five-, eight- or 12-week old *P. floridana* plants, either healthy or PLRV-infected. The total number of honeydew droplets as well as the moment at which honeydew excretion started was monitored. The nymphs were individually tested for their ability to transmit PLRV in serial transfers to *P. floridana* seedlings using IAP's of 16 h for the first transfer, 12 h for the next six and 96 h for the last transfer.

Table 1. Mean number of honeydew droplets produced by *M. persicae* nymphs during an AAP of 8 h on *P. floridana* plants.

	Honeydew droplets produced by *M. persicae* ± S.E. (n)			
	PLRV-infected		Healthy	
Plant age (w)	Top leaves[a]	Bottom leaves[b]	Top leaves	Bottom leaves
5	3.6 ± 0.7 (41)	6.3 ± 1.1 (42)	3.3 ± 0.7 (27)	2.6 ± 0.8 (26)
8	5.5 ± 0.8 (37)	7.3 ± 1.3 (42)	1.3 ± 0.3 (28)	0.9 ± 0.2 (34)
12	4.3 ± 0.6 (42)	6.2 ± 1.1 (39)	2.1 ± 0.5 (27)	1.9 ± 0.5 (26)

[a] Leaves, hardly showing symptoms of PLRV infection.
[b] Leaves with pronounced PLRV symptoms.

Factorial analysis showed that only the main effect PLRV infection (D.F., 1; F, 74.22; P < 0.0001) was significant (Table 1), but not that of leaf position (D.F., 1; F, 0.78; P > 0.38) or plant age (D.F., 2; F, 1.86; P > 0.16). Nymphs produced significantly more honeydew droplets on infected than on healthy *P. floridana* plants. The interactions between plant age and PLRV infection (P < 0.0002; D.F., 2; F, 8.83), and between PLRV infection and leaf position (P < 0.019; D.F., 1; F, 9.25) were both significant but the interaction between leaf position and plant age was not (P > 0.10; D.F., 2; F, 0.29). The number of honeydew droplets excreted by *M. persicae* nymphs on bottom leaves of infected plants was higher than that on top leaves of the same plants (P < 0.01) or on top or bottom leaves of healthy plants (P < 0.001), at any plant age tested (Table 1). The nymphs also produced more honeydew droplets on top leaves of PLRV-infected than on healthy *P. floridana* plants; the differences on plants which were infected 8 (P < 0.001) and 12 w (P < 0.01) before were significant. On healthy plants, the number of honeydew droplets produced was the same on top and bottom leaves (P > 0.10), for each plant age.

Results from the same experiment showed that almost all nymphs transmitted the virus after an AAP of 8 h on top leaves of PLRV-infected *P. floridana* plants of different age (Table 2). The LP_{50} found after virus acquisition on top leaves (Fig. 2) was the same for all plant ages (P < 0.001). When nymphs acquired PLRV from bottom leaves, the percentage of viruliferous nymphs decreased with plant age (Table

Table 2. Percentage of viruliferous *M. persicae* nymphs after an AAP of 8 h on top and bottom leaves of PLRV-infected *P. floridana* plants.

Plant age (w)	Percentage viruliferous nymphs	
	Top leaves	Bottom leaves
5	100	79
8	92	69
12	98	41

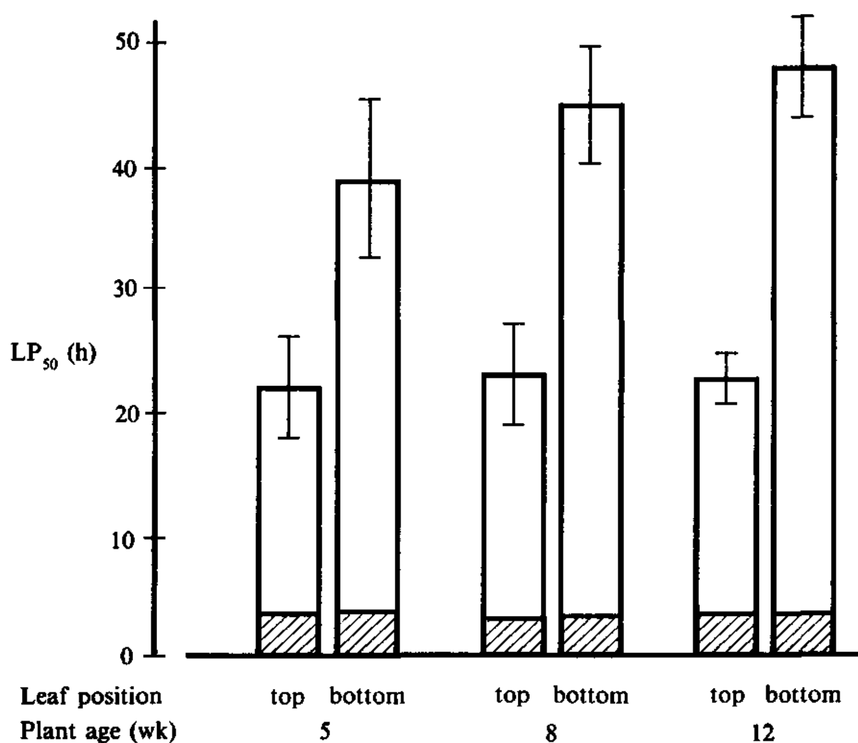

Fig. 2. The median latency period (LP_{50}) of viruliferous *M. persicae* nymphs after an AAP of 8 h on top or bottom leaves of PLRV-infected *P. floridana* plants. Vertical bars represent the S.E. The shaded areas represent the time interval between the start of the AAP and production of the first honeydew droplet.

Table 3. Number of honeydew droplets produced on PLRV-infected *P. floridana* plants by *M. persicae* nymphs with latency periods (LP), shorter and longer than the estimated median LP[a].

	Number of honeydew droplets ± S.E.			
	Top leaves		Bottom leaves	
Plant age (w)	Short LP	Long LP	Short LP	Long LP
5	3.5 ± 0.7[b]	4.1 ± 1.3	6.1 ± 1.3	6.9 ± 2.0
8	8.0 ± 1.4	4.5 ± 1.1	7.9 ± 1.3	6.5 ± 1.4
12	4.6 ± 0.7	3.1 ± 0.8	6.5 ± 2.0	4.4 ± 1.2

[a] The median latency periods for the various combinations are shown in Fig. 2.
[b] Back-transformed values are presented.

2) but the LP_{50}'s on these leaves did not differ significantly ($P > 0.10$) (Fig. 2). The LP_{50}'s of nymphs acquiring virus on bottom leaves were longer than those on top leaves for all plant ages ($P < 0.05$). The time elapsing before production of the first honeydew droplet was not affected by plant age or leaf position (Fig. 2, shaded areas).

For all combinations of plant age and leaf position, the numbers of honeydew droplets excreted by the nymphs that accomplished their first transmission before or after the LP_{50} were compared (Table 3). Factorial analysis showed that only the main effect leaf position was significant (D.F., 1; F, 5.3;. $P < 0.02$). The main effects plant age and length of the LP, and all interactions were not significant ($P > 0.05$). The number of honeydew droplets produced by nymphs with short LP's on top or bottom leaves of infected plants of varied age did not differ significantly from the number produced by nymphs with long LP's.

The diameter of the honeydew droplets was estimated with a stereomicroscope after collecting them in a Petri dish, filled with mineral oil. The honeydew droplets, which are almost spherical, produced by one-day old nymphs ranged from 9.9 to 10.7 nl. The size of the honeydew droplets was not affected by PLRV infection, leaf position or age of the plants.

Table 4. Influence of leaf position and plant age on the absorbance values obtained with cocktail-ELISA in PLRV-infected *P. floridana* plants.

Plant age (w)	A_{405} ± S.E. Leaf position Top	Bottom
5	0.27 ± 0.08	1.30 ± 0.05
8	0.46 ± 0.11	0.92 ± 0.11
12	0.62 ± 0.08	1.48 ± 0.10

Virus concentration in leaves

In two parallel experiments, samples from bottom and top leaves of five-, eight- and 12-week old PLRV-infected and healthy *P. floridana* plants were tested in cocktail-ELISA. Of each combination of leaf position and plant age, leaves from 5 to 10 plants were collected and pooled. After freezing, leaf material was crushed and 1 g from each sample was homogenized and diluted 20-fold before testing (Table 4). Leaves of healthy *P. floridana* plants were used as controls.

Factorial analysis of the absorbance values showed that both main effects, leaf position (D.F. 1; F, 73.40; $P < 0.0001$) and plant age (D.F., 2; F, 4.85; $P < 0.01$) were significant. The interaction between leaf position and plant age was not significant ($P > 0.05$). The absorption values measured with samples of bottom leaves were significantly higher than those of top leaves of PLRV-infected *P. floridana* for all plant ages tested, and tended to increase with plant age. The absorption values at 405 nm of the controls were lower than 0.01.

DISCUSSION

Many workers have pointed out the wide variation that occurs in the interactions between viruses, plants and aphids (Rochow, 1974; Pirone & Thornbury, 1988; Fargette, Jenniskens & Peters, 1982). In order to limit the variability as much as possible, the production of nymphs (Van den Heuvel & Peters, 1989), plant material and environmental conditions was standardized.

The results obtained in experiments with AAP's of various lengths and done on

comparable virus sources (Fig. 1) suggest that the transmission of PLRV depends on the dose acquired. The longer the AAP, the more honeydew droplets were excreted and the higher was the percentage of transmitting nymphs. Dose sensitivity in persistent virus transmission has already been reported by Fargette, Jenniskens & Peters (1982), Sylvester (1967; 1980), and Paliwal & Sinha (1970). With phloem-restricted viruses, virus acquisition by aphids from infected plants occurs as a result of phloem sap imbibition. Thus, a higher number of honeydew droplets excreted is probably correlated with a higher amount of virus ingested by the aphid. However, this did not effect the length of the LP, since the difference in the amount of honeydew excreted by nymphs with short and long LP's was not significant (Table 3).

Nymphs feeding on bottom leaves of PLRV-infected *P. floridana* excreted more honeydew droplets during the AAP than those on top leaves (Table 1). As transmitting nymphs produced more honeydew droplets (Fig. 1) than non-transmitters, one would expect nymphs that use bottom leaves as a virus source to be more efficient virus transmitters than those which acquire PLRV from top leaves. However, nymphs fed on top leaves were in fact more efficient in virus transmission (Table 2). In addition, on bottom leaves, the percentage of viruliferous nymphs declined with plant age (Table 2), although the concentration of viral antigen in those leaves did not decrease (Table 4).

MacKinnon (1963) also observed less frequent virus transmission by *M. persicae* when he used old leaves PLRV-infected *P. floridana* as compared with young ones. Peters & Elderson (1984) found less virus transmission when using PLRV-infected *P. floridana* plants showing severe symptoms than when using plants with mild symptoms. So it is probable that the availability of virus to aphids declines with increasing age of the infection and symptom severity. Barker & Harrison (1986) showed that *M. persicae* was unable to acquire and transmit PLRV from potato plants cv Maris Piper later in the season and that aphids transmitted much less frequently from older plants than from younger plants of the same cultivar. These differences in virus transmission could not be explained in terms of differences in virus concentration, because the virus was readily detected by ELISA (Barker & Harrison, 1986). Although we used *P. floridana* as virus source, our findings are in agreement with Barker & Harrison (1986). Our results also demonstrate that differences in feeding behaviour between the nymphs on top and bottom leaves of PLRV-infected *P. floridana* do not explain the better virus acquisition from top leaves since nymphs imbibed more phloem sap on the bottom leaves. Therefore, availability rather than virus concentration or feeding behaviour determines virus

acquisition by aphids.

Barker (1987) reported that PLRV invades non-phloem tissue in *Nicotiana clevelandii* when in mixed infection with potato virus Y. Presumably, disease-associated changes in *P. floridana* plants infected with PLRV induce the invasion and replication of PLRV in parenchymatous tissue in later stages of the infection, such that the virus concentration in the bottom leaves is higher (Table 4) without a concomitant improvement of virus acquisition by nymphs from those leaves. In ELISA the antigen concentration in the whole of the leaf is determined, while it is, the concentration in the phloem which determines the availability for acquisition.

The low transmission efficiency of nymphs feeding on bottom leaves could also be explained by a loss of infectivity of the virus in those leaves, or changes at the surface of the viral capsid. Eweida, Oxelfelt & Tomenius (1988) recently demonstrated that barley yellow dwarf luteovirus can occur in aggregated forms, may be linked to membrane constituents in the phloem in oats. Such a clumping and associating could have reduced the availability of PLRV for acquisition in our experiments, resulting in lower percentages of viruliferous nymphs which fed on bottom leaves (Table 2). If aggregated or membrane-linked virus particles are imbibed by M. persicae nymphs, the difference in LP_{50} between nymphs acquiring virus on bottom as compared with top leaves may represent the time needed to set free the particles within the alimentary tract of the aphid.

Studies on the presence of PLRV in leaves, and in aphids using those leaves as a virus source, combined with electron microscopical studies on the localisation of the virus are needed for a better understanding of the factors determining the efficiency by which aphids acquire and transmit PLRV.

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CHAPTER 2

IMPROVED DETECTION OF POTATO LEAFROLL VIRUS IN PLANT MATERIAL AND IN APHIDS

SUMMARY

To improve detection of potato leafroll virus (PLRV) in plant material and viruliferous aphids, the enzyme-linked immunosorbent assay (ELISA) was modified by simultaneous incubation of sample and conjugate (cocktail-ELISA) and by amplification of the enzyme reaction. Absorbance values of PLRV-containing samples in the cocktail-ELISA were higher than those of comparable samples in a sandwich ELISA procedure in which sample and conjugate were incubated sequentially. Addition of sodium diethyldithiocarbamate or ethylenediaminetetraacetic acid to the sample buffer in the cocktail-ELISA significantly increased the absorbance values of infected plant material, while the background signals were reduced. Amplification of the enzyme reaction, in which dephosphorylated substrate catalytically triggered an enzyme-mediated redox cycle, further increased sensitivity. Using this technique, 50-100 pg of PLRV could be detected per sample, and the virus could be detected in highly diluted leaf sap and in single *Myzus persicae* nymphs after a 12-h acquisition access period on PLRV-infected *Physalis floridana* plants. In addition, when coated plates were used, the total assay time for PLRV detection in plant material could be reduced to 20 minutes.

INTRODUCTION

Potato leafroll virus (PLRV), a member of the luteovirus group, causes a widespread and economically important disease in potatoes. The virus is transmitted

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in a persistent manner by aphids and occurs in low titers in infected plants and in its vectors. To study virus-vector relationships for PLRV, a highly sensitive method for detecting the virus is therefore required.

The microplate method of the sandwich form of enzyme-linked immunosorbent assay (ELISA), introduced by Voller *et al* (1976) has been adapted by Clark and Adams (1977) for use in routine testing of plant viruses. Although hybridization techniques are developing rapidly, ELISA is still considered a major assay for the detection of potato viruses, especially when no labelled probes are available or a quantitative interpretation of results is required.

Since the introduction of ELISA, numerous reports have been published on improvements of the assay conditions for many host-virus combinations (Clark & Adams, 1977; McLaughlin *et al*, 1981). Modifications were made to maximize the sensitivity of the assays and to reduce nonspecific background signals. In most cases the improvements have involved only slight modifications of the original ELISA procedure.

Little attention has been paid to the cocktail-ELISA technique, in which sample and conjugate are incubated simultaneously in the wells of a microtiter plate. Van Vuurde & Maat (1985) demonstrated the potential of the cocktail method in the routine testing of lettuce mosaic virus (LMV) and pea early-browning virus (PEBV). Furthermore, apple chlorotic leafspot virus (CLSV) could only be detected by using simultaneous incubation of sample and conjugate (Flegg & Clark, 1979).

Recently, amplification of the alkaline phosphatase reaction in ELISA (Stanley, Johannsson & Self, 1985) has been applied to detect barley yellow dwarf virus in oat plants and in individual vector aphids (Torrance, 1987). Using the sandwich method, assay time could be reduced to less than 2 h if coated and blocked microtiter plates were used. Whether simultaneous incubation of sample and conjugate (cocktail-ELISA) followed by amplification of the enzyme reaction can increase both sensitivity and efficiency of ELISA has not been tested.

This paper illustrates first of all the advantages of cocktail-ELISA over the sandwich method for the detection of PLRV in infected plant material and in viruliferous aphids. Second, the addition of chelating agents to the sample buffer is shown to reduce background signals. Finally, we demonstrate that sensitivity was enhanced by combining cocktail-ELISA with a procedure in which the bound enzyme is visualized by amplification of the enzyme reaction.

MATERIALS AND METHODS

Aphid rearing

Myzus persicae (Sulz.) was maintained on *Brassica napus* L. subsp. *oleifera* (oilseed rape) in a glasshouse compartment at 20°C under a regime of 16 h light and 8 h of darkness. To ensure a regular supply of aphids and to produce cohorts of similar-aged aphids, mature apterae, confined to leaf cages, were transferred daily to leaves of oilseed rape plants. Nonviruliferous nymphs used in the experiments were deposited onto *Vicia faba* L. cultivar 'Drie x Wit' by mature apterae derived from a cohort.

Virus sources

Physalis floridana Rydb. plants were inoculated in their seedling stage by single viruliferous nymphs of *M. persicae* in a two-day inoculation access period. For symptom development the seedlings were kept in a glasshouse (16 h light, 8 h darkness) at 25°C. PLRV-infected and virus-free potatoes (*Solanum tuberosum* L. 'Bintje') were raised from tubers and were kept under the same conditions as mentioned for *P. floridana* seedlings.

Purification and enzyme conjugation of gamma-globulins

Antiserum was kindly provided by Mr D.Z. Maat. The gamma-globulin fraction was partially purified by ammonium sulphate precipitation as described by Clark & Adams (1977). Gamma-globulins at 1 mg/ml were conjugated with 2,000 units of alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline pH 7.4 as described by Avrameas (1969). Conjugate was not further purified. Gamma-globulin and conjugate were stored with 0.05% sodium azide at 4°C.

ELISA procedures

The following ELISA procedure was used as a reference. Wells of Nunc-Immunoplate IF plates (Nunc, Denmark) were sensitized by adding 250 μl of 2 μg/mlgamma-globulin in coating buffer (0.05 M sodium carbonate, pH 9.6). After a 3-h incubation at 37°C, wells were vigorously rinsed with tap water and filled with leaf material or aphids (200 μl per well) triturated in 0.02 M sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, 2% polyvinylpyrrolidone (M.W. about 44,000), 0.05% Tween 20 and 0.2% ovalbumin (sample buffer). Stock suspensions of leaf material and aphids consisting of 1 g of leaf material in 4 ml of

sample buffer or 100 aphids in 1 ml of sample buffer were prepared. Dilutions of these suspensions and relevant controls were incubated overnight at 4°C. The wells were flushed with tap water, then filled with 200 μl of 1,000-fold with sample buffer diluted conjugate, and incubated for 3 h at 37°C. After the plates were washed, 200 μl of 2.7 mM p-nitrophenyl phosphate disodium salt (p-NPP) in 10% diethanolamine, pH 9.8, was added to each well. The immobilized enzyme concentration was measured by monitoring substrate conversion to coloured end product.

The same kind of plates and buffers used in the sandwich method were used in cocktail-ELISA, but sample and enzyme conjugated gamma-globulins were added together and incubated overnight at 4°C. The total volume of sample and conjugate was 200 μl when alkaline phosphatase concentration was determined using p-NPP and 100 μl when the enzyme reaction was amplified.

The amplification of the enzyme reaction was performed following Stanley, Johannsson & Self (1985). All chemicals used, unless otherwise stated, were purchased from Sigma Chemical Co., St. Louis, MO. We added 100 μl 0.2 mM nicotinamide-adenine dinucleotide phosphate monosodium salt (NADP) in 0.05 M diethanolamine buffer, pH 9.5, to each well. After incubation at 20°C for 30 min, remaining alkaline phosphatase activity was blocked by adding 15 μl of 0.5 M 4-nitrophenyl disodium orthophosphate (o-NPP) (BDH Chemicals Ltd., Poole, England) in 0.025 M phosphate buffer, pH 7.0, to each well (Self, 1985). Subsequently, we added 150 μl per well of the amplification reaction mixture. The stock amplification mixture consisted of 700 units of alcohol dehydrogenase, 100 units of lipoamide dehydrogenase (type VI), 3% (v/v) ethanol, and 1 mM p-iodonitrotetrazolium violet in 15 ml 0.025 M phosphate buffer, pH 7.0.

In both enzyme assays, colour development was allowed to proceed at room temperature. Absorbance values were read on a Titertek Multiskan colorimeter (Flow Laboratories Ltd., Irvine, Scotland) at 405 nm when p-NPP was used, and at 492 nm when NADP was used. The plates were blanked against wells that did not contain sample.

RESULTS

Detection of PLRV with cocktail-ELISA

Cocktail-ELISA was compared with the sandwich method in sensitivity tests with PLRV-infected potato and *P. floridana* leaf tissue, PLRV-carrying *M. persicae* adults, and appropriate virus-free controls. A dilution series of a purified PLRV suspension was included as a positive control.

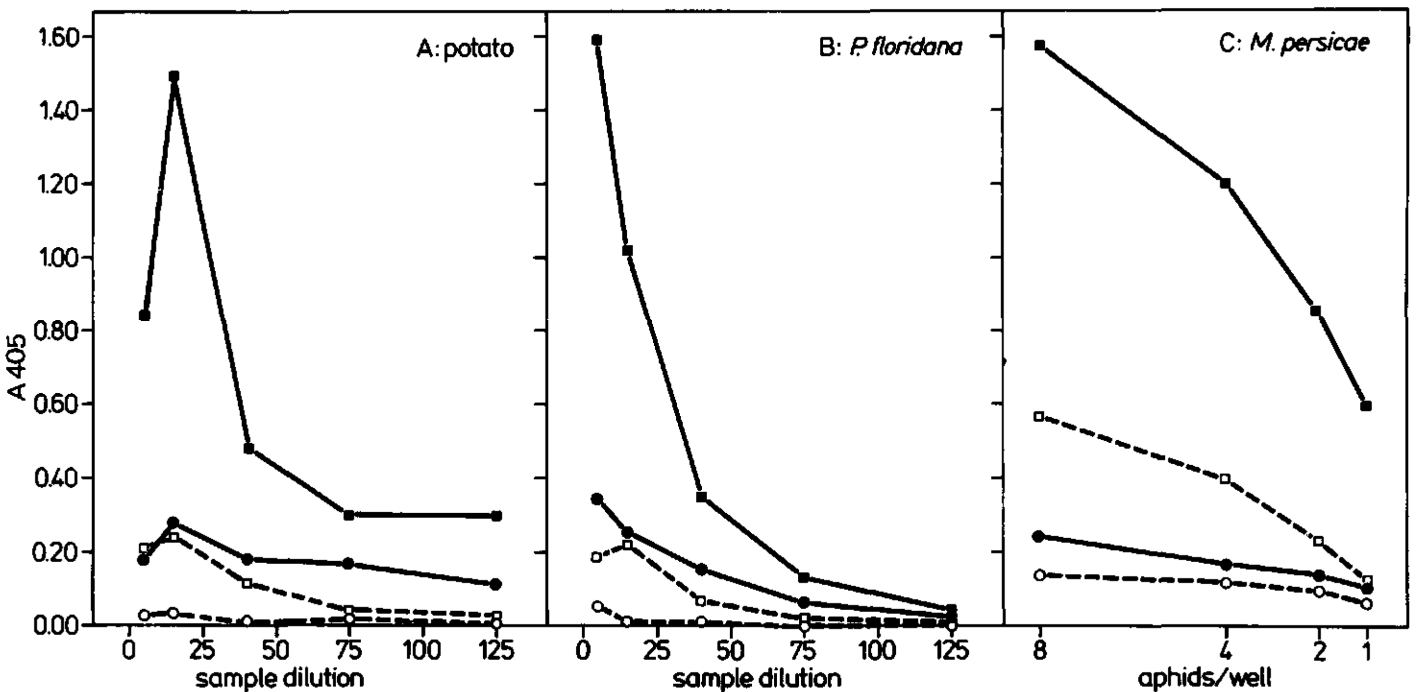

Fig. 1. Absorbance values at 405 nm (A_{405}) obtained with healthy (open symbols) and PLRV-infected (solid symbols) potato leaf material (A), *P. floridana* (B) leaf material, and *M. persicae* aphids (C) in the cocktail (□,■) and sandwich (○,●) ELISA procedures. Samples of leaf material and aphids were prepared and diluted in sample buffer. Sandwich procedure consisted of overnight sample incubation (200 μl per well) at 4°C and 2-h conjugate incubation (1 μg/ml of sample buffer; 200 μl per well) at 37°C. In the cocktail procedure, 200 μl of sample and 0.2 μg of conjugate per well were incubated simultaneously overnight at 4°C. Absorbance values of A, B, and C were measured after 4, 0.5, and 2 h of substrate incubation at room temperature, respectively. Each point in the graph is the average of five absorbance values.

Al dilutions prepared from PLRV-infected leaf tissue and virus-containing aphids had higher absorbance values in the cocktail-ELISA than in the sandwich method (Fig. 1). With most sample dilutions, absorbance values were four to seven times higher in the cocktail-ELISA. The improvement in values in PLRV-infected potato and *P. floridana* at five- to 40-fold dilutions was remarkable (Fig. 1A and 1B).

Background absorbance values were higher in cocktail-ELISA than in the sandwich method when the absorbance values of a particular sample were measured in both procedures after the same period of colour development (Fig. 1). However, when the absorbance values of potato, *P. floridana*, and *M. persicae* in sandwich ELISA were allowed to develop to values, comparable to those obtained with cocktail-ELISA after 4, 0.5, and 2 h respectively, background values were higher in the sandwich method.

With purified PLRV, 1 ng per well could readily be detected in the cocktail-ELISA. An absorbance value of 0.14 was obtained after 2.5 h of substrate incubation at room temperature, while the virus-free buffer gave a value of 0.01. Under the same conditions, 8 ng of virus per well was necessary to obtain similar values in the sandwich method. On the other hand, at PLRV concentrations above 50 ng per well, the sandwich method yielded higher readings than the cocktail-ELISA. In both ELISA systems, the mean absorbance values of 20-fold diluted leaf material of potato and 40-fold diluted leaf material of *P. floridana* corresponded approximately with those of 50 ng of purified PLRV per well.

Influence of chelating agents on the enzyme reaction

To reduce background signals in the cocktail-ELISA, sodium diethyldithiocarbamate (NaDIECA) or ethylenediaminetetraacetic acid (EDTA) was added to the sample buffer in concentrations of 6.25, 12.5, 25, and 50 mM. NaDIECA significantly diminished absorbance values of healthy *P. floridana* leaf tissue and virus-free *M. persicae* (Table 1). The absorbance values obtained with healthy potato leaf tissue were slightly, but not significantly ($P = 0.05$), affected by NaDIECA. The values with PLRV-infected potato and *P. floridana* leaf tissue were significantly enhanced when 6.25 or 12.5 mM of this chelating agent was used; higher NaDIECA concentrations inhibited colour development. Although the values for virus-carrying aphids decreased with increasing NaDIECA concentrations, the discriminative capacity of the system was improved overall because the absorbance values of virus-free aphids fell more than those of virus-carrying aphids.

When applied in the sandwich procedure, NaDIECA reduced the absorbance values of PLRV-containing samples without affecting background signals.

The effects of adding EDTA were analogous to those demonstrated for NaDIECA.

Table 1. Influence of sodium diethyldithiocarbamate (NaDIECA) on the detection of potato leafroll virus (PLRV) in leaf material[t] and in aphids[u] by cocktail enzyme-linked immunosorbent assay.

NaDIECA concentration (mM)	*Physalis floridana*[w]		Potato[x]		*Myzus persicae*[y]	
	PLRV	Healthy	PLRV	Healthy	PLRV	Healthy
0	0.817 (0.039)a[z]	0.049 (0.038)a	1.315 (0.110)b	0.038 (0.012)a	0.821 (0.235)a	0.216 (0.017)a
6.25	0.970 (0.082)ab	0.029 (0.018)b	1.501 (0.052)a	0.043 (0.013)a	0.544 (0.102)ab	0.117 (0.046)b
12.5	1.023 (0.066)b	0.018 (0.014)bc	1.404 (0.093)a	0.035 (0.014)a	0.514 (0.090)b	0.098 (0.042)b
25	0.623 (0.107)c	0.012 (0.008)c	0.856 (0.118)c	0.027 (0.006)a	0.351 (0.042)b	0.063 (0.013)bc
50	0.216 (0.080)d	0.014 (0.011)c	0.430 (0.075)d	0.023 (0.006)a	0.042 (0.013)c	0.038 (0.007)c
Standard deviation	0.102	0.008	0.060	0.014	0.121	0.023

[t] For each NaDIECA concentration and source of leaf material, 30 samples from two different extracts were divided into six samples per plate. Ground *P. floridana* leaf material was diluted 100-fold (w/v); ground potato leaf material was diluted 18-fold (w/v).

[u] For each NaDIECA concentration and source of aphid material (PLRV-carrying and virus-free), 18 samples from two different extracts were divided into 6 samples per plate. The aphids were given an acquisition access period of 3 days on PLRV-infected *P. floridana*. Aphids were homogenized, and each sample corresponded to two aphids.

[v] Gamma-globulins and conjugate were used at concentrations of 2 and 1 μg/ml, respectively. Samples and conjugate were incubated together overnight at 4°C.

[w] Absorbance at 405 nm after 2 h of substrate incubation; values are the average absorbance values of six wells of five microtiter plates tested ($N = 5$); standard error in parentheses.

[x] Absorbance at 405 nm after 4 h of substrate incubation; values are the average absorbance values of six wells of five microtiter plates tested ($N = 5$); standard error in parentheses.

[y] Absorbance at 405 nm after 3.5 h of substrate incubation; values are the average of six wells of three microtiter plates tested ($N = 3$); standard error in parentheses.

[z] Values within a column not followed by the same letter are significantly different ($P = 0.05$) according to Tukey's test.

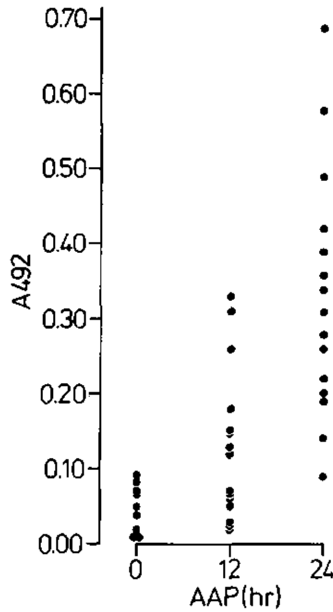

Fig. 2. Absorbance values at 492 nm (A_{492}) of individual six-day old *M. persicae* nymphs, obtained with amplification of the enzyme reaction after simultaneous incubation of sample and conjugate. Nymphs were individually homogenized in 100 µl of sample buffer containing 12.5 mM sodium diethyldithiocarbamate and 1 µg/ml conjugate. The cocktail was incubated overnight at 4°C. The amplification of the enzyme reaction was done at room temperature by sequentially adding 100 µl of 0.2 mM nicotinamide-adenine dinucleotide phosphate monosodium salt in 0.05 M diethanolamine buffer (30 min incubation), 15 µl 0.5 M 4-nitrophenyl disodium orthophosphate in 0.025 M phosphate buffer (5 min incubation), and 150 µl of the amplification reaction mixture. A_{492} was measured after 25 min of colour development.

Amplification of the enzyme reaction

The enzyme reaction was amplified to enhance the detection of PLRV in single viruliferous aphids. Six-day old *M. persicae* nymphs were individually triturated in small Elvejhem-Potter tubes in 100 µl of sample buffer containing 12,5 mM NaDIECA, after an acquisition access period of 12 or 24 h on PLRV-infected *P. floridana*. The triturates were centrifuged for 15 min at 10,000 rpm in an Eppendorf centrifuge. After the simultaneous incubation of conjugate and supernatant at 4°C overnight, the enzyme reaction was amplified.

The absorbance values (Fig. 2) show that PLRV antigen could be detected even in nymphs after an acquisition access period of 12 h. Individual nymphs varied

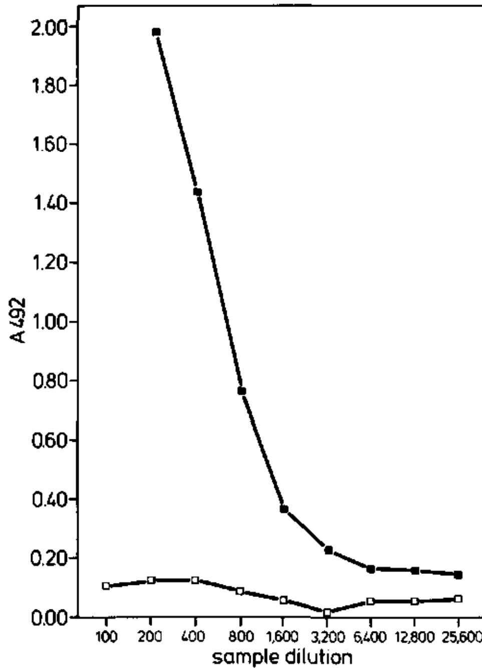

Fig. 3. Absorbance values at 492 nm (A_{492}) of highly diluted PLRV-infected (■) and healthy (□) potato leaf material, obtained with amplification of the enzyme reaction after the simultaneous incubation of sample and conjugate. Leaf material was ground and diluted in sample buffer containing 12.5 mM NaDIECA and 1 µg/ml conjugate. The cocktail (100 µl) was incubated overnight at 4°C. The amplification of the enzyme reaction was done at room temperature by sequentially adding 100 µl of 0.2 mM nicotinamide-adenine dinucleotide phosphate monosodium salt in 0.05 M diethanolamine buffer (30 min incubation) and 150 µl of the amplification reaction mixture. A_{492} was measured after 15 min of colour development. Each point in the graph is the average of 3 absorbance values.

widely in the efficacy with which they acquired PLRV during an acquisition access period.

The amount of purified PLRV that could be detected using the amplification of the enzyme reaction following cocktail incubation was between 50 and 100 pg of virus per well. The enhanced sensitivity of ELISA obtained with amplification, in comparison with p-NPP, enabled us to detect PLRV in highly diluted leaf material samples (Fig. 3) and to develop a faster assay (Table 2). For the latter purpose, discs (5 mm in diameter) were punched out of healthy and PLRV-infected potato leaves and individually ground in 100 µl of sample buffer containing 12,5 mM

Table 2. Effect of amplification of the enzyme reaction following cocktail incubation for short periods on the detection of potato leafroll virus (PLRV) in crushed potato leaf discs[a] by enzyme-linked immunosorbent assay.

Cocktail incubation[b] (min)	NADP incubation[b] (min) 5		10		15	
	PLRV	Healthy	PLRV	Healthy	PLRV	Healthy
1	0.125[c]	0.038	0.204	0.046	0.280	0.051
5	0.127	0.016	0.223	0.018	0.312	0.015
10	0.169	0.000	0.302	0.015	0.429	0.023
15	0.183	0.018	0.353	0.036	0.516	0.029

[a] Samples were made by grinding single potato leaf discs in 100 μl of extraction buffer containing 12.5 mM sodium diethyldithiocarbamate and 1 μg/ml conjugate.
[b] The simultaneous incubation of sample and conjugate (cocktail) and the incubation of nicotinamide-adenine dinucleotide phosphate monosodium salt (NADP) solution (0.2 mM NADP in 0.05 M diethanolamine buffer; 100 μl per well) were done at room temperature.
[c] Values (absorbance at 492) are the averages of five potato leaf discs. Absorbance values were measured 10 min after the amplification reaction mixture was added to the NADP solution.

NaDIECA and 0.1 μg of conjugate. These homogenates were incubated for 1, 5, 10 or 15 min in the wells of a coated microtiter plate, followed by 5, 10 or 15 min of NADP incubation and 10 min of colour development. Within a total assay time of 16 min - 1 min of cocktail incubation, 5 min of NADP incubation and 10 min of colour development - an absorbance value at 492 nm of 0.125 (blank: 0.038) was obtained (Table 2). Values over 0.1 could be scored as positives by visual evaluation. The o-NPP step could be omitted because of rapid colour development. The amplification procedure enabled us to perform the assay in less time than the procedure using p-NPP as substrate. When p-NPP (100 μl 2.7 mM in 10% diethanolamine, pH 9.8) was used after short incubation periods of the cocktail, absorbance values at 405 nm obtained after 6 h of colour development were comparable to those obtained with enzyme amplification after about 20 min.

DISCUSSION

The recently developed technique of the amplified alkaline phosphatase reaction in ELISA (Stanley, Johannsson & Self, 1985) has been used to detect the enzyme immobilized in the simultaneous incubation of samples and conjugate (cocktail-ELISA). Cocktail-ELISA was more sensitive than sandwich ELISA and less time consuming, as one step could be omitted. The absorbance values of virus-containing samples were higher for all sample dilutions prepared from extracts of both potato and *P. floridana* leaves and of *M. persicae* vector aphids (Fig. 1). This effect was also observed for CLSV in petals, leaves and fruit of various apple cultivars (Flegg & Clark, 1979).

The improved detection may be caused by immobilization of a larger proportion of antigen-conjugate complexes on the solid phase, binding of more conjugate molecules to PLRV antigen or the fact that the omission of one washing step reduces release of antigen-antibody complexes from the solid phase. Application of the cocktail-ELISA also resulted in reduced background reaction. This reduction could be demonstrated when the absorbance values in the sandwich ELISA were allowed to develop to similar levels to those obtained with the same samples in the cocktail-ELISA (results not shown). Antigenic sites on plant proteins that compete with immune complexes for binding to the solid phase may be obstructed by the conjugate molecules in the solutions during simultaneous incubation.

The view that the cocktail method can be applied only with smaller antigens (Clark, 1981) seems at least questionable in view of previous results with CLSV and apple stem grooving virus (closteroviruses), PEBV (tobraviruses), and LMV (potyvirus) (Van Vuurde & Maat, 1985). Moreover, good results were also obtained in the detection of beet necrotic yellow vein virus (furovirus) (unpublished data).

Reduced sensitivity of the cocktail-ELISA in concentrated plant extracts compared to diluted ones, as found by Flegg and Clark (1979), was also observed for extracts of PLRV-infected potato leaf material (Fig. 1A) and purified PLRV suspensions at concentrations over 50 ng per well. *P. floridana* leaf material, on the other hand, did not react in this way (Fig. 1B), although virus yield after purification was almost equivalent to that obtained from potato leaf material (unpublished data). The question arises whether this reduced sensitivity when a relatively high virus concentration was tested was caused by an unfavourable conjugate-antigen ratio (Clark, 1981) or by negative influences of components in the leaf extract of potato. Sample composition can also affect virion stability or interfere with the formation of immune complexes or with binding of those complexes to the solid phase.

Adding NaDIECA or EDTA to the sample buffer in the cocktail-ELISA further improved the detection of PLRV; a NaDIECA or EDTA concentration of 12,5 mM was most effective (Table 1). Absorbance values for PLRV-infected potato and *P. floridana* leaf samples were higher, and background signals in healthy aphids were suppressed significantly. Gibbs & Harrison (1976) and Smith & Banttari (1987) discuss the possible functions of these chelating agents in stabilizing virus nucleocapsid structure by reducing oxidized phenolic compounds or the release of viruses from cell organelles.

Amplification of the enzyme reaction increased the ultimate detection level of PLRV about 15-fold. This enabled the serological detection of PLRV antigen in single vector aphids within an hour, which included a 30-min NADP incubation and 25 min of colour development (Fig. 2). Tamada & Harrison (1981) also succeeded in detecting PLRV in single aphids, but in their experiments the most clear-cut results were obtained only after an overnight incubation period with substrate.

A quantitative interpretation of absorbance values obtained from amplification of the enzyme reaction is possible because the blockage of remaining phosphatase activity with o-NPP excludes any interference from newly dephosphorylated NADP while colour development is proceeding. Hence, the colour development caused by the reduction of p-iodonitrotetrazolium violet is proportional to the NAD originally formed by alkaline phosphatase in the bound conjugate (Self, 1985). This accurate and sensitive enzyme assay permits the analysis of various factors that play a role in the acquisition of PLRV by aphids, such as the determination of amounts of virus involved when PLRV is acquired from different virus sources.

For qualitative purposes, amplification of the enzyme reaction offers the possibility of a field test which can be completed within 20 minutes and the detection of PLRV in highly diluted samples. The o-NPP step can be omitted because of rapid colour development. Moreover, the use of glutaraldehyde coupling of alkaline phosphatase with gamma-globulin, without any further purification of the conjugate, makes the enzyme amplification assay economically attractive.

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CHAPTER 3

TRANSMISSION OF POTATO LEAFROLL VIRUS FROM PLANTS AND ARTIFICIAL DIETS BY *MYZUS PERSICAE*

SUMMARY

To analyse quantitatively characteristics of potato leafroll virus (PLRV) transmission by *Myzus persicae*, acquisition from purified virus-containing artificial diets and transmission efficiency were investigated. Multiple regression of the data revealed that the amount of viral antigen in the nymphs after feeding on PLRV-containing artificial diets is linearly related to the $\log_{10}$ transformed virus concentration in the diet and the length of the acquisition access period (AAP). The percentage of viruliferous nymphs was also linearly related to the logarithm of the virus concentration in the diets. These relationships indicate that the amount of viral antigen present in aphids can be used as a parameter in deducing the amount of virus available in a source for acquisition by aphids. The PLRV concentration in the diets did not influence the length of the median latency period (LP_{50}) in *M. persicae*. However, the LP_{50} was affected by intrinsic properties of the virus and the age of the *M. persicae* nymphs used. PLRV purified from top leaves of *Physalis floridana* was transmitted with a significantly shorter LP_{50} than virus purified from bottom leaves. This difference was also observed when aphids acquired the virus from top or bottom leaves, hence showing that purification did not affect the relative transmissibility of the virus. After acquiring purified PLRV from artificial diet, one-day old *M. persicae* nymphs were more efficient in transmitting the virus than four-day old nymphs. To obtain 50% viruliferous nymphs, the virus concentration needed in the diet was significantly lower for young nymphs than for older ones. Furthermore, the LP_{50} of the virus was significantly shorter in the younger nymphs.

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INTRODUCTION

Persistent virus transmission by aphids encompasses the processes of acquisition, circulation and inoculation. To describe virus transmission in a quantitative manner, the concepts of acquisition access period (AAP), latency period (LP) and inoculation access period (IAP) have been introduced (for a recent review see Peters, 1986). The LP of potato leafroll virus (PLRV) recorded in *Myzus persicae* (Sulz.) ranges from 5 min, measured as the minimum LP, to 123 h, measured as the maximum LP (Kirkpatrick & Ross, 1952: Ponsen, 1972; Tanaka & Shiota, 1970). Differences in the minimum or maximum values of the LP are difficult to analyse statistically. In these studies, the length of the AAP and IAP, the PLRV source, the test plant species, the number of aphids per test plant and the age of the vectors involved varied considerably (Ponsen, 1972). Sylvester (1965) introduced the median LP (LP_{50}) of which the fiducial limits are more readily determined. The LP_{50} can be estimated from repeated aphid transfers (Sylvester, 1965) and may be affected by the experimental design (Toros, Schotman & Peters, 1978). The LP_{50} is commonly used to characterize virus isolates with respect to their transmissibility (e.g. Bath & Tsai, 1969; Kojima, Niitsu & Takahashi, 1987; Mellor & Frazier, 1970). However, there is no consensus as to whether differences in the LP are caused by the virus concentration in the sap ingested (Sylvester, 1965 and 1980) or injected into the aphids (Clarke & Bath, 1973), or by intrinsic biological properties of the virus.

The length of the LP_{50} of PLRV in *M. persicae* nymphs has been shown to increased with the severity of symptoms on the *Physalis floridana* plants used as the virus source (Peters, 1986). Similarly, Van den Heuvel & Peters (1990) observed that the LP_{50} was significantly longer when *M. persicae* nymphs acquired this virus from *P. floridana* bottom leaves with pronounced symptoms rather than from symptomless top leaves. Additionally, a lower percentage of the nymphs that fed on bottom leaves was viruliferous as compared with those that fed on the top leaves. However, the concentration of viral antigen measured by an enzyme-linked immunosorbent assay (ELISA) was higher in the bottom leaves than in the top leaves (Van den Heuvel & Peters, 1990). These results indicate that the acquisition of the virus by aphids declines with increasing infection age and symptom severity.

This study aims to quantify the amount of virus ingested by the aphids that fed on leaves with different symptoms caused by PLRV, and to elucidate whether the dose of virus acquired is responsible for the observed differences in the transmission characteristics. For this purpose, we studied PLRV acquisition and transmission by *M. persicae* from artificial diets with known concentrations of virus. The amount of

viral antigen present in *M. persicae* nymphs, and the transmission characteristics like the LP_{50} and the percentage of viruliferous nymphs after an AAP on virus-containing artificial diets were compared with those of nymphs after similar AAPs on top or bottom leaves of *P. floridana* infected with PLRV. Furthermore, the transmission of the virus purified from top and bottom leaves of infected *P. floridana* plants and acquired by *M. persicae* from artificial diets was investigated.

MATERIALS AND METHODS

Aphids

M. persicae biotype WMp2 (Reinink *et al*, 1989) was reared on *Brassica napus* L. subsp. *oleifera* (oilseed rape) in a glasshouse compartment at 20±3°C with a photoperiod of 16 h per day. Cohorts of similar-aged nymphs were produced by daily transfer of mature apterae, confined to leaf cages, to new oilseed rape plants.

Virus maintenance and purification

The Wageningen isolate of PLRV was maintained by repeated aphid transfers on seedlings of *P. floridana*. They were inoculated by one viruliferous *M. persicae* nymph in a two-day IAP. After inoculation, seedlings were kept in a glasshouse at 25±3°C for symptom development.

PLRV was purified from *P. floridana* leaf material infected with PLRV using a modified enzyme-assisted purification procedure (Van den Heuvel *et al*, 1990). Frozen leaf material was homogenized with 0.1 M sodium citrate buffer (2 ml/g), pH 6.0, containing 0.5% cellulase Onozuka R-10, 0.5% macerozyme R-10 (Yakult Honsha Co. Ltd, Tokyo), 0.1% thioglycollic acid and 0.5% ethanol in a blender. The homogenate was stirred for 4 h at 25°C and emulsified with one-half of the volume of a 1:1 (v/v) mixture of chloroform and 1-butanol. After low-speed centrifugation, 1% Triton X-100, 8% polyethylene glycol, and 0.4 M sodium chloride were added to the aqueous phase. The precipitate was collected by centrifugation, resuspended in 0.1 M sodium citrate buffer, pH 6.0 (containing 5% ethanol), layered on 30% sucrose, and subjected to high-speed centrifugation. The pellets were resuspended in 0.1 M sodium citrate buffer, pH 6.0, and loaded on a 20 - 50% sucrose gradient. After high-speed centrifugation, the virus-containing zones were collected and the virus was sedimented by centrifugation. The pellets were resuspended in 0.1 M sodium citrate buffer, pH 6.0, and the virus concentration was measured spectrophotometrically assuming a specific absorbance of 8.6 units at 260 nm

(Takanami & Kubo, 1979) for 1 mg of virus per ml. When purified PLRV was used in membrane-feeding experiments, the pellets were resuspended in artificial diet MP148 (Harrewijn, 1983).

Virus acquisition from artificial diet and from plants

Membrane-feeding experiments were carried out with purified virus in an artificial diet. To prevent the virus from adhering to the Parafilm membranes, 1% bovine serum albumin was added to the diet. The virus concentration in the diet was determined with an ELISA by comparing a dilution series of the virus in diet with a stock suspension of known PLRV concentration. Sachets made of two stretched Parafilm membranes with 100 μl of the virus-containing diet sealed between them were prepared under sterile conditions. Membrane feeding chambers were made by attaching these sachets to a plastic ring (2.5 cm diameter). A group of approximately 30 nymphs was caged in a chamber and placed in a controlled environment at 20 ± 0.1°C, 80% relative humidity, and continuous illumination (8,000 lx) to acquire the virus.

Aphids were allowed to acquire virus from top leaves that did not show symptoms and from bottom leaves that showed pronounced symptoms; the *P. floridana* used were inoculated with PLRV five or 12 weeks before, respectively. Groups of about 30 nymphs were put in cages clipped on these leaves and the plants were placed in a controlled environment.

Virus transmission

After acquisition from the virus-containing sachets or from *P. floridana* infected with PLRV, the nymphs were individually transferred to seedlings of *P. floridana* to determine the percentage of nymphs that transmitted PLRV in an IAP of 5 days.

In parallel experiments, the LP of the virus in the nymphs was determined by transferring them at constant intervals of 24 h to new seedlings of *P. floridana*. The LP was defined as the time interval from the start of the AAP to the end of that IAP in which the first transmission occurred. A log-probit transformation was used to estimate the LP_{50}, which represents the time at which 50% of the nymphs that transmitted the virus completed their LP (Sylvester, 1965). The LP_{50} and its 95% fiducial limit (f.l.) were calculated following Finney (1962) using the SAS program (SAS Institute Inc., Cary, NC).

Recording honeydew excretion

The number of honeydew droplets excreted by one-day old *M. persicae* nymphs feeding on sachets and on different leaves of *P. floridana* infected with PLRV was recorded with a honeydew clock (Van den Heuvel & Peters, 1990). The volume of the honeydew droplets excreted by these nymphs was estimated by measuring the diameter of the almost spherical droplets after collecting them in petri dishes filled with mineral oil.

Cocktail-ELISA and enzyme amplification

The amount of viral antigen present in nymphs after acquisition on virus-containing artificial diet or PLRV-infected *P. floridana* was determined using the cocktail-ELISA followed by enzyme amplification (Van den Heuvel & Peters, 1989). Nunc-Immunoplate Maxisorp F96 plates (Nunc, Denmark) were coated by adding 250 μl of 2 μg/ml anti-PLRV rabbit gamma-globulin in coating buffer (0.05 M sodium carbonate, pH 9.6) to each well. After an incubation period of 3 h at 37°C, plates were washed with PBS-Tween (0.02 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl, 2 mM KCl, and 0.05% Tween 20). Nymphs were individually homogenized in 100 μl of sample buffer (PBS-Tween containing 2% polyvinylpyrrolidone and 0.2% ovalbumin) and the extract was added to the wells with 1 μg/ml of anti-PLRV mouse gamma-globulin. The plates were incubated overnight at 4°C, washed with PBS-Tween, and 100 μl of a 5,000-fold diluted goat anti-mouse- alkaline phosphatase conjugate (Sigma Chemical Co, St. Louis, MO) was added to the wells. After 3 h of incubation at 37°C, plates were washed again and 100 μl of 0.2 mM ß-nicotinamide adenine dinucleotide phosphate monosodium salt in 0.05 M diethanolamine buffer, pH 9.5, was added to each well. After an 45-min incubation period at 20°C, remaining alkaline phosphatase activity was blocked by the addition of 15 μl of 0.5 M 4-nitrophenyl disodium orthophosphate (BDH Chemicals Ltd., Poole, England) in 0.025 M phosphate buffer, pH 7.0 to each well. Subsequently, 150 μl per well of the amplification mixture, consisting of 700 units of alcohol dehydrogenase, 100 units of lipoamide dehydrogenase, 3% (v/v) ethanol, and 1 mM *p*-iodonitrotetrazolium violet in 15 ml of 0.025 M phosphate buffer, pH 7.0, was added.

Absorbance values were read on a Titertek Multiskan colorimeter (Flow Laboratories Ltd., Irvine, Scotland) at 492 nm (A_{492}). A dilution series of purified PLRV was incorporated in each plate.

RESULTS

Quantification of virus acquisition by *M. persicae* nymphs

The amount of virus acquired by *M. persicae* nymphs was analysed after an AAP of 1 - 5 days on PLRV-containing artificial diets and on top and bottom leaves of *P. floridana* infected with PLRV. PLRV was purified from seven-week old infected *P. floridana* plants and the artificial diets contained 3.75, 7.5, 15, 30, 60 and 120 µg of virus per ml. For each combination of virus concentration and AAP, 20 nymphs were individually tested for their virus content by cocktail-ELISA followed by enzyme amplification. The average absorbance values are presented in Fig. 1.

On all virus sources tested, either artificial diets or infected leaves, an increase of the AAP resulted in a greater amount of viral antigen in individual nymphs. Also, the amount of virus detected in the aphids increased with increasing PLRV concentration in the artificial diets. Multiple regression of the data showed that the A_{492} was linearly related with the $\log_{10}$ transformed virus concentration (µg/ml) in the diet and the length of the AAP (days). The regression equation was $A_{492} = -0.507 + 0.491X + 0.129Z$ ($r^2 = 0.82$), in which X represents the $\log_{10}$ transformed virus concentration (µg/ml) in the diet, ranging from 3.75 to 120 µg/ml; and Z is the AAP, ranging from 1 to 5 days.

Nymphs that had fed on bottom leaves of 12-week old PLRV-infected plants with pronounced symptoms acquired considerably less virus in the same AAP than nymphs from diets with the lowest concentration of virus. The amount of virus detected in nymphs from top leaves equalled that in nymphs from artificial diets containing 3.75 or 7.5 µg purified PLRV per ml.

Positive control samples of 0.5, 1, 2, 4, and 8 ng purified PLRV by cocktail-ELISA and enzyme amplification gave A_{492} values of 0.02, 0.12, 0.32, 0.64, and 1.30, respectively. Control virus-free aphids yielded A_{492} values ranging from 0.00 to 0.02.

Relationships among virus concentration, vector age, and virus transmission

Membrane-feeding experiments were carried out to determine the effect of the PLRV concentration in the diet on the percentage of nymphs transmitting the virus and on the LP_{50}. The virus concentrations in the artificial diets were as mentioned before. For each virus concentration, four sachets were used on which two cohorts of one- and four-day old *M. persicae* nymphs were allowed to acquire virus for a period of 24 h. The percentage of nymphs transmitting PLRV was determined by placing 30 nymphs of each cohort individually on test plants for five days. To estimate the LP_{50}, another group of 30 nymphs was transferred to fresh test plants

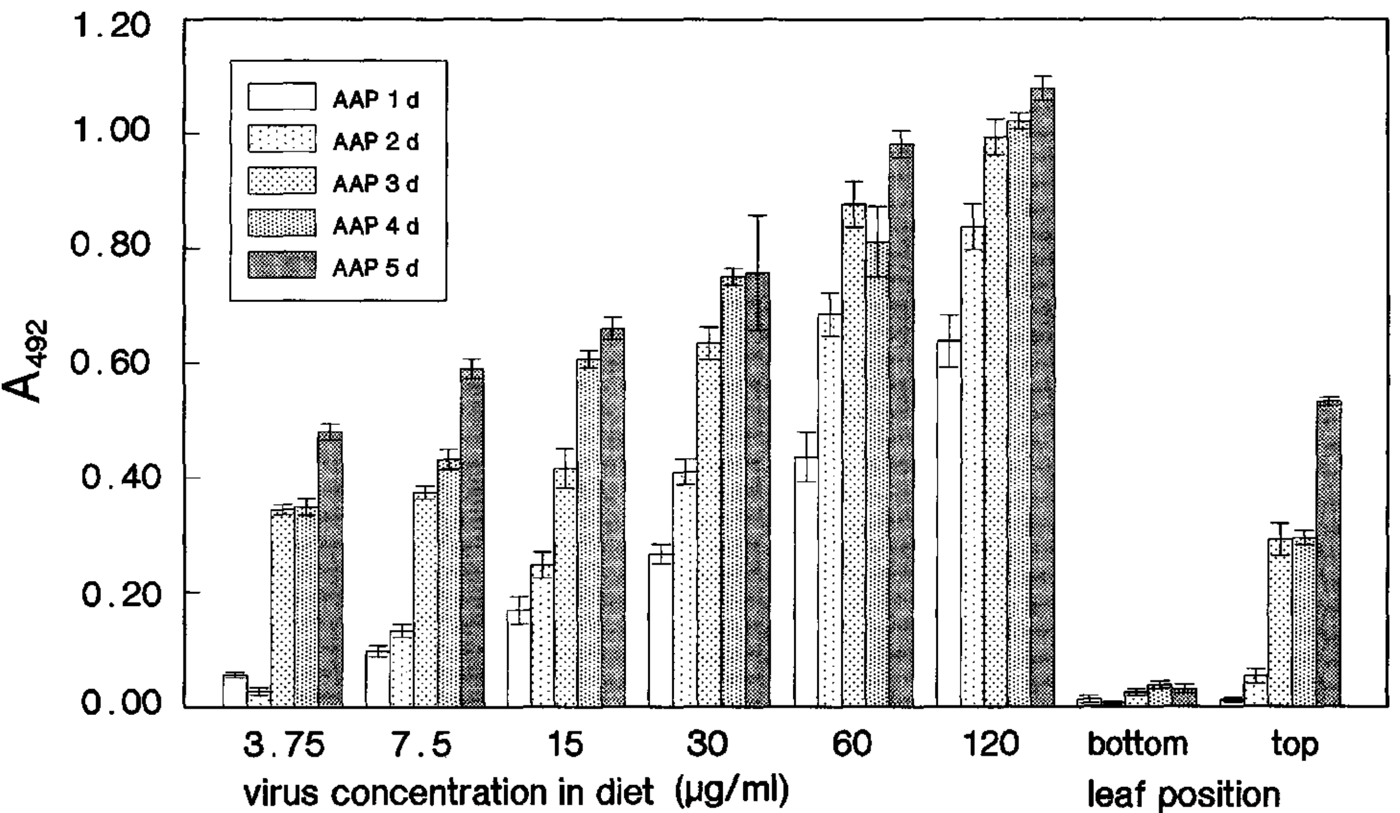

Fig. 1. Virus acquisition by *Myzus persicae* nymphs during an acquisition access period of 1 - 5 days (d) on artificial diets that contained increasing amounts of potato leafroll virus (PLRV), and on leaves of *Physalis floridana* infected with PLRV. The nymphs were individually tested by cocktail-ELISA followed by enzyme amplification. Colour development was measured at 492 nm (A_{492}) after 30 min. Each bar represents the mean value for 20 nymphs. The standard error of a mean is indicated in each bar.

Table 1. Efficiency of potato leafroll virus (PLRV) transmission[a] of one- and four-day old *Myzus persicae* nymphs after an acquisition access period (AAP) of 24 hours on artificial diet containing purified PLRV.

PLRV concentration (μg/ml)	One-day old nymphs			Four-day old nymphs		
	Transmitted (%)	LP_{50}[b] (h)	95% f.l.[c] (h)	Transmitted (%)	LP_{50} (h)	95% f.l. (h)
3.75	8	n.d.[d]	n.d.	0	n.d.	n.d.
7.5	23	79	66 - 95	10	n.d.	n.d
15	70	58	37 - 70	17	94	75 - 124
30	76	71	60 - 87	31	99	90 - 114
60	83	45	24 - 57	60	98	89 - 113
120	97	61	45 - 73	83	94	83 - 107

[a] After the AAP, the aphid nymphs were transferred five times to fresh *Physalis floridana* seedlings at inoculation access periods (IAP) of 24 h to determine the percentage of nymphs that transmitted the virus and the period after which the first virus transmission occurred.

[b] Median latency period (LP_{50}): the time interval at which 50% of the nymphs completed their latency period (LP). The LP is defined as the period of time between the start of the AAP and the end of the IAP in which the first virus transmission was accomplished.

[c] Fiducial limits of the LP_{50}.

[d] Not determined.

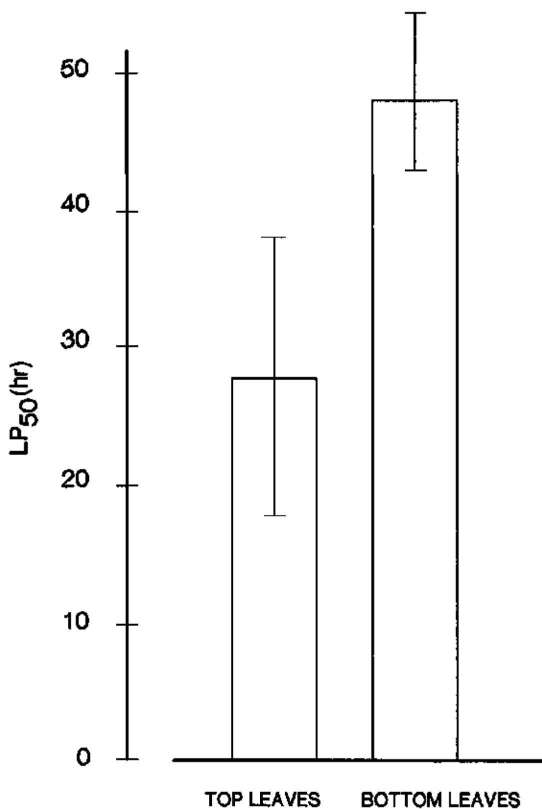

Fig. 2. The median latency period (LP_{50}) of potato leafroll virus (PLRV) in one-day old *Myzus persicae* nymphs that have been feeding on an artificial diet that contains 60 μg PLRV per ml, purified from top or bottom leaves of infected *Physalis floridana* plants. After the acquisition access period, the nymphs were transferred at constant intervals of 12 h to new *P. floridana* seedlings. The 95% fiducial limits are presented with each bar.

five times at intervals of 24 h. The data are presented in Table 1. Young nymphs transmitted the virus more efficiently than older nymphs, as was reflected in a shorter LP_{50} and a higher percentage of virus-transmitting nymphs at each virus concentration tested. The virus concentration in the diet at which 50% of the nymphs transmitted PLRV was 14 μg/ml (95% f.l.: 11 - 18 μg/ml) and 45 μg/ml (95% f.l.: 35 - 60 μg/ml) for young and old nymphs, respectively. The percentage of nymphs transmitting (Y) was linearly related with the $\log_{10}$ transformed virus concentration in the diet (X). The regression equations were $Y = 60X - 20$ ($r^2 = 0.90$) and $Y = 55X - 39$ ($r^2 = 0.94$) for one- and four-day old *M. persicae* nymphs, respectively. The length of the LP_{50} was independent of the virus concentration present in artificial diets.

Table 2. Honeydew excretion by one-day old *Myzus persicae* nymphs feeding on artificial diets and on different leaves of *Physalis floridana* infected with potato leafroll virus.

Source	Number of nymphs	Honeydew excretion rate (droplets/nymph/hour)
Artificial diet	20	0.6
Top leaves	28	1.3
Bottom leaves	28	1.9

Transmission of virus purified from bottom or top leaves

PLRV was purified from top leaves that did not have any symptoms and from bottom leaves showing pronounced interveinal chlorosis of *P. floridana* plants that had been inoculated with PLRV eight weeks previously. The percentage of nymphs that transmitted the virus and the LP_{50} were determined after feeding for 24 h on an artificial diet containing 60 μg of purified virus per ml from either top or bottom leaves. One-day old *M. persicae* nymphs were transferred to fresh *P. floridana* seedlings every 12 h for five days. The results are presented in Fig. 2.

All nymphs transmitted PLRV from either preparation. However, the LP_{50} of nymphs that had been feeding on virus purified from top leaves was significantly shorter ($P < 0.05$) than that from nymphs feeding on virus purified from bottom leaves.

Honeydew excretion

The number of honeydew droplets produced by nymphs feeding on artificial diets and on different leaves of *P. floridana* infected with PLRV was recorded during a defined period (Table 2). Nymphs on bottom leaves excreted three times as many and nymphs on top leaves about twice as many honeydew droplets as those feeding on the diet. The volume of the collected honeydew droplets varied between 9 and 11 nl and did not depend on the source.

DISCUSSION

When PLRV was acquired by *M. persicae* from virus-containing artificial diets,

the amount of viral antigen detected by ELISA in the nymphs was a function of the $\log_{10}$ transformed virus concentration and the length of the AAP. There is also a linear relationship between the percentage of transmitting nymphs and the logarithm of the virus concentration in the diet. These relationships indicate that the virus charge in the aphid can play a role as a parameter in estimating the amount of virus available in a source. A dosage dependency of the LP, as suggested by others for persistent virus transmission (Clarke & Bath, 1973; Sylvester, 1965, 1980), could not be confirmed in the membrane-feeding experiments in which different amounts of virus were offered to the aphids (Table 1).

Previous work on the relationship between PLRV transmission and feeding behaviour of *M. persicae* nymphs on comparable leaves of infected *P. floridana* plants, in which the honeydew excretion of aphids was used as an indicator of the amount of PLRV imbibed, showed that aphids in which the virus had a long LP excreted as much honeydew as aphids with a short long LP (Van den Heuvel & Peters, 1990). This observation confirms the present findings that the LP does not depend on the amount of virus acquired by an aphid or present in its body. Indeed, it is unlikely that the number of particles will determine the speed of circulation within the aphid, unless cooperative action between virus particles is required for initiating or completing virus circulation.

The LP seems to be influenced by the age of the *M. persicae* nymphs involved and the intrinsic properties of the virus. Younger nymphs are more efficient virus transmitters than older ones (MacKinnon, 1962; Peters, 1986; Robert, Maury & Quemener, 1969), although some workers observed the contrary (Kirkpatrick & Ross, 1952) or did not notice any difference (Day, 1955; Hovey & Bonde, 1948). In our experiments, four-day old *M. persicae* nymphs were less efficient at transmitting the virus than one-day old nymphs (Table 1), this being reflected in longer LP_{50} and a lower percentage of transmitting nymphs.

PLRV purified from top leaves of infected *P. floridana* plants and given to nymphs in the artificial diet, had a shorter LP_{50} than PLRV purified from bottom leaves (Fig. 2). This observation is in accordance with the results on PLRV transmission with intact leaves as the virus source (Van den Heuvel & Peters, 1990), and indicates that the virus purification did not change the relative transmissibility of the virus. As similar amounts of virus were fed to the aphids it is likely that the transmissibility of the virus is affected by changes in the coat protein at the surface of the viral capsid or proteins purified along with PLRV.

The availability of PLRV for acquisition by *M. persicae* in infected plants was determined by comparing the acquisition of the virus by the aphid on top and

bottom leaves of infected *P. floridana* during an AAP of one to five days with that of aphids on artificial diets containing purified PLRV (Fig. 1). The amount of viral antigen detected in nymphs feeding on top leaves matched well with that of those on a diet containing 3.75 - 7.5 μg virus per ml. However, in making an estimation of the actual virus concentration available in these leaves, it should be noted that nymphs feeding on top leaves excreted twice as much honeydew as those on artificial diets (Table 2). Although the nymphs that had fed on bottom leaves excreted three times as much honeydew as those on the artificial diets, the amount of viral antigen present in these nymphs fell below that of nymphs from diets containing 3.75 μg of PLRV per ml. This was not expected as the viral antigen concentration in bottom leaves of PLRV-infected *P. floridana* was higher than in top leaves of the same plants (Van den Heuvel & Peters, 1990). This finding and the presence of more viral antigen in nymphs which that fed on top leaves than in those that fed on bottom leaves of *P. floridana* (Fig. 1), suggest that the amount of virus available for acquisition by aphids is considerably lower in bottom leaves than in top leaves. This conclusion emphasizes that the virus charge of the aphid is a more reliable parameter for determining the potency of a plant as a virus source than the concentration of viral antigen in the source itself.

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CHAPTER 4

POTATO LEAFROLL VIRUS TITER IN SECONDARILY INFECTED POTATO PLANTS AND THE AVAILABILITY OF VIRUS FOR ACQUISITION BY *MYZUS PERSICAE*.

SUMMARY

The concentration of potato leafroll virus (PLRV) antigen in young leaves of eight potato genotypes with different levels of field resistance, and the uptake of virus by *Myzus persicae* nymphs from these leaves was determined. The PLRV antigen concentration in young leaves of three-week old secondarily-infected potato plants was negatively correlated with the levels of field resistance of the genotypes. The concentration of viral antigen in *M. persicae* nymphs which fed on the young leaves of three-week old plants was positively correlated with the concentration of viral antigen in the leaves and with the level of field resistance. With the age of the plants, as symptoms started to develop, the initially high concentrations of viral antigen in the susceptible genotypes Astarte and Desiree, and in the moderately resistant genotype Aquila, decreased to levels found in the other genotypes. Moreover, the amount of PLRV acquired by aphids from top leaves of older potato plants of all genotypes dropped significantly although the virus was still readily detectable in those leaves by ELISA. As a result, the relationships previously observed disappeared two to three weeks later in the growing season.

The feeding behaviour of *M. persicae* nymphs, reflected in the number of honeydew droplets excreted, did not account for the reduced virus acquisition later in the season. Therefore, it is concluded that the availability of virus declines with increasing plant age and symptom development.

INTRODUCTION

Potato leafroll virus (PLRV), a member of the luteovirus group, occurs worldwide

and causes heavy economic losses in potato. The virus is transmitted in a persistent manner by several aphid species. *Myzus persicae* (Sulz.) is considered to be the principal and most efficient vector. Resistance to PLRV is present in several potato genotypes and is polygenically controlled (Ross, 1958). Several interacting components like the replication potential of the virus in the host, the ability of the host to sustain feeding activities by aphids and colonization, and the potency of a host to function as a virus source, may control this resistance (Peters, 1986).

Several workers found under field or glasshouse conditions a negative correlation between the PLRV antigen concentration in primarily- or secondarily-infected potato plants of the studied genotypes and their field resistance levels (Dziewonska & Was, 1987; Souza-Dias & Slack, 1987; Barker & Harrison, 1985; Gase, Moeller & Schenk, 1988; Swiezynski, Dziewonska & Ostrowska, 1988). However, Dziewonska & Was (1987) noticed in some preliminary experiments that this could not be detected anymore when infection age of primarily-infected potato plants of different genotypes increased.

A positive correlation between the concentration of PLRV in potato plants and the transmission efficacy of aphids which fed on them was reported by Souza-Dias & Slack (1987). Since the probability of virus transmission increases with greater amounts of PLRV antigen present in the vector aphid (Van den Heuvel, Boerma & Peters, 1991), their findings contradict those of Harrison (1981) who did not find a link between the PLRV charge in aphids and the virus titer in potato plants on which they were kept.

The dissimilarity between the conclusions on the relationships among the field resistance level, the virus titer in potato plants, and the uptake of virus by aphids might well be explained by the fact that different stages of plant development or infection age were involved in the studies referred. Therefore, the present paper aims to investigate these relationships in secondarily-infected plants at different moments during their growth using eight potato genotypes with different levels of field resistance to PLRV.

MATERIALS AND METHODS

Aphids

Myzus persicae (Sulz.) biotype WMp2 (Reinink *et al*, 1989) was cultured, in cohorts differing by one day in age, on *Brassica napus* L. subsp. *oleifera* at 20 ± 2°C and a 16-h photoperiod (Van den Heuvel & Peters, 1989).

Table 1. Resistance ratings of potato genotypes used in the experiments.

Potato genotype	Resistance rating[a]
Astarte (Ast)	4
Desiree (Des)	4.5
Nicola (Nic)	6
Bintje (Bin)	6.5
Aquila (Aqu)	7
Bevelander (Bev)	7
Burmania (Bur)	8
MPI 49.540.2 (MPI)	9

[a] Ratings on a scale of 4 (most susceptible) to 9 (most resistant) derived from the '64[e] Beschrijvende Rassenlijst voor Landbouwgewassen 1989'. The rating for MPI 49.540.2 is provisional (Miss L. Colon, personal communication).

Plant material

Eight potato genotypes with different resistance ratings to PLRV (Table 1) were used. Secondarily infected plants were produced in the glasshouse. To this end, virus-free tubers, kindly provided by Miss L. Colon (CPRO, Wageningen), were potted in May 1987 and 1988 in soil-less compost and grown at about 20-25°C. Plants of three to five cm in height were inoculated with PLRV-Wageningen (Van der Wilk *et al*, 1989) from *P. floridana* by ten viruliferous *M. persicae* nymphs. Tubers from infected plants were harvested by the end of September and stored until the beginning of May next year at 4°C. After incubating the tubers at room temperature for two weeks, single eye cuttings were excised and potted in soil-less compost. The secondarily-infected potato plants were kept in glasshouses under different temperature and light regimes. In 1988 temperature varied between 20 and 25°C and the light intensity was low (6,000-8,000 lx), whereas in 1989, plants were held at lower temperature (17-20°C) and at higher light intensities (9,000-50,000 lx).

Recording of honeydew excretion

The honeydew excretion of one-day old *M. persicae* nymphs was monitored in a climate chamber at 20 °C as previously reported by Van den Heuvel & Peters (1990) using a honeydew clock similar to the one described and illustrated by Ajayi & Dewar (1982). The total number of honeydew droplets and the time elapsing

before production of the first honeydew droplet were recorded on full-grown young leaves of healthy and secondarily-infected potato plants. Statistical analysis of the data was done using the SAS program (SAS Institute Inc., Cary, NC).

Detection of viral antigen

To detect viral antigen in leaf extracts of potato plants cocktail-ELISA was used (Van den Heuvel & Peters, 1989). The different incubation steps were separated by washing the plates with 0.02 M sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride and 0.05% Tween 20 (PBS-Tween). Wells of Nunc-Immuno Maxisorp (Nunc, Denmark) plates were sensitized by incubating with 250 µl of 2 µg/ml rabbit anti-PLRV globulins in coating buffer (0.05 M sodium carbonate, pH 9.6) for 3 h at 37°C. Homogenized leaf material, diluted in sample buffer (PBS-Tween containing 2% polyvinylpyrrolidone and 0.2% ovalbumin), and rabbit anti-PLRV alkaline phosphatase conjugate diluted to a final concentration of 1 µg/ml were simultaneously added (150 µl per well). After an overnight incubation period at 4°C, 200 µl of 1 mg p-nitrophenyl phosphate disodium salt per ml of 10% diethanolamine, pH 9.8, was added to each well. Substrate conversion was measured at 405 nm with a Titertek Multiskan colorimeter (Flow Laboratories Ltd., Irvine, Scotland).

To detect viral antigen in individual *M. persicae* nymphs, the cocktail-ELISA was followed by enzyme amplification (Van den Heuvel & Peters, 1989). Coating of the wells was done as described above. Nymphs were individually triturated in 100 µl of sample buffer containing 12.5 mM sodium diethyldithiocarbamate and 1 µg/ml rabbit anti-PLRV alkaline phosphatase conjugate. The cocktail was incubated overnight at 4°C. Subsequently, 100 µl of 0.2 mM nicotinamide-adenine dinucleotide phosphate monosodium salt in 0.05 M diethanolamine buffer, pH 9.5, was added. After an incubation period of 30 min at room temperature, 15 µl of 0.5 M nitrophenyl o-phosphate disodium salt was added to the NADP solution. Followed by the addition of 150 µl of the amplification mixture which consisted of 700 units of alcohol dehydrogenase, 100 units of lipoamide dehydrogenase (type VI), 3% ethanol, and 1 mM p-iodonitrotetrazolium violet per 15 ml of 0.025 M phosphate buffer, pH 7.0. Colour development was measured at 492 nm.

In both assays, the plates were blanked against wells that were not incubated with plant material. Each plate contained two identical dilution series of a purified PLRV suspension. To determine the amount of viral antigen in the samples, their absorbance values were compared by intrapolation with those of the dilution series of the purified PLRV suspension. The data were statistically evaluated using the SAS program.

Sampling leaf material and PLRV acquisition by *M. persicae*

Leaf material was sampled by two different methods from either healty or secondarily PLRV infected plants of the potato genotypes. In 1988, at three two-week intervals, starting three weeks after planting, one leaf disk of about 25 mg was removed from the center of a distal leaflet of a completely expanded young leaf at the top of a plant. Three plants of each genotype were tested. Each leaf disk was homogenized in 500 μl sample buffer, and three aliquots of 150 μl were assayed in cocktail-ELISA. In 1989, at three three-week intervals from the moment of planting, leaf disks of seven to ten mg, were punched out from the center of a distal leaflet of an completely expanded young leaf at the top. One leaf disk was taken per plant and 20 plants of each genotype, arranged in four blocks of five plants, were sampled. Each disk was triturated in 100 μl sample buffer and tested with the cocktail-ELISA. Leaf disks of healthy plants served as controls.

The acquisition of PLRV from secondarily infected potato plants by *M. persicae* was analysed in a climate chamber of 20°C by placing cohorts of two-day old nymphs, confined to leaf cages, on the leaflets from which the disks were taken for sampling. After an acquisition access period (AAP) of four days, 20 nymphs per genotype were individually tested in cocktail-ELISA followed by enzyme amplification.

RESULTS

Concentration of viral antigen in leaf disks and in nymphs

Due to high temperatures and low light intensity in 1988, symptom development was weak and only after seven weeks very mild interveinal chlorosis could be observed. In 1989, when plants were held at lower temperature and at higher light intensities than in 1988, a strong chlorosis, and purpling and rolling of the leaf margins was already apparent five weeks after planting.

In both years, the highest concentration of viral antigen was found in the susceptible genotypes Astarte and Desireee, and in the moderately resistent genotype Aquila, three weeks after planting the tubers. The moderately resistent genotypes Nicola and Bintje, and the resistent genotypes Bevelander, Burmania and MPI 49.540.2 contained significantly less viral antigen. The virus titer in plants of these genotypes was not affected by plant age (Fig. 1 A and B). During the growing season, the initially high concentrations of viral antigen in Astarte, Desiree and Aquila decreased at different rates to levels found for the other potato genotypes.

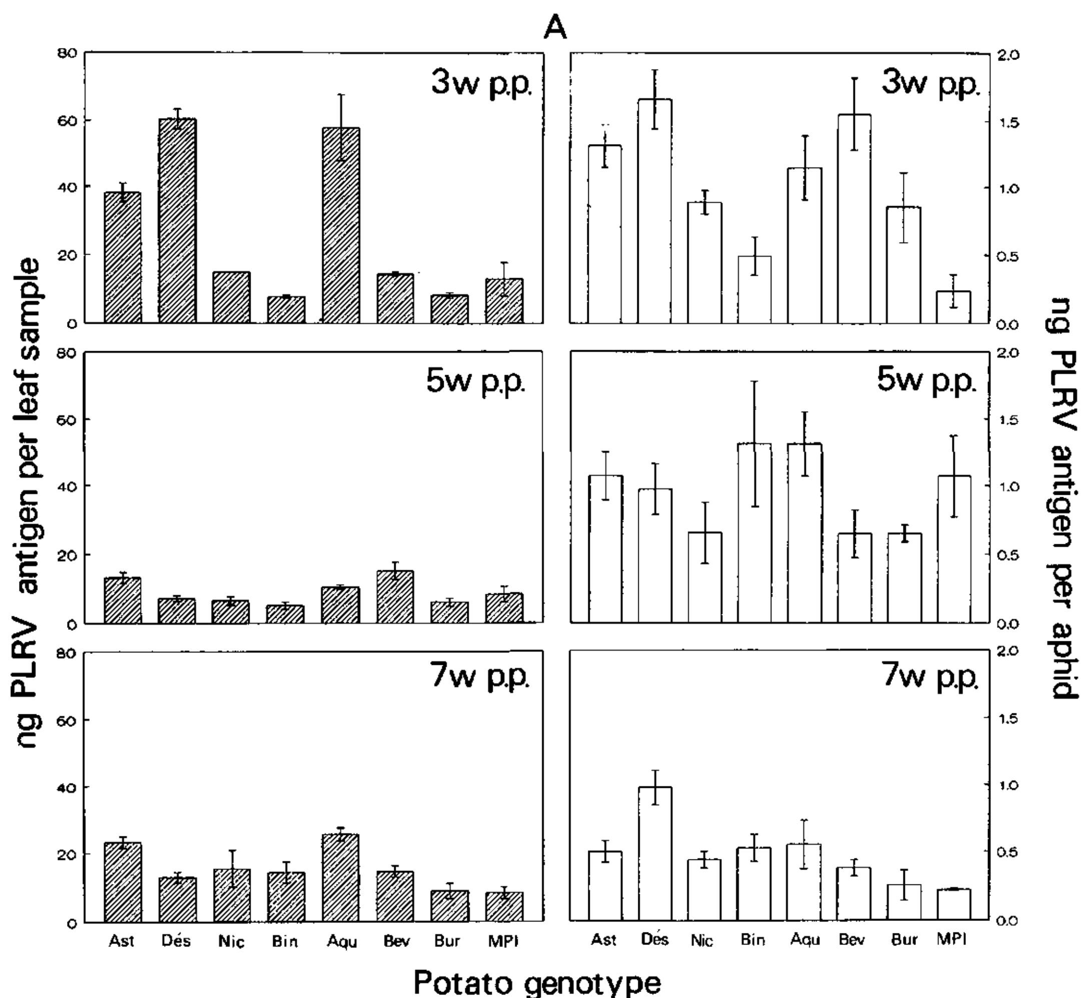
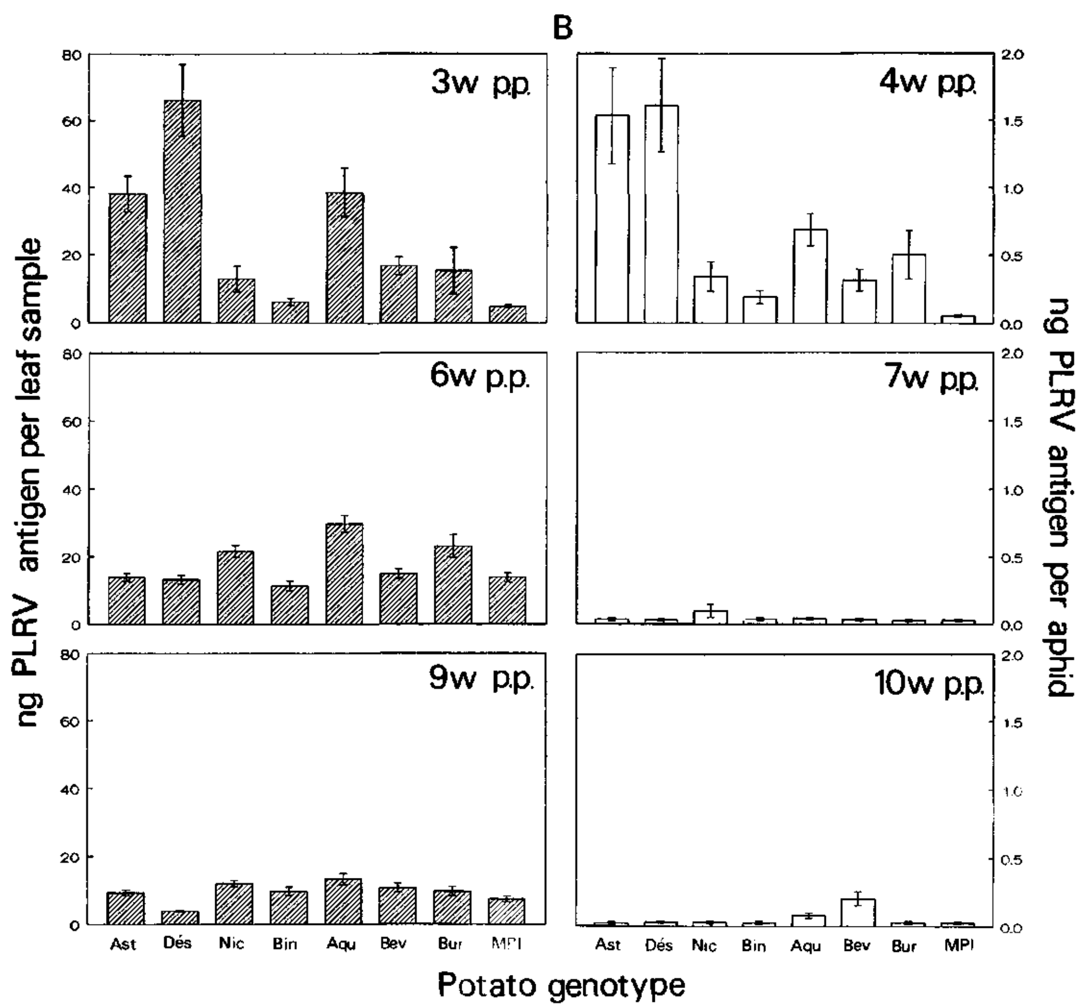


Fig. 1. Amount of PLRV antigen in samples of young leaves of secondarily infected potato genotypes (shaded area) and single *M. persicae* nymphs which fed on them for 4 days (dotted area). Potato genotypes are ranked according to their resistance rating; left: most susceptible, right: most resistant (Table 1). Samples were taken at different moments (w) post planting (p.p.) during the growing season in 1988 (A) and 1989 (B). The vertical bars represent the standard error.


B
3w p.p.
4w p.p.
6w p.p.
7w p.p.
9w p.p.
10w p.p.
ng PLRV antigen per leaf sample
ng PLRV antigen per aphid
Ast
Dés
Nic
Bin
Aqu
Bev
Bur
MPI
Potato genotype

A correlation between the resistance ratings of the genotypes and the concentration of viral antigen in the young leaves was found only three weeks after planting [Spearman, $P < 0.08$ (1988) and $P < 0.05$ (1989)]. It could not be observed later in the growing season (Spearman, $P > 0.08$).

In both years, the young potato plants were found to be the best sources for PLRV acquisition by *M. persicae*, irrespective of the genotype. At the first sampling dates, three to four weeks after planting the tubers, infected plants of the genotypes which are more resistent than Aquila were generally poorer sources for virus acquisition by *M. persicae* than those of the susceptible genotypes. The amount of viral antigen found in the *M. persicae* nymphs was correlated with the concentration of viral antigen in the leaves on which they had been feeding [Spearman, $P < 0.05$ (1988) and $P < 0.005$ (1989)], and with the resistance ratings [Spearman, $P < 0.06$ (1988) and $P < 0.025$ (1989)]. The amounts of virus that could be acquired by the nymphs from the plants of the various genotypes decreased with plant age. The rates of decrease differed in both years and were strongly associated with the severity of symptom development. In 1988, when symptom development was weak, 0.2 to 1.0 ng of viral antigen was detected in nymphs which had been feeding on top leaves of seven-week old plants. At the same moment in 1989, when plants showed severe symptoms, only 50 - 100 pg was found per nymph after a similar AAP.

Aphid feeding behaviour on potato plants

The total number of honeydew droplets produced by three-day old *M. persicae* nymphs, and the time interval between the start of the experiment and the production of the first honeydew droplet was recorded on young leaves of healthy and secondarily infected potato plants during a period of 16 h. Twenty honeydew excretion patterns on either healthy and infected plants of each genotype were recorded. Recordings were evenly spaced over a period of seven weeks, starting with three-week old potato plants.

Some characteristics of honeydew excretion by *M. persicae* are presented in Table 2. The number of honeydew droplets produced in 16 h on the infected plants showed a positive correlation (Spearman, $P < 0.025$) with the resistance ratings of the potato genotypes. On the susceptible genotypes Astarte, Desiree and the moderately resistant genotype Nicola, the nymphs produced almost twice as much honeydew as on MPI 49.540.2. On leaves of healthy potato plants, no correlation was found between the number of honeydew droplets produced and the resistant ratings, but nymphs excreted about two times less droplets on the resistant genotype Burmania than on the other genotypes. On Astarte, Desiree, Bintje, and Burmania

Table 2. The average number of honeydew droplets ± S.E. produced by *M. persicae* nymphs during a feeding period of 16 h and the average period ± S.E. after which *M. persicae* nymphs started honeydew excretion.

Potato genotype	Average number of honeydew droplets		Average period after which honeydew excretion started (h)	
	Healthy	Infected	Healthy	Infected
Astarte	17.6 ± 3.4	31.4 ± 3.6	5.0 ± 0.6	4.1 ± 0.4
Desiree	15.9 ± 2.2	26.0 ± 4.3	4.2 ± 0.5	4.5 ± 0.8
Nicola	n.t.[a]	27.0 ± 2.8	n.t.	3.6 ± 0.4
Bintje	14.4 ± 3.1	22.9 ± 3.0	4.4 ± 0.5	4.5 ± 0.5
Aquila	22.8 ± 3.1	23.0 ± 3.6	4.5 ± 0.5	4.1 ± 0.4
Bevelander	n.t.	19.3 ± 3.5	n.t.	4.9 ± 0.6
Burmania	8.9 ± 2.0	26.4 ± 5.4	6.4 ± 0.8	3.4 ± 0.3
MPI 49.540.2	15.4 ± 2.4	16.4 ± 3.1	4.6 ± 0.5	5.6 ± 0.8

[a] Not tested.

nymphs produced more honeydew droplets on secondarily-infected plants than on healthy ones ($P < 0.05$). However, on healthy plants of the genotypes Aquila and MPI 40.540.2, the honeydew excretion of *M. persicae* was similar to that on the infected plants (Table 2).

Honeydew excretion by *M. persicae* on healthy and secondarily-infected potato plants of the various genotypes started approximately between 3.4 and 6.4 h after placing the nymphs on the leaves (Table 2). The observed differences were not significant ($P > 0.10$).

The number of honeydew droplets and the time interval that elapsed before the production of the first honeydew droplet were not influenced by the age of the potato plants (Spearman, $P > 0.10$).

DISCUSSION

Much attention has been attributed in literature to the relative importance of the various components underlying the polygenic resistance to PLRV. The viral antigen content in leaves of virus infected plants was in several cases found to be

a measure for the field resistance levels of potato genotypes. Wen (1985) and Barker & Harrison (1985) already pointed out that this relation could be used to develop more efficient strategies in resistance breeding. The results described in this chapter indicate that the viral antigen concentration in young leaves of secondarily-infected potato genotypes matched only well with their field resistance ratings ($P < 0.08$ (1988); $P < 0.05$ (1989)) at the first sampling date, three weeks after planting the tubers. The correlation disappeared when plants became older ($P > 0.08$). In both years, the results displayed a high degree of similarity although the experiments were differently designed and carried out under different growing conditions. Our findings support the preliminary observations made by Dziewonska & Was (1987) with various potato genotypes, primarily inoculated with PLRV. They, therefore, indicate that screening potato genotypes for field resistance on base of the virus titer in infected plants has to be done at an early stage of plant development.

The honeydew excretion of *M. persicae* nymphs was recorded on the potato genotypes because it is an appropriate measure of their feeding activities on plants (Sylvester, 1988). It supplies information about the amount of phloem sap imbibed by the aphids. The uptake of phloem sap by nymphs on leaves of secondarily-infected potato plants decreased with increasing field resistant ratings (Table 2). As a result, the amount of PLRV antigen in these nymphs was even stronger correlated with the resistance levels [$P < 0.06$ (1988); $P < 0.025$ (1989)] than the amount of PLRV antigen detected in the leaf disks [$P < 0.08$ (1988); $P < 0.05$ (1989)].

The availability of virus to be acquired by aphids diminished during the growing season, although PLRV antigen in the source leaves was still readily detectable by ELISA. This decline was strongly influenced by the severity of symptoms. In 1988, when symptom development on the potato plants was very slow, relatively high amounts of virus were acquired by aphids even later in the growing season. In contrast, when symptom development was severe (1989), the PLRV antigen titer in the source leaves did not result in notable virus acquisition by the nymphs. A lower virus titer found in the infected plants resulted only at the first sampling date in a lower efficacy with which aphids acquired the virus [$P < 0.05$ (1988); $P < 0.005$ (1989)]. In both years, the relationships found early in the season between the amount of viral antigen in aphids, and that in the leaves or the resistance ratings disappeared with the development of the plants ($P > 0.08$).

In other studies it has also been reported that aphids acquire and transmit PLRV less readily from older than from younger potato plants of the same cultivar although virus was still readily detectable by ELISA (Barker & Harrison, 1986). In addition, a reduced availability for acquisition of virus by aphids was observed with

increasing infection age and symptom development on *P. floridana* (MacKinnon, 1962; Peters & Elderson, 1984; Van den Heuvel, Boerma & Peters, 1991). Therefore, the conclusion that the amount of viral antigen present in aphids represents a more reliable parameter in deducing the potential of a plant to act as a virus source than the viral antigen concentration of the source itself, seems to be justified.

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CHAPTER 5

CHARACTERIZATION OF EPITOPES ON POTATO LEAFROLL VIRUS COAT PROTEIN

SUMMARY

A panel of ten stable hybridoma cell lines secreting monoclonal antibodies (MAbs) specific for potato leafroll virus (PLRV) antigen, was produced in two fusion experiments with murine splenic and myeloma cells. Using different ELISA procedures and Western blotting it was shown that one MAb detected a continuous epitope and nine MAbs reacted with conformational dependent ones. The conformational dependent epitopes could be separated into two groups after an alkaline treatment of the virus. The MAbs were further differentiated in competitive binding assays. Within the group of MAbs reacting with epitopes not sensitive to alkaline degradation, only two MAbs showed to be directed to the same epitope. The MAbs detecting epitopes formed by the quaternary protein structure or by a protein subunit configuration sensitive to alkaline degradation, displayed positive cooperative binding among each other. In total, a minimum number of nine different, though overlapping, epitopes on the PLRV capsid could be revealed.

The immune response to PLRV antigen in rabbit appeared to be directed mainly towards epitopes recognized by three MAbs.

Most MAbs displayed heterologous reactivity to other luteoviruses i.e. tomato yellow top virus (TYTV), beet western yellows virus (BWYV), beet mild yellowing virus (BMYV), bean leafroll virus (BLRV) and different strains of barley yellow dwarf virus. Three MAbs solely reacted with PLRV and TYTV. Six MAbs gave different reaction patterns in these tests; one of these MAbs differentiated BMYV from BWYV, and another detected a common epitope on PLRV and BLRV, a serological relationship not reported previously to our knowledge.

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INTRODUCTION

Luteoviruses, persistently transmitted by aphids, cause worldwide heavy losses in a considerable number of economically important crops. They occur in low concentrations in their host plants in which they are mainly confined to phloem tissue (Waterhouse, Gildow & Johnstone, 1988).

Monoclonal antibodies (MAbs) have been produced to several members of the luteovirus group, viz. barley yellow dwarf virus (BYDV) strains (Hsu, Aebig & Rochow, 1984; Pead & Torrance, 1988; Torrance *et al*, 1986), beet western yellows virus (BWYV) (P. Ellis in D'Arcy, Torrance & Martin, 1989), soybean dwarf virus strains (R.R. Martin in D'Arcy, Torrance & Martin, 1989), tobacco necrotic dwarf virus (Ohshima, Uyeda & Shikata, 1989) and potato leafroll virus (PLRV) (Martin & Stace-Smith, 1984; Massalski & Harrison, 1987; Ohsima, Uyeda & Shikata, 1988). These MAbs have been mainly used to study their potential use in diagnosis and detection, and to differentiate luteovirus strains and isolates. Along with these studies common epitopes among luteoviruses were identified as well as a minimum number of one to five epitopes on the coat protein of different luteoviruses (D'Arcy, Torrance & Martin, 1989; Massalski & Harrison, 1987). In these studies the minimum number of epitopes present on a luteovirus coat protein was assessed by determining the homologous and heterologous reactivity of MAbs, generated to a luteovirus. MAbs with distinct reactivity patterns in these tests were considered as being directed against different epitopes. Since many MAbs responded similarly in these tests, it is still equivocal whether these MAbs are actually the same. Therefore, the MAbs raised to the Wageningen isolate of PLRV (PLRV-Wag) were analysed by competitive binding experiments (Yewdell & Gerhard, 1981). The minimum number of epitopes as well as their topological relationships on the coat protein of PLRV were investigated. Competitive binding studies were also done between the MAbs and polyclonal antibodies to PLRV raised in a rabbit to determine the antibodies prevailing in the serum. Furthermore, new serological relationships with other luteoviruses were disclosed with our panel of MAbs to PLRV.

MATERIALS AND METHODS

Virus purification

PLRV-Wag was purified from infected *Physalis floridana* Rydb. leaves using a modification of the enzyme-assisted purification procedure described by Takanami

& Kubo (1979). Frozen leaves (300 g) were homogenized with 600 ml 0.1 M sodium citrate buffer (2 ml/g), pH 6.0 (citrate buffer), containing 0.5% macerozyme R-10 (Yakult Honsha Co. Ltd, Tokyo), 0.5% cellulase 'Onozuka' R-10 (Yakult Honsha Co. Ltd, Tokyo), 0.1% thioglycollic acid and 0.5% ethanol in a blender. The homogenate was stirred at 25°C for 4 h and then emulsified with a mixture of 150 ml chloroform and 150 ml 1-butanol. The emulsion was vigorously stirred for 5 min and broken by centrifugation at 10,000 rpm for 15 min in a Sorvall GSA rotor. The aqueous phase was collected and Triton X-100, polyethylene glycol 6,000 and sodium chloride were added to final concentrations of 1%, 8% and 0.4 M, respectively. After stirring at room temperature for 1 h the mixture was allowed to precipitate for at least 45 min followed by a centrifugation at 10,000 rpm for 15 min in a Sorvall GSA rotor. The precipitate was resuspended in 90 ml citrate buffer containing 5% ethanol. The supernatant was clarified by centrifugation at 7,000 rpm for 15 min in a Sorvall SS-34 rotor. The partially purified virus suspension was divided into aliquots of 30 ml, layered on 15 ml 30% sucrose (w/v) in citrate buffer, and centrifuged at 30,000 rpm for 4 h in a Beckman R 35 rotor. Each pellet was resuspended in 1 ml citrate buffer, loaded on a sucrose gradient prepared with equal volumes of 20 and 50% sucrose (w/v) in citrate buffer using an LKB Varioperpex II pump, and centrifuged for 5 h at 40,000 rpm in a Beckman SW 41 rotor. The PLRV containing zone was collected using an ISCO density gradient fractionator connected with an LKB Uvicord III. The virus was pelleted in a MSE R 60 rotor at 40,000 rpm for 4 h. All centrifugations were performed at 15°C except for the sucrose density gradient centrifugation which was done at 4°C.

Immunization

Two female BALB/c mice, designated as A and B, were primarily stimulated with an intraperitoneal (i.p.) injection of an emulsion of 50 μg PLRV in 100 μl citrate buffer and 100 μl of Freund's complete adjuvant on day 0. Mouse A received a second injection of 25 μg PLRV in 100 μl citrate buffer intravenously (i.v.) on day 56. Mouse B was injected (i.p.) with 25 μg purified PLRV emulsified with Freund's incomplete adjuvant on day 32, and 25 μg PLRV in 100 μl citrate buffer on day 124 (i.v.).

Production of hybridomas

Hybridomas were derived from fusion experiments between splenocytes of mouse A and B, immunized with purified PLRV-Wag and the mouse myeloma line SP 2/O-Ag 14. The fusions were carried out essentially after Lane (1985) using polyethylene

glycol 4,000 GA (Merck, Darmstadt) as fusion agent. The spleen cells of mouse A and B were fused with the myelomas four days after the last injection. Cell culture and cloning were done following Schots (1989).

Screening of cell culture supernatants

A triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) was applied to detect the presence of PLRV-specific antibodies produced by the fusion products from donor mouse A. Nunc-Immunoplate IF plates (Nunc, Denmark) were used throughout the experiments and after each incubation step, plates were washed with PBS-Tween (2 mM KH_2PO_4, 8 mM $Na_2HPO_4.2H_2O$, 0.14 M NaCl, and 2 mM KCl, pH 7.4, containing 0.05% Tween-20). The plates were sensitized by adding 150 µl of 2 µg per ml polyclonal rabbit anti-PLRV antibodies in 0.05 M sodium carbonate, pH 9.6 (coating buffer) to the wells. After an incubation period of 3 h at 37°C, 50 µl of 20-fold diluted extracts of ground leaf material of PLRV-infected or healthy *P. floridana* plants was added per well. The extracts were prepared in sample buffer (Van den Heuvel & Peters, 1989) and incubated overnight at 4°C. Subsequently, 50 µl of undiluted tissue culture supernatants was added to each well and incubated overnight at 4°C, followed by the addition of 50 µl of 2,000-fold diluted goat anti-mouse IgG alkaline phosphatase conjugate (Sigma, St. Louis) in sample buffer. Plates were incubated for 3 h at 37°C. The immobilized alkaline phosphatase was monitored by adding 150 µl of 1 mg/ml para-nitrophenyl phosphate disodium salt (pNPP) in 10% diethanolamine, pH 9.8. Substrate conversion was measured at 405 nm, using a Titertek multiscan (Flow Laboratories, Irvine).

The fusion products from donor mouse B were analysed by TAS-ELISA and by an indirect ELISA using antigen-coated plates (ACP). In the ACP-ELISA, wells were coated with 50 µl of 1 µg purified PLRV per ml coating buffer and incubated overnight or longer at 4°C, followed by the addition of 150 µl of 0.5% bovine serum albumin (BSA) in sample buffer for 0.5 h at 37°C. The procedure was continued as described for TAS-ELISA.

Isotyping

The isotypes of the MAbs were determined in a sandwich ELISA based on rat MAbs to the mouse isotypes IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM (Sanbio BV, the Netherlands) using hybridoma culture supernatants.

Production of ascitic fluid

Adult BALB/c mice were primed by an i.p. injection of 200 µl 2,6,10,14-

tetramethylpentadecane (Sigma) followed by an i.p. injection of approximately $5x10^6$ hybridoma cells two weeks later. After the development of an ascitic tumour, ascitic fluid was withdrawn from the peritoneal cavity and centrifuged for 5 min at 3,000 rpm. MAbs were partially purified from the ascitic fluids by precipitation with 45% saturated ammonium sulphate, centrifugation for 15 min at 10,000 rpm in a Sorvall SS-34 rotor and resuspension in PBS containing 0.05% sodium azide. The protein concentration was adjusted to 2 mg/ml prior to testing.

Biotinylation

Biotinyl N-hydroxysuccinimide ester (E-Y Laboratories, Inc. San Mateo, CA) (0.2 mg) was added dropwise to 1 mg purified mono- or polyclonal antibodies. The mixtures were incubated while gently shaking for 3 h at room temperature after which the reaction was stopped by adding 10 μl 1 M ammonium chloride per ml (Zrein, Burckard & Van Regenmortel, 1986). Subsequently, the mixture was dialysed extensively versus PBS at 4°C.

Competitive binding assay

In the competitive binding assay, the coating of the microtiter plates and the antigen incubation was done as described for TAS-ELISA. Subsequently, 50 μl of a mixture of a 10,000-fold diluted biotinylated antibody and 10-fold dilutions of the unlabelled antibodies to compete for binding were added to the wells. Biotinylated WAU-A5 was applied 4,000-fold diluted. Following incubation for 3 h at 37°C, the plates were washed with PBS-Tween and incubated overnight with 50 μl of 2,000-fold diluted streptavidin alkaline phosphatase conjugate in sample buffer. Subsequently, the plates were washed and filled with 150 μl of 1 mg/ml p-NPP in 10% diethanolamine, pH 9.8. Substrate conversion was measured at 405 nm. The maximum amount of bound biotinylated antibody was determined in the presence of anti-thyroglobulin antibodies. Incubation with biotinylated antibodies in wells treated with healthy *P. floridana* leaf material served as controls.

Western blotting

A sample of 400 ng purified virus per lane was electrophoresed on a 12% (w/v) acrylamide separation gel and a 6% (w/v) stacking gel. Transfer of proteins from the gels onto nitrocellulose was done following Towbin, Staehelin & Gordon (1979). Specific binding between the denatured coat protein and the selected monoclonal antibodies was detected by incubating the blots with the MAbs for 4 h at room temperature. The blots were washed with PBS-Tween containing 0.05% BSA and

incubated overnight with goat anti-mouse IgG alkaline phosphatase conjugate (1 μg/ml). To visualize the bands the blots were treated with a mixture of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride in a substrate buffer consisting of 0.1 M Tris-HCl, 0.1 M NaCl and 5 mM $MgCl_2$, pH 9.5. The reaction was terminated by washing the blots in 20 mM Tris-HCl (pH 7.5) containing 5 mM sodium diethyldithiocarbamate.

Virus isolates

BWYV isolated from lettuce and beet mild yellowing virus (BMYV) from sugar beet were maintained at our laboratory. Bean leafroll virus (BLRV) was obtained from Mr N. Huyberts (Research Institute for Plant Protection (IPO), Wageningen, the Netherlands), transmitted by *Acyrthosiphon pisum* (Harris) and propagated in *Pisum sativum* L. 'Onyx'. Desiccated BLRV-infected leaf material was provided by Miss L. Katul (FBRC, Braunschweig, Federal Republic of Germany). Tomato yellow top virus (TYTV) on *Lycopersicon esculentum* L. was supplied by Mr A. Dusi (National Center for Horticultural Research (EMBRAPA), Brasilia, Brazil) and was transferred to *P. floridana* by *Myzus persicae* (Sulzer). Desiccated leaf material containing BYDV strain MAV and PAV was provided by Dr W. Huth (FBRC, Braunschweig, FRG). BYDV strains MAV, PAV and RPV from Canada were provided by Dr S. Haber (Agriculture Canada, Winnipeg).

Antibodies

Rabbit anti-PLRV, anti-BWYV, anti-BLRV, and anti-BYDV sera were kindly provided by Mr D.Z. Maat (IPO, Wageningen, the Netherlands), Dr S. Marco (Volcani Center, Bet Dagan, Israel), Miss L. Katul, and Dr W. Huth and Dr S. Haber, respectively. Rabbit anti-BMYV serum was prepared in our department.

RESULTS

Fusion experiments A and B yielded a total of ten stable hybridoma cell lines secreting MAbs specific for PLRV antigen. Their designation, isotypes, reactivity and usefulness in different serological assays is presented in Table 1. Three distinct groups of MAbs could be distinguished; six MAbs (WAU-A2, -A5, -A6, -A7, -A13, and -A47) reacted with equal strength in the two ELISA formats using unlabelled MAbs, three MAbs (WAU-A12, -A24, and -B9) reacted only strongly in TAS-ELISA, and one MAb WAU-B10 was detected in ACP-ELISA but not in TAS-

Table 1. The isotypes, and the reactivity of the monoclonal antibodies against potato leafroll virus in different ELISA formats and in Western blotting.

		Titer[a] in ELISA				
		Unlabelled MAbs		Biotinylated MAbs		
Designation	Isotype	TAS[b]	ACP[c]	DAS[d]	ACP[e]	Western blotting
WAU-A2	IgG1	>160,000	>160,000	>160,000	80,000	-
WAU-A5	IgG1	1,000	1,000	5,000	5,000	-
WAU-A6	IgG1	>160,000	>160,000	40,000	20,000	-
WAU-A7	IgG1	>160,000	>160,000	20,000	10,000	-
WAU-A12	IgG1	>160,000	5,000	>160,000	1,000	-
WAU-A13	IgG2a	40,000	20,000	40,000	10,000	-
WAU-A24	IgG2b	>160,000	1,000	20,000	1,000	-
WAU-A47	IgG2a	>160,000	>160,000	>160,000	>160,000	-
WAU-B9	IgG2b	>160,000	<100	>160,000	1,000	-
WAU-B10	IgM	<100	5,000	100	10,000	+

[a] ELISA titer represents the dilution of the MAbs in the different ELISA formats that yielded an A_{405} of at least 0.10 (control <0.010) after 1 h of colour development at room temperature.
[b] ELISA format: polyclonal rabbit anti-PLRV - antigen - MAbs - goat anti-mouse conjugate - pNPP.
[c] ELISA format: antigen - MAbs - goat anti-mouse conjugate - pNPP.
[d] ELISA format: polyclonal rabbit anti-PLRV - antigen - biotinylated MAbs - streptavidin conjugate - pNPP.
[e] ELISA format: antigen - biotinylated MAbs - streptavidin conjugate - pNPP.

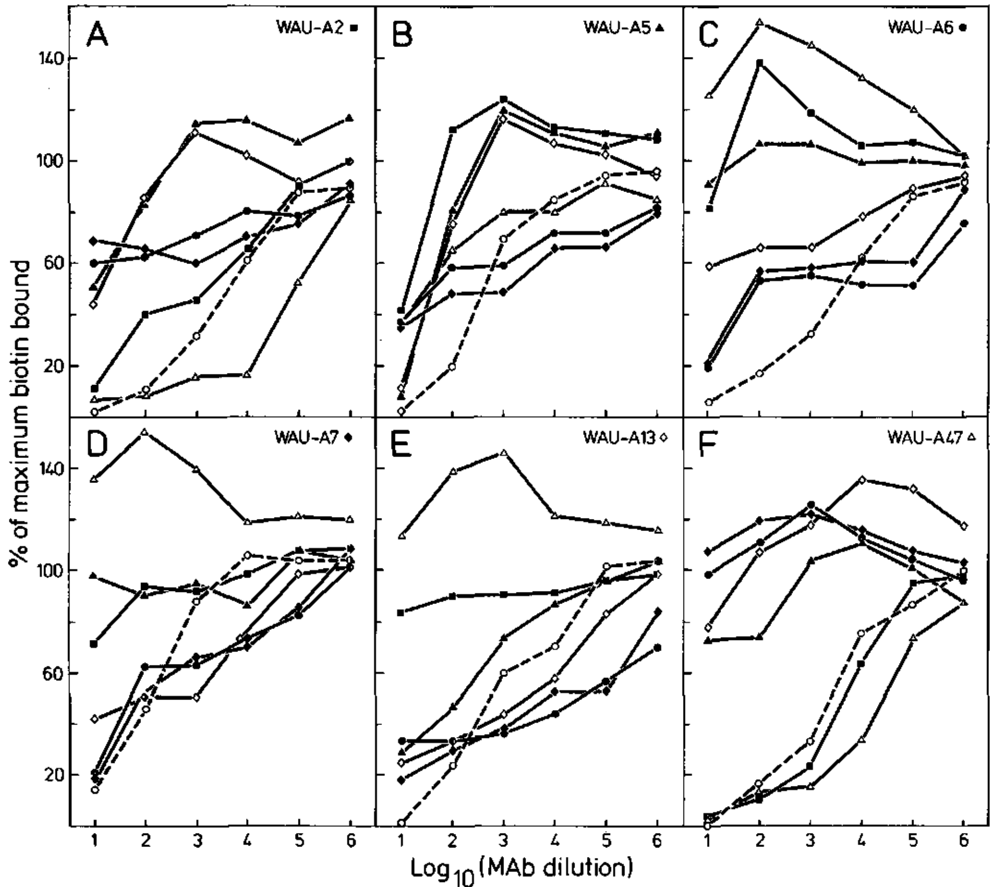

Fig. 1. Results of the competitive binding assay with MAbs reacting in TAS and ACP-ELISA. The following biotinylated MAbs were used: WAU-A2 **(A)**, WAU-A5 **(B)**, WAU-A6 **(C)**, WAU-A7 **(D)**, WAU-A13 **(E)**, and WAU-A47 **(F)**. The unlabeled competitors were WAU-A2 (■), WAU-A5 (▲), WAU-A6 (●), WAU-A7 (◆), WAU-A13 (◇), WAU-A47 (△), and polyclonal rabbit PLRV antibodies (○)

ELISA (Table 1).

Samples of all MAbs were biotinylated and tested in assays, comparable to the TAS and ACP formats, in which the goat anti-mouse conjugate was replaced by a streptavidin conjugate (Table 1). The results show that biotinylation did not alter the behaviour of these MAbs in general, although in some cases their reactivity was impaired. The reaction of only one, WAU-A5, was enhanced.

The reactivity of the 100-, 500-, and 1,000-fold diluted stock suspensions of the MAbs with denatured PLRV coat protein was tested in Western blotting. Only WAU-B10 reacted in this test.

Competitive binding assay

In the competitive binding assays, the binding of biotinylated MAbs to PLRV antigen was challenged by unlabelled MAbs, present in 10-fold serial dilutions. WAU-B10 was not included in these experiments since it did not react in DAS-ELISA when biotinylated (Table 1).

The results with the six MAbs reacting at high titers in both TAS- and ACP-ELISA (Table 1), are depicted in Figure 1A-F. Three pairs of MAbs were found to inhibit strongly each other's binding when the competing antibody was applied at 10-fold dilutions in the reciprocal tests, viz. WAU-A2 with WAU-A47 (Fig. 1A and F), WAU-A5 with WAU-A13 (Fig. 1B and E) and WAU-A6 with WAU-A7 (Fig. 1C and D). Each pair may recognize the same epitope. However, the enhanced binding of the biotinylated MAbs WAU-A6, -A7 and -A13 (Fig. 1C, D and E) in the presence of 10- to 1,000-fold diluted WAU-A47 stock suspension, distinguished this Mab from WAU-A2. Unlabelled WAU-A2, at 10-fold dilutions of the stock suspension, partially inhibited (20-30%) the binding of biotinylated WAU-A6, -A7 and -A13. Also WAU-A5 and -A13 could be differentiated. At 10-fold dilutions, the unlabelled MAbs WAU-A2 and -A47 strongly inhibited (60%) the binding of biotinylated WAU-A5, but not of WAU-A13 (Fig. 1B and E). On base of binding characteristics, WAU-A6 and -A7 could not be discriminated in these assays.

Figure 2 displays the results obtained in the competitive binding assays with the MAbs WAU-A12, -A24 and -B9 which reacted strongly in TAS-ELISA, but not in ACP-ELISA. These MAbs did not compete with each other in the reciprocal tests, indicating that they were directed against different epitopes. When WAU-A12 was used as a competitor, it strongly reduced the binding of labelled WAU-A24 (Fig. 2B). This effect was not observed when the binding of biotinylated WAU-A12 was challenged with unlabelled WAU-A24. A positive cooperative binding among the antibodies WAU-A12, -A24 and -B9 was consistently observed. WAU-A24 and -B9 applied at 10-fold dilutions enhanced the binding of probe WAU-A12 about 20% (Fig. 2A). The binding of labelled WAU-A24 (Fig. 2B) was enhanced by the presence of 10^2- to 10^5-fold dilutions of WAU-B9 with 20-40%; that of labelled WAU-B9 was about 20-80% higher when together with 10^2- to 10^5-fold dilutions of WAU-A12 and -A24 (Fig. 2C).

The MAbs of the two groups were also reciprocally challenged with each other in these competitive binding assays. It appeared that almost all MAbs when present at high concentrations inhibited the binding of the biotinylated antibodies of the other group for 25 to 75% (results not shown).

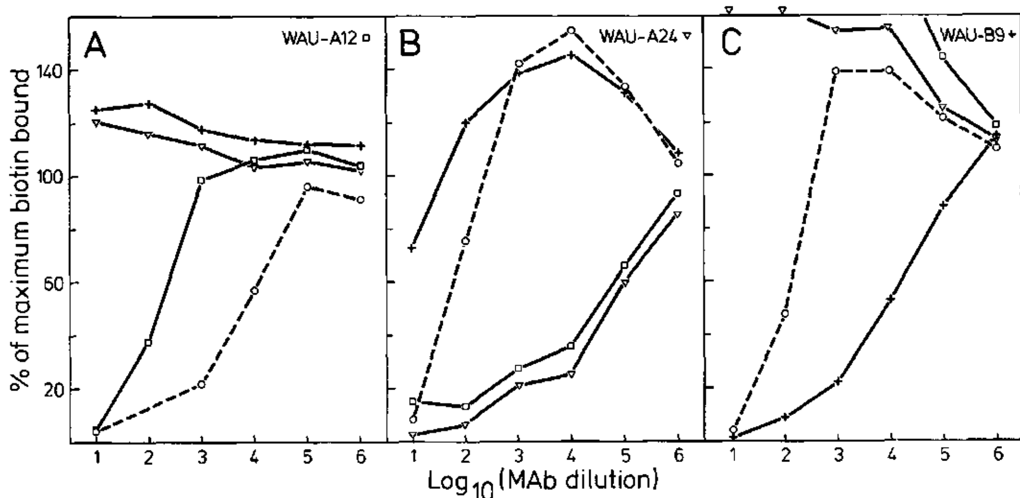

Fig. 2. Results of the competitive binding assay with MAbs only reacting in TAS-ELISA. The following biotinylated MAbs were used: WAU-A12 **(A)**, WAU-A24 **(B)**, WAU-B9 **(C)**. The unlabeled competitors were WAU-A12 (□), WAU-A24 (▽), WAU-B9 (+), and polyclonal rabbit PLRV antibodies (○)

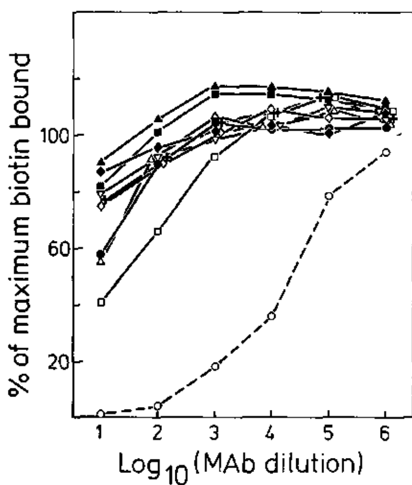

Fig. 3. Results of the competitive binding assay with biotinylated polyclonal antibodies raised against PLRV in rabbit. The unlabeled competing antibodies were: WAU-A2 (■), WAU-A5 (▲), WAU-A6 (●), WAU-A7 (◆), WAU-A12 (□), WAU-A13 (◇), WAU-A24 (▽), WAU-A47 (△), WAU-B9 (+), and polyclonal rabbit PLRV antibodies (○)

Titer of antibodies in rabbit anti-PLRV serum

The titer of the antibodies occurring in an anti-PLRV serum produced in rabbit and reacting with the epitopes distinguished by our panel of MAbs was determined. To this end, the biotinylated polyclonal anti-PLRV antibodies were tested at 50,000-fold

Table 2. Homologous and heterologous reactivity[a] to a number of luteoviruses in TAS-ELISA of MAbs generated to PLRV.

MAbs	Dilution	PLRV	TYTV	BWYV	BMYV	BLRV	BYDV		
							PAV	MAV	RPV
WAU-A2	16,000	S	S	-	-	-	-	-	-
WAU-A5	100	S	S	W	W	W	W	W	W
WAU-A6	4,000	S	S	-	-	-	S	S	-
WAU-A7	2,000	S	S	-	-	-	S	S	-
WAU-A12	16,000	S	S	S	S	-	-	-	-
WAU-A13	4,000	S	S	W	S	-	-	-	-
WAU-A24	2,000	S	S	M	M	S	S	-	-
WAU-A47	16,000	S	S	-	-	-	-	-	-
WAU-B9	16,000	S	S	-	-	-	-	-	-

[a] A_{405} after overnight substrate incubation at 4°C. S Strong = > 1.0; M moderate = 0.5 - 1.0; W weak = < 0.5; - = < 0.1.

dilution with a 10-fold dilution series of the MAbs in a competitive binding assay (Fig. 3). These assays showed that the anti-PLRV antibody population in the serum consisted mainly of antibodies which are probably directed against the same epitope as WAU-A12 (Fig. 3). This MAb, applied at 10-fold dilution, inhibited the binding of the rabbit antibodies when biotinylated by more than 55%. WAU-A6 and -A47 inhibited the binding of the labelled polyclonal antibodies by approximately 40%.

Reactivity of the MAbs with different luteoviruses

In a TAS-ELISA, the panel of MAbs raised to PLRV antigen was tested with 20-fold diluted extracts of leaf material of plants infected with different luteoviruses or luteovirus strains. The MAbs were used as detecting antibody in a 10-fold higher concentration than the titers of these MAbs as found in TAS-ELISA (Table 1). WAU-B10 was not included in these tests since it did not give a specific reaction in TAS-ELISA. The homologous and heterologous reactions of the MAbs are shown in Table 2. All MAbs reacted in a similar way with TYTV and PLRV. Three of these MAbs, i.e. WAU-A2, -A47, and -B9, reacted exclusively with PLRV and

TYTV. WAU-A5, -A6, -A7, -A12, -A13, and -A24 showed heterologous reactivity with one or more of the other viruses. These latter MAbs, except WAU-A6 and -A7, gave markedly different reaction patterns. The BLRV isolates from Wageningen and Braunschweig reacted weakly with WAU-A5 and strongly with WAU-A24. The BYDV strains PAV (FRG and Canada) and MAV reacted similarly with MAbs WAU-A5, -A6, and -A7, but could be distinguished from each other by the failure of MAV to react with Mab WAU-A24. The BYDV strain RPV reacted only weakly with WAU-A5. BWYV could be discriminated from BMYV in this test with WAU-A13.

On protein blots, samples of 400 ng purified PLRV, BWYV, BMYV and BLRV were tested with WAU-B10. This MAb detected one single band of 22-25 kDa with all viruses used in this test.

DISCUSSION

Ten stable hybridoma cell lines secreting MAbs specific for PLRV antigen were obtained in two fusions experiments. The MAbs were differentiated by testing them with PLRV in different ELISA formats, Western blotting, by competitive binding assays and by analyzing the reactions with different luteoviruses and luteovirus strains in TAS-ELISA.

Testing the MAbs in different ELISA formats and Western blotting (Table 1) showed that the panel could be split into three groups. The MAbs WAU-A2, -A5, -A6, -A7, -A13 and -A47 formed one group, all of which reacted with high titers in both TAS- and ACP-ELISA, but not in Western blotting. They might be directed against discontinuous epitopes present on a subunit of the capsid, which are not degraded by the adsorption of the antigen to the wells under alkaline conditions.

A second group of MAbs, WAU-A12, -A24 and -B9, reacted with high titers in TAS-ELISA, but at low titers when the virus was bound to the microtiter plates under alkaline conditions. The loss of reactivity can be explained by extensive degradation of the virus particles as shown by Massalski & Harrison (1987). The MAbs of this group did not detect denatured protein. Therefore, it is concluded that these MAbs are also directed to discontinuous epitopes, presumably formed by the quaternary protein structure or by a coat protein subunit configuration sensitive to alkaline degradation.

The third group comprises only WAU-B10. This MAb was unique as it reacted in Western blotting and it detected viral antigen only in ACP-ELISA. This behaviour

indicates that WAU-B10 is directed to a continuous epitope exposed on the surface of the viral coat protein subunit, which is not accessible when the virus is intact.

The MAbs were further differentiated in competitive binding assays, in which the antibodies, occurring in one group, were reciprocally challenged (Figs. 1 and 2). MAbs were considered as being directed against, operationally defined (Yewdell & Gerhard, 1981), different epitopes when they show different reaction profiles in reciprocal tests. Since the differences of the ELISA titers in TAS-ELISA (Table 1) might indicate non-homogeneity of the affinity of MAbs to antigen, unlabelled antibodies were applied in 10-fold dilution series to compensate. Within the first group of MAbs (Table 1) WAU-A6 and -A7 displayed almost identical competition profiles (Fig. 1), indicating that they might react with the same epitope. All other MAbs in this group showed different profiles and will, therefore, recognize different epitopes.

The results of competitive binding assays can, within certain limits (Yewdell & Gerhard, 1981), supply information on the topological relationships of the epitopes, especially when the antibodies do not compete with each other. The MAbs WAU-A2 and -A47 hardly competed with WAU-A6 and -A7 for binding to the antigen. As a result, the epitopes delineated with WAU-A2 and WAU-A47 differed from the one identified by WAU-A6 and -A7. However, the epitopes reacting with WAU-A5 and -A13 seem to overlap with those recognized by WAU-A6 and -A7, WAU-A2, and WAU-A47 (Fig. 1).

The data obtained with the three MAbs of the second group (Fig. 2) show that they all react with different epitopes. The epitope detected by WAU-B9 did not interfere with those recognized by WAU-A12 and -A24 (Fig. 2C), and WAU-B9 and -A24 did not inhibit the binding of biotinylated WAU-A12 (Fig. 2A). The unidirectional blocking of the non-overlapping epitopes WAU-A12 and -A24 could suggest a conformational change of an epitope (Cepica, Yason & Ralling, 1990) distant to the attachment side. The differences in affinities of these two MAbs were, however, too small to explain the unidirectional blocking (Table 1). Furthermore, positive cooperative binding among the antibodies WAU-A12, -A24 and -B9 might also be an indication for the conformational plasticity of epitopes interacting with antibodies of this group.

Cooperative binding was also induced by rabbit anti-PLRV antibodies in tests with biotinylated WAU-A24 and WAU-B9 (Fig. 2B and C). This effect could be explained by the high titer of antibodies in the polyclonal rabbit antiserum reacting with the same epitope as WAU-A12 does (Fig. 3).

In total, a minimum number of nine different epitopes (including WAU-B10)

was established on the PLRV coat protein by the competitive binding assays. When testing the MAbs of the two distinct groups for competitive binding, it was shown that almost all MAbs inhibited the binding of the biotinylated antibodies. As a result, no distinct antigenic regions on the viral coat protein could be assigned.

In tests with other luteoviruses and luteovirus strains (Table 2), six distinct reaction patterns were observed. The MAbs WAU-A6 and -A7, and WAU-A2, -A47 and -B9 gave identical reaction patterns with all viruses. Additionally, new information about serological relations among luteoviruses was obtained. All MAbs reacting with PLRV also did with TYTV; an observation which corroborates the classification of TYTV as a PLRV strain infecting tomatoes (Casper, 1988; D'Arcy, Torrance & Martin, 1989; Harrison, 1984; Waterhouse, Gildow & Johnstone, 1988). BMYV and BWYV which have a close serological relationship (Casper, 1988) could be differentiated by WAU-A13. Since WAU-A5 and WAU-A24 were reactive to BLRV it can be concluded that PLRV and BLRV have epitopes in common; an observation which has not been reported before.

MAbs, produced against luteoviruses, have so far been differentiated solely by tests in which different viruses or virus strains were used (D'Arcy, Torrance & Martin, 1989; Massalski & Harrison, 1987). Following this approach five different epitopes on PLRV coat protein were distinguished using a panel of 27 and 10 MAbs, respectively. The tests described in this paper indicate conclusively that differentiating MAbs by this approach, as compared with competitive binding assays results in an underestimation of the minimum number of epitopes present on the coat protein and capsid of PLRV.

The fact that in our study nine different epitopes were distinguished by competitive binding assays using a panel of only ten MAbs, suggests that one would arrive at a greater number of epitopes when various states of the coat protein are analysed and different selection criteria after fusion are used.

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CHAPTER 6

EPITOPES ON THE CAPSID MAY BE INVOLVED IN THE TRANSMISSION OF POTATO LEAFROLL VIRUS

SUMMARY

Potato leafroll virus (PLRV) isolates which are readily transmitted by *Myzus persicae*, reacted in ELISA significantly stronger with the monoclonal antibodies (MAbs) WAU-A5, -A6, -A7 and -A13 than poorly transmitted PLRV isolates or tomato yellow top virus. Furthermore, these MAbs reduced the probability of PLRV transmission by aphids and significantly increased the length of the median latency period of the virus in the vector when suspensions of purified virus and MAbs in three particle ratios were fed to *M. persicae*. Therefore, the epitopes delineated by these MAbs might be involved in passage of the virus particles through the aphid's body.

Polyclonal antibodies raised in rabbits against these MAbs were specifically detected by rabbit, rat and mouse anti-PLRV antibodies. These suspensions will thus contain anti-idiotypic antibodies which may be considered as blueprints of epitopes on the PLRV capsid. Ingestion of these antibodies prior to PLRV acquisition by *M. persicae* obstructed virus transmission up to 71%. This could be a result of blocking specific sites in the aphid which have a function in transcellular transport of virus particles.

INTRODUCTION

Potato leafroll virus (PLRV) is transmitted in a persistent manner by several aphid species of which *Myzus persicae* (Sulz.) is the principal one (Sylvester, 1980). During circulation through the vector's body, the virus particles are transcellularly transported across the aphid hindgut and accessory salivary gland membranes. Ultrastructural localization studies of luteovirus particles in aphids have led to the development of a receptor-mediated endocytosis-exocytosis model to describe

transcellular virus transport (Gildow, 1987). In this model, the ability of a luteovirus to be transmitted by an aphid is determined by attachment of the particles to the membranes by receptor molecules (Adam, Sander & Shepherd, 1979; Gildow, 1987). This implies that surface domains of the viral capsid are recognized by these proposed aphid membrane-associated receptors.

Massalski & Harrison (1987) showed that two PLRV isolates poorly transmitted by *M. persicae*, reacted weakly with two MAbs to PLRV as compared with PLRV isolates readily transmitted by this aphid. They concluded that the poorly transmitted isolates lack one or more antigenic determinants, presumably dependent on the quaternary protein structure, which might be involved in initial recognition and transcellular transport of PLRV particles through the aphid's body. Harrison & Robinson (1988) suggested that the specificity of geminiviruses and their ability to be transmitted by their vectors is also linked to the antigenic specificity of the virus particles.

In order to identify epitopes which have a function in initial recognition of PLRV in *M. persicae*, PLRV isolates (Van den Heuvel & Peters, 1990) and a tomato yellow top virus (TYTV) isolate which differ strongly in their transmissibility by aphids, have now been immunologically compared with a panel of nine MAbs against PLRV. Moreover, it is investigated whether the simultaneous or sequential acquisition of MAbs or anti-idiotypic antibodies (AiAbs), and PLRV interferes with virus transmission by *M. persicae* nymphs.

MATERIALS AND METHODS

Aphids

M. persicae biotype WMp2 (Reinink *et al*, 1989) was cultured on *Brassica napus* L. subsp. *oleifera* (oilseed rape) in a greenhouse compartment at 20±3°C and a photoperiod of 16 h per day. Cohorts of nymphs differing less than 24 h in age were produced by transferring daily mature apterae, confined to leaf cages, to fresh plants.

Virus isolates and maintenance

PLRV-Wag was maintained on *P. floridana* as previously described (Van den Heuvel & Peters, 1990). The virus was purified using a modified enzyme-assisted procedure (Van den Heuvel *et al*, 1990).

To generate a highly aphid-transmissible PLRV isolate, PLRV-Wag was

transferred every two weeks to fresh *P. floridana* seedlings. In these transfers, one-day old *M. persicae* nymphs had a 24-h acquisition access period (AAP) on infected *P. floridana* plants and subsequently a 6-h inoculation access period (IAP) on the seedlings. Thirty seedlings were inoculated at each aphid transfer and three nymphs were used per plant.

A PLRV isolate from Brazil on potato (PLRV-Brazil) and TYTV on *Lycopersicon esculentum* L. were supplied by Mr A. Dusi (National Centre for Horticultural Research (EMBRAPA), Brasilia), and upon receipt transferred by *M. persicae* nymphs to *P. floridana* seedlings.

Virus acquisition and transmission experiments were done at 20±0.1°C. Plants were kept in a glasshouse at 25±3°C for symptom development.

MAbs and polyclonal antibodies

Nine MAbs specific for PLRV purified from ascitic fluids of mice, previously characterized (Van den Heuvel *et al*, 1990) were used. Mouse anti-PLRV serum was produced as described before (Van den Heuvel *et al*, 1990). Rabbit anti-PLRV, mouse anti-thyroglobulin and rabbit anti-BlCMV polyclonal antibodies were kindly provided by Mr D.Z. Maat (Research Institute for Plant Protection (IPO), Wageningen), Dr A. Schots (Laboratory for Monoclonal Antibodies, Wageningen) and Mr H. Lohuis respectively.

Production of AiAbs

AiAbs were produced against the MAbs WAU-A5, -A6, -A7 and -A13. Rabbits were immunized by injecting subcutaneously 1 mg antibodies in 1 ml of 0.5xPBS emulsified with 1 ml Freund's incomplete adjuvant at multiple sites on the back every two weeks. Two weeks after the third injection, blood was collected. Antibodies against non-idiotypic epitopes of the MAbs were removed by incubating (Hu & Rochow, 1988) 500 μl of the rabbit serum with 100 μl of 10-fold diluted mouse anti-thyroglobulin serum at 37°C for 3 h. After centrifugation the AiAbs were isolated from the supernatant by precipitation with 45% saturated ammonium sulphate and resuspended in 0.5xPBS. The protein concentration of the AiAb suspension was determined.

Acquisition and transmission experiments

The effect of antibodies on PLRV transmission by *M. persicae* nymphs was studied in experiments in which the antibodies and the virus were either simultaneously or sequentially fed to the nymphs. In the first type of experiments,

a known concentration of purified PLRV was mixed with three different concentrations of MAbs in 100 μl of artificial diet MP148 (Harrewijn, 1983) and offered between Parafilm membranes (Van den Heuvel, Boerma & Peters, 1990) to one-day old *M. persicae* nymphs during an AAP of 24 h. Subsequently, nymphs were individually placed on *P. floridana* seedlings to determine the percentage of the nymphs that could successfully transmit the virus in an IAP of four days. In parallel experiments, nymphs were every 24 h transferred to fresh *P. floridana* seedlings to determine the latency period (LP) of the virus in the vector (Van den Heuvel, Boerma & Peters, 1990). The LP_{50} and its 95% fiducial limit were calculated according to Finney (1962) using the SAS program (SAS Institute Inc., Cary, NC).

In the sequential acquisition experiments, the nymphs were first fed for two days on Parafilm sachets containing a known concentration of MAbs or AiAbs in 100 μl MP148 and could then acquire the virus on top leaves of seven-week old *P. floridana* plants infected with PLRV in an AAP of 24 h. After this, the nymphs were individually transferred to *P. floridana* seedlings to test their ability to transmit PLRV in an IAP of 4 to 5 days. Thirty nymphs were used for each virus-antibody mixture tested. The experiments were carried out in a climate chamber at 20 ± 0.1°C and continuous illumination (8,000 lx).

ELISA

All enzyme-linked immunosorbent assay (ELISA) experiments were performed using Nunc-immunoplate Maxisorp F96 (Nunc, Denmark) plates and the different incubation steps were separated by washing the plates with 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.14 M NaCl, and 2 mM KCl, pH 7.4, containing 0.05% Tween-20 (PBS-Tween).

Virus-infected leaf material was tested in a triple antibody sandwich (TAS) ELISA procedure. The wells of the plates were sensitized with 150 μl of 2 μg/ml rabbit anti-PLRV antibodies in coating buffer (0.05 M sodium carbonate, pH 9.6) during 3 h at 37°C. Subsequently, 100 μl of homogenized virus-infected *P. floridana* plant material, 20-fold diluted in sample buffer (Van den Heuvel & Peters, 1989), was incubated overnight at 4°C. Viral antigen was detected by incubating either MAbs to PLRV or mouse anti-PLRV antibodies, diluted in sample buffer, for 3 h at 37°C. Then, the wells were filled with 100 μl of 2,000-fold diluted goat anti-mouse IgG alkaline phosphatase (Sigma, St. Louis) and incubated for 3 h at 37°C.

The antibody content of *M. persicae* nymphs which fed on MP148 with MAbs was tested in the same ELISA procedure. Macerates of these nymphs replaced the suspension with antibodies to detect the virus in TAS-ELISA.

The reactivity of the AiAbs was tested in a direct ELISA procedure. The wells were incubated with 150 μl of 1 μg AiAbs per ml coating buffer for 3 h at 37°C. Then 100 μl of biotinylated MAbs in sample buffer were added for an overnight incubation period at 4°C, which was followed by an incubation of 100 μl of 4,000-fold diluted streptavidin alkaline phosphatase conjugate for 3 h at 37°C. Macerates of *M. persicae* nymphs which had fed on AiAbs-containing MP148 were also tested for their AiAb content by this ELISA procedure.

The immobilized alkaline phosphatase in the assays was monitored by measuring the conversion of 1 mg/ml para-nitrophenyl phosphate disodium salt in 10% diethanol amine, pH 9.8 (150 μl per well) at 405 nm after incubation at room temperature, using a Bio-Kinetics Reader EL312.

Gel electrophoresis and Western blotting

To detect antibodies in *M. persicae*, nymphs were fed for 3 days on MAbs- or AiAbs-containing MP148 sachets and macerated. The extracts were electrophoresed under denaturating conditions on a 12% (w/v) polyacrylamide separation gel and a 4% (w/v) stacking gel. The proteins were transferred onto nitrocellulose sheets essentially following Towbin, Staehelin & Gordon (1979). Bands were visualized by incubating the sheets with goat anti-mouse or goat anti-rabbit IgG alkaline phosphatase conjugate in PBS-tween containing 0.05% BSA, followed by a mixture of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride in 0.1 M Tris-HCl buffer pH 9.5, containing 0.1 M NaCl and 5 mM $MgCl_2$. The colour reaction was terminated by the addition of 20mM Tris-HCl (pH 7.5) containing 5 mM diethyldithiocarbamate.

RESULTS

Transmissibility of the PLRV isolates and TYTV

PLRV acquired by *M. persicae* nymphs from top leaves of *P. floridana* infected with PLRV-Wag was more efficiently transmitted than that from bottom leaves of the same plants (Van den Heuvel & Peters, 1990; Van den Heuvel, Boerma & Peters, 1991).

A highly aphid transmissible PLRV isolate (PLRV-hat) was obtained in serial transfers of PLRV-Wag. *M. persicae* nymphs were given a 6-h IAP on *P. floridana* seedlings after an AAP of 24 h. During the first five transfers, 5 to 15% of the test plants became infected. After the fifth cycle, the percentage of infected plants

jumped to 55% and was maintained at this level in five successive transfers.

The efficiency by which PLRV-Brazil and TYTV were transmitted was studied in single aphid transfer experiments. One-day old *M. persicae* nymphs were placed on *P. floridana* plants infected with PLRV-Brazil or TYTV and individually transferred to *P. floridana* test plants for an IAP of four days. PLRV-Brazil was readily transmitted by *M. persicae* nymphs; 29 out of 30 *P. floridana* seedlings became infected. TYTV was poorly transmitted; 6 of the 30 test plants became infected.

Immunological differentiation of the PLRV isolates and TYTV

To study the relation between the transmissibility of PLRV and its surface structures, the reactivity of three pairs of isolates each consisting of a readily and a poorly transmissible one was tested in TAS-ELISA with the MAbs and mouse anti-PLRV antibodies. The pairs of isolates compared were PLRV-Wag in top and bottom leaves of eight-week old infected *P. floridana* plants, PLRV-Brazil and TYTV in four-week old infected plants, and PLRV-Wag and PLRV-hat in four-week old infected plants. Of each isolate, samples from three to nine different leaves or plants were tested. The MAbs were applied in a 10-fold higher concentration in TAS-ELISA than their previously determined titers (Van den Heuvel *et al*, 1990). Mouse anti-PLRV antibodies were 5,000-fold diluted. The absorbance value of a MAb with a sample, measured at 405 nm (A_{405}) after 2 h of substrate incubation, was related to that found with mouse anti-PLRV antibodies with the same sample. The relative reactivity, expressed as the quotient of the A_{405}'s obtained with a MAb and mouse anti-PLRV antibodies x 100% is presented in Table 1.

The relative reactivities of the MAbs WAU-A5, -A6, -A7 and -A13 were significantly lower for the poorly transmitted virus from bottom leaves of PLRV-Wag- and TYTV-infected *P. floridana* than for the readily transmitted virus from top leaves of PLRV-Wag and PLRV-Brazil ($P < 0.05$). These MAbs also reacted weaker with the less efficiently transmitted PLRV-Wag isolate than with the highly transmissible PLRV-hat isolate ($P < 0.05$) (Table 1). Hence, these result suggest that the epitopes on the viral capsid to which they are directed are involved in passage of PLRV through the aphid's body.

Table 1. The relative reactivity of nine monoclonal antibodies (MAbs) to PLRV in TAS-ELISA with PLRV isolates and TYTV differing in their transmissibility by *Myzus persicae*.

	Relative reactivity[a] (%) ± S.E.					
	PLRV-Wag					
	Top leaves (7)[b]	Bottom leaves (7)	PLRV-Brazil (9)	TYTV (3)	PLRV-hat (6)	PLRV-Wag (6)
Antibodies	Readily[c]	Poorly[c]	Readily	Poorly	readily	poorly
WAU-A2	11 ± 1	18 ± 2	17 ± 1	42 ± 3	7 ± 1	6 ± 1
WAU-A5	16 ± 4	1 ± 1	32 ± 7	4 ± 4	11 ± 1	7 ± 1
WAU-A6	40 ± 2	16 ± 2	65 ± 4	44 ± 4	136 ± 14	109 ± 7
WAU-A7	46 ± 2	13 ± 4	62 ± 3	46 ± 5	124 ± 12	98 ± 8
WAU-A12	100 ± 16	158 ± 17	104 ± 12	151 ± 24	124 ± 8	108 ± 9
WAU-A13	29 ± 6	3 ± 3	52 ± 7	29 ± 16	114 ± 10	93 ± 6
WAU-A24	130 ± 24	169 ± 28	132 ± 6	210 ± 36	47 ± 4	52 ± 3
WAU-A47	79 ± 10	100 ± 13	75 ± 7	153 ± 20	30 ± 5	31 ± 3
WAU-B9	52 ± 11	83 ± 14	67 ± 12	134 ± 28	40 ± 5	41 ± 2
mouse anti-PLRV	100	100	100	100	100	100
A_{405}[d]	0.362	0.410	0.695	0.617	0.458	0.472

[a] The relative reactivity is expressed as A_{405} MAb/A_{405} mouse anti-PLRV x 100%.

[b] Number of different samples tested.

[c] Aphid transmissibility.

[d] The mean absorbance value of the different samples with mouse anti-PLRV.

Table 2. The effect of simultaneous acquisition of either monoclonal (MAb) or polyclonal antibodies and purified PLRV from a suspension made with diet MP148 by *M. persicae* on the virus transmission.

Antibody	Inhibition of virus transmission (%) [a] Ratio of MAbs per virus particle 400	200	40
WAU-A2	3	7	0
WAU-A5	27	32	11
WAU-A6	47	30	32
WAU-A7	13	23	19
WAU-A12	7	3	0
WAU-A13	23	30	0
WAU-A24	17	10	11
WAU-A47	30	27	0
WAU-B9	23	13	0
rabbit anti-PLRV	100	100	68

[a] The percentage of inhibition of virus transmission by aphids was related to the number of infected *P. floridana* plants obtained with rabbit anti-BlCMV in the same experiment. At particle ratios of 400 and 200, all plants became infected, and 93% at a ratio of 40.

On the contrary, the poorly transmissible PLRV-Wag from bottom leaves and TYTV reacted significantly better with the MAbs WAU-A2 and -A12 than PLRV-Wag from top leaves and PLRV-Brazil. However, significant differences between the relative reactivity with these MAbs were not found when PLRV-Wag was compared with PLRV-hat. The MAbs WAU-A24, -A47 and -B9 did not differentiate between readily and poorly transmissible isolates. The epitopes on the viral capsid to which WAU-A2, -A12, -A24, -A47, and -B9 are directed, therefore, can not be associated with aphid transmissibility.

Blocking PLRV transmission by MAbs

Samples of the MAbs, and rabbit anti-PLRV and rabbit anti-BlCMV antibodies were mixed with purified PLRV in artificial diet MP148. The virus concentration was 6 μg per 100 μl MP148. The antibodies were added in amounts

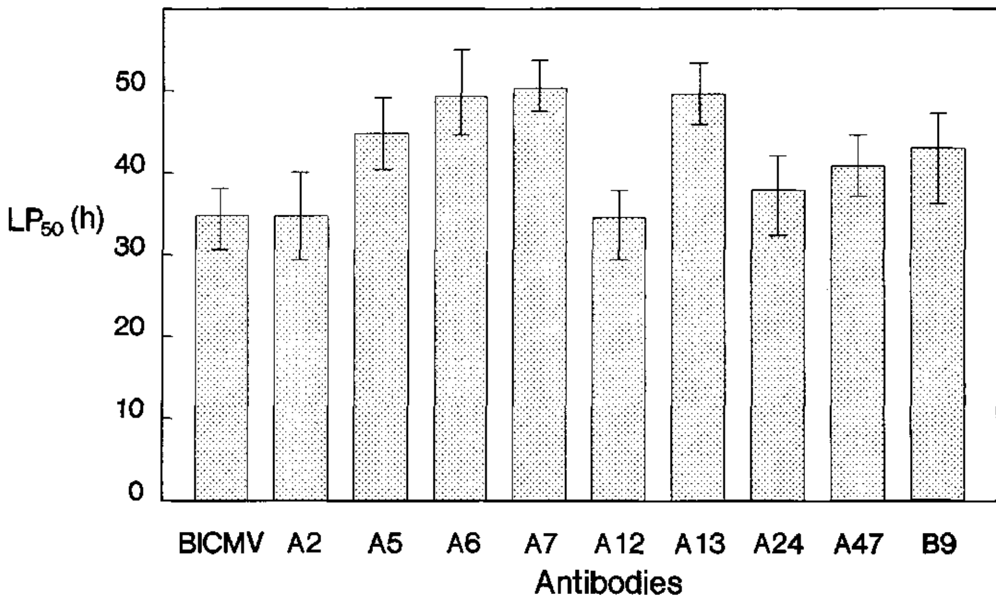

Fig. 1. The influence of simultaneous acquisition of a mixture of monoclonal antibodies and purified PLRV in a particle ratio of 200 from artificial diets by *Myzus persicae* on the median latency period (LP_{50}).

of 134, 67 and 13 μg per 100 μl of virus-containing MP148. The ratio of MAbs to the virus in these mixtures is about 400, 200 and 40 antibodies per virus particle. To calculate this ratio it was assumed that 50% of the protein in the MAb suspensions consisted of antibodies, and that the virus has a particle mass of about 6.3×10^6 kDa and antibodies of 150 kDa. The suspensions were placed between Parafilm membranes and fed to one-day old *M. persicae* nymphs for 24 h. The transmission of PLRV from the suspension with rabbit anti-BICMV antibodies was used as a reference to calculate the inhibition of virus transmission from suspensions with MAbs or rabbit anti-PLRV antibodies. The virus transmission was reduced by 13% (WAU-A7) to 47% (WAU-A6) when the aphids fed on a suspension with a MAb-virus ratio of 400 (Table 2). A similar inhibition of PLRV transmission was found, when suspensions of 200 MAbs per virus particle were tested. The inhibitory effect was much lower when 40 MAbs per virus particle were added. In this case the MAbs WAU-A5, -A6, -A7, and -A24 exerted some effect. WAU-A2 and WAU-A12 hardly had any inhibitory effect on virus transmission, which corroborates the results of the immunological comparison of readily and poorly transmissible isolates (Table 1).

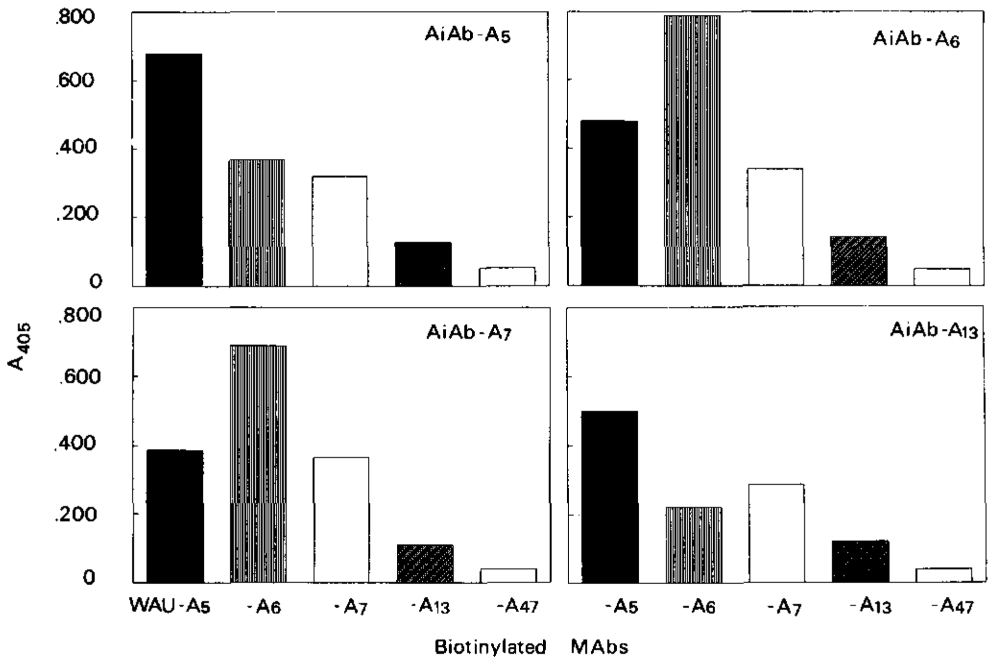

Fig. 2. Reactivity of the biotinylated MAbs WAU-A5, -A6, -A7, -A13 and -A47 with anti-idiotypic antibodies (AiAbs) raised to WAU-A5, -A6, -A7 and -A13.

The LP_{50} was determined in aphids which acquired the virus from suspensions with an antibody-virus particle ratio of 200. Three identical experiments were carried out and their results were combined (Fig. 1). The results show that only the addition of WAU-A5, -A6, -A7 or -A13 significantly increased the length of the LP_{50}. The decrease of transmission efficiency caused by these MAbs further supports the involvement of the corresponding epitopes in virus transmission.

Sequential acquisition of MAbs suspended in MP148 and PLRV from infected plants by *M. persicae* did not consistently affect virus transmission (results not shown).

To examine whether MAbs accumulated in the aphids, macerates, each consisting of 5 nymphs which fed for 48 h on 100 μl of MP148 containing 100 μg MAbs, were tested in ELISA and Western blotting. Antibodies could not be detected in these aphid macerates (results not shown).

Table 3. Inhibition of PLRV-Wag transmission after ingestion of anti-idiotypic antibodies (AiAbs) to selected monoclonal antibodies from artificial diets (100 μg AiAb per sachet) prior to PLRV acquisition by *Myzus persicae*.

	PLRV transmission by *M. persicae*					
	Exp. I		Exp. II		Exp. III	
Antibody	Transmission[a]	Inhibition[a](%)	Transmission	Inhibition (%)	Transmission	Inhibition (%)
AiAb-A5	12/37 (32)	43	16/33 (48)	49	20/30 (67)	28
AiAb-A6	25/48 (52)	7	17/28 (61)	35	20/30 (67)	28
AiAb-A7	1/2 (50)	11	11/20 (55)	41	20/30 (67)	28
AiAb-A13	4/10 (40)	29	8/30 (27)	71	15/30 (50)	46
rabbit anti-PLRV	22/39 (56)	0	15/16 (94)	0	28/30 (93)	0

[a] n infected test plants/n tested (%).
[b] % inhibition is related to the number of test plants infected after rabbit anti-PLRV uptake.

Reactivity of antibodies to MAbs and their effect on PLRV transmission

Because the MAbs WAU-A5, -A6, -A7, and -A13 reacted significantly stronger with readily transmissible PLRV isolates than with poorly transmitted ones or TYTV (Table 1), and impaired virus transmission (Table 2 and Fig. 1), they were selected to raise antisera to in rabbits. Immunoglobulins from these antisera were readily detected by rabbit and rat anti-PLRV antibodies in a direct ELISA (results not shown), indicating their anti-idiotypic nature.

The specificity of the anti-idiotypic antibodies in the sera was tested by comparing their reactivity with the biotinylated MAbs WAU-A5, -A6, -A7, -A13, and -A47 in a direct ELISA. The biotinylated MAbs were used as detecting antibodies. WAU-A47 was incorporated in these tests since it is known from competitive binding studies that this MAb interfered only marginally with the binding of the other MAb to PLRV antigen (van den Heuvel *et al*, 1990). WAU-A47 therefore is most probably directed to an epitope which does not or only marginally overlap with those detected by WAU-A5, -A6, -A7, and -A13. The A_{405} values obtained after 30 min substrate incubation are shown in Fig. 2. The AiAbs all reacted and cross-reacted with the MAbs to which they were raised to, but hardly with WAU-A47. A strong heterologous reactivity was observed between AiAb-A7 and WAU-A6, AiAb-A13 and WAU-A5, and AiAb-A6 and WAU-A5.

Virus transmission was impaired when the AiAb-A5, -A6, -A7, and -A13 were ingested by *M. persicae* nymphs from suspensions containing 100 μg of protein from the anti-MAb sera per 100 μl MP148 followed by PLRV-Wag acquisition from top leaves of *P. floridana* plants infected with PLRV (Table 3). The transmission inhibiting effect fluctuated strongly among the experiments and was on average 40, 20, 29 and 49% for AiAb-A5, -A6, -A7, and -A13, respectively. In experiments in which different concentrations of AiAbs suspended in MP148 were fed to *M. persicae*, the virus transmission inhibiting effect declined with decreasing AiAb concentration (results not shown).

No detectable amounts of AiAbs were found in macerates of five *M. persicae* nymphs that had been feeding for 3 days on MP148 containing 100 μg of AiAbs.

DISCUSSION

The MAbs WAU-A5, -A6, -A7, and -A13 reacted much weaker with PLRV isolates and TYTV which were poorly transmitted by *M. persicae* than with PLRV isolates efficiently transmitted by this aphid. The other MAbs reacted stronger with

these isolates, or did not differentiate between poorly and readily aphid-transmissible isolates (Table 1). When suspensions of purified PLRV and MAbs at different particle ratios were offered to *M. persicae*, the MAbs WAU-A5, -A6, -A7, -A13, -A24, -A47, and -B9 considerably reduced the percentage of nymphs that successfully transmitted the virus to test plants, especially at higher molecule ratios (Table 2). The MAbs WAU-A5, -A6, -A7, and -A24 contributed to this virus transmission inhibiting effect even at a molecule ratio of 40 antibodies per virus particle. However, mixing purified PLRV with antibodies could have changed the virus concentration in the sachets as a result of aggregation and/or precipitation of virus. The reduced transmission may then be the result of a lower effective virus concentration and not by blocking of epitopes involved in transmission. For that reason, the LP_{50} of the virus after being mixed with antibodies was investigated since it was previously shown that this transmission parameter does not depend on the dose of virus acquired by aphids (Van den Heuvel, Boerma & Peters, 1991). Only the MAbs WAU-A5, -A6, -A7 and -A13 significantly increased the length of the LP_{50} (Fig. 2). The other MAbs did not have a significant effect on the LP_{50}. These findings are in agreement with those of the immunological comparison of virus isolates (Table 1). Two lines of evidence, therefore, indicate that the epitopes delineated by the MAbs WAU-A5, -A6, -A7 and -A13, or protein domains closely related to them, might be involved in transcellular transport of luteoviruses in their vector aphids.

Although we do not have sufficient information about the topological relationship of these epitopes, the results of the competitive binding assays indicate that they all strongly overlap with each other. WAU-A6 and -A7 are most likely directed against the same epitope, whereas WAU-A5 and WAU-A13 reacted with different epitopes (Van den Heuvel *et al*, 1990). ELISA experiments in which the virus was caught onto the solid phase under conditions which are known to partially degrade the viral capsid showed that these epitopes are supposedly dependent on the tertiary protein structure (Van den Heuvel *et al*, 1990). This type of protein conformation is essentially different from the protein structures on the PLRV capsid associated with aphid-transmission as revealed by Massalski & Harrison (1987).

MAbs ingested by *M. persicae* form artificial diets did not react with the virus which was acquired by the aphid thereafter. Moreover, MAbs could not be detected by ELISA or Western blotting in the nymphs, indicating that they do not accumulate in the gut lumen nor in the hemocoel of *M. persicae*. Although transport of antibodies across the gut epithelial lining and accumulation in the hemocoel has been reported for some other insects (Nogge & Giannetti, 1980; Schlein, Spira &

Jacobson, 1976; Vaughan & Azad, 1988), it seems that immunoglobulins are rapidly degraded in the gut lumen of *M. persicae*, when not associated to other proteins.

Antisera raised to WAU-A5, -A6, -A7 and -A13 were readily detected by the corresponding MAbs and by polyclonal antibodies to PLRV. Part of the antibodies in the sera is therefore anti-idiotypic and may be considered as blueprints of epitopes on the PLRV capsid. The specificity of the AiAbs is illustrated by the fact that biotinylated WAU-A47 hardly recognized these AiAbs (Fig. 2). A strong heterologous reactivity among the AiAbs and the MAbs to which they were raised was observed and could be expected from the topological relationships of the epitopes they are substituting. The ingestion of AiAbs prior to virus acquisition inhibited virus transmission up to 71% (Table 3). The fact that no detectable amounts of AiAbs were found in the macerates of aphids after an AAP of 3 days on artificial medium containing the AiAbs using ELISA and Western blotting, might indicate that AiAbs do not accumulate in the hemocoel and that virus transport was impeded at gut level. Most likely the AiAbs block the sites at the gut epithelium where initial recognition of virus takes place, and thus obstruct transcellular virus transport. The obstruction of virus transport in a vector by PLRV-specific antibodies could well prove to be a basis for a novel control strategy of protein-mediated protection in genetically-engineered plants against circulative viruses. Acquisition of those antibodies along with the virus from infected plants may result in a lower transmission efficiency. Such an acquisition may be feasible, since it has already been demonstrated that antibodies can be expressed at high levels in transgenic tobacco plants (Hiatt, Cafferkey & Bowdish, 1989).

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SUMMARY

Studying the transmission of potato leafroll virus (PLRV) by *Myzus persicae* from infected *Physalis floridana* plants, revealed that the ability of aphids to transmit the virus differed widely among individuals and strongly depended on the biotype of the vector aphid and the age of the virus source. Virus transmission was more frequent by *M. persicae* from top leaves that hardly showed any disease symptom than from bottom leaves with pronounced symptoms. The percentage of successful PLRV transmission decreased with the infection age. Furthermore, the median latency period (LP_{50}) of the virus was significantly shorter in aphids that fed on top leaves than on bottom leaves. The differences in virus transmission from bottom and top leaves could not be explained by the amount of viral antigen in the sources, since a higher concentration of viral antigen was detected in bottom leaves than in top leaves. The feeding behaviour of aphids on the virus sources also did not account for the observed differences; the honeydew excretion rate of *M. persicae* nymphs feeding on bottom leaves was higher than on top leaves of infected plants (Chapter 1).

To investigate to what extent the amount of virus acquired by *M. persicae* contributed to differences in virus transmission, the sensitivity of the double antibody sandwich (DAS) variant of the enzyme-linked immunosorbent assay (ELISA) had to be increased. To this end, the procedure was modified by incubating sample and conjugate simultaneously (cocktail-ELISA), and by amplifying the enzyme reaction in which a dephosphorylated substrate catalytically triggers an enzyme-mediated redox cycle. Cocktail-ELISA preceding enzyme amplification was 10- to 15-fold more sensitive than DAS-ELISA and could easily be applied to detect viral antigen in individual nymphs which had been feeding for only a short period of time on infected *P. floridana* plants (Chapter 2).

Nymphs that had fed on bottom leaves with pronounced symptoms of *P. floridana* plants infected with PLRV acquired considerably less virus in the same acquisition access period than nymphs feeding on top leaves. The observed dissimilarity between the viral antigen content in the virus sources and in the aphids feeding on them suggests that the availability of the virus for acquisition by aphids is considerably lower in bottom than in top leaves (Chapter 3). Changes in availability of virus for acquisition were also noticed on eight potato genotypes with different levels of field resistance to PLRV. Early in the growing season, a lower virus titer in the secondarily-infected potato plants resulted in a lower capacity of the plants to act

as a virus source. As plants aged, and symptoms became apparent, the virus was still readily detectable in the plants but virus acquisition by *M. persicae* was impaired and did not correlate anymore with the amount of viral antigen in the source (Chapter 4). PLRV acquisition and transmission by *M. persicae* from artificial diets containing purified PLRV demonstrated that the amount of viral antigen in the nymphs, and the percentage of viruliferous nymphs were linearly related to the $\log_{10}$ transformed virus concentration in the diet. Therefore, the amount of viral antigen present in aphids is a more reliable parameter in deducing the potential of a plant to act as a virus source, than the viral antigen concentration of the plant itself.

Artificial diet studies furthermore demonstrated that the LP_{50} of the virus in *M. persicae* was not influenced by the virus concentration in the diet, but by interactions between the virus and its vector. Four-day old *M. persicae* nymphs displayed a longer LP_{50} than one-day old nymphs. PLRV purified from top leaves of infected *P. floridana* was transmitted with a significantly shorter LP_{50} than virus purified from bottom leaves. This finding shows that the previously observed differences in virus transmission from intact top and bottom leaves of *P. floridana* can not be solely explained by differences in the virus concentration available for acquisition. As similar amounts of purified virus from top and bottom leaves in artificial diet were fed to the aphids, it is likely that intrinsic properties of the virus also determine the transmissibility of the virus occurring in top and bottom leaves. This may concern changes at the surface of the viral capsid (Chapter 3).

To study possible relationships between the transmissibility of PLRV and protein structures at the surface of the viral capsid, monoclonal antibodies (MAbs) were generated to PLRV. After two fusion experiments, nine different MAbs to PLRV were selected, and the topological relationships and the nature of the epitopes, to which they were directed, determined (Chapter 5). PLRV isolates which differed in their transmissibility by *M. persicae*, were tested in a triple antibody sandwich ELISA with this panel of MAbs. It was shown that four MAbs reacted significantly stronger with isolates which were readily transmitted than with the poorly transmitted isolates. Moreover, when mixtures of PLRV and MAbs suspensions were fed to *M. persicae*, these four MAbs reduced the probability of virus transmission and significantly increased the latency period of the virus in its vector. Hence, two lines of evidence indicate that the epitopes to which these MAbs were directed might be functionally involved in virus transmission by *M. persicae* (Chapter 6). The four MAbs reacted with conformation-dependent epitopes which are supposedly dependent on the tertiary protein structures. Competitive binding assays indicated that these epitopes strongly overlap which each other (Chapter 5). Anti-idiotypic antibodies

(AiAbs) were raised to the four MAbs in rabbits. They may be considered as blueprints of the epitopes to which the MAbs are directed. When suspensions containing these AiAbs were fed to *M. persicae* prior to virus acquisition, PLRV transmission was reduced by up to 71% (Chapter 6). AiAbs may have blocked specific sites in the aphid which have a function in transcellular transport of virus particles.

SAMENVATTING

In de efficiëntie van overdracht van het aardappelbladrolvirus ('potato leafroll virus'; PLRV) door *Myzus persicae* vanuit geïnfecteerde *Physalis floridana* planten wordt een grote variatie waargenomen tussen individuele bladluizen, zelfs als men van een kloon uitgaat. Hoofdstuk 1 beschrijft in hoeverre het stadium van de virusinfectie, de concentratie viraal antigeen in de bronplant en het voedingsgedrag van de bladluis elk hun invloed op deze variatie doen gelden. Jonge topbladeren, nog zonder symptomen, van een *P. floridana* plant geïnfecteerd met PLRV, zijn, onafhankelijk van de leeftijd van de plant, een betere bron voor virusverwerving door *M. persicae* dan bladeren aan de basis van een plant met duidelijke symptomen. Dit werd weerspiegeld in een hoger percentage infectieuze bladluizen na een bepaalde verwervingsperiode op topbladeren in vergelijking tot basisbladeren. In de basisbladeren nam dit percentage verder af met toenemende leeftijd van de virusinfectie en de daarmee gepaard gaande symptoom ontwikkeling. Voorts bleek de mediane latentie periode (LP_{50}) van het virus in de bladluis significant korter te zijn wanneer het virus verworven was van topbladeren dan van basisbladeren. De waargenomen verschillen in de overdrachtsefficiëntie konden niet verklaard worden door de concentratie van viraal antigeen aanwezig in de bladeren, daar juist een hogere concentratie viraal antigeen aangetroffen werd in basisbladeren dan in topbladeren. Ook het voedingsgedrag van de bladluizen op deze bladeren kon niet verantwoordelijk gesteld worden want de honingdauwexcretie van bladluizen die zich op de basisbladeren gevoed hadden lag duidelijk hoger dan die op topbladeren (Hoofdstuk 1).

Om te kunnen bestuderen in hoeverre de hoeveelheid virus verworven door bladluizen uit de bladeren verantwoordelijk was voor de waargenomen verschillen, werd de 'enzyme-linked immunosorbent assay' (ELISA) aangepast teneinde de gevoeligheid te vergroten. De modificaties bestonden uit een simultane incubatie van het monster en het conjugaat (cocktail-ELISA) en amplificatie van de enzymreactie. Dit laatste houdt in dat de substraatreactie versterkt wordt doordat het gedefosforyleerde substraat als katalysator voor een cyclische redox reactie gaat fungeren waarbij steeds een intensief kleurend eindprodukt vrijkomt. Cocktail-ELISA gevolgd door enzym-amplificatie bleek 10 tot 15 keer gevoeliger te zijn dan de standaard ELISA procedure. Het was nu mogelijk om PLRV aan te tonen in individuele bladluizen na een korte verwervingsperiode op geïnfecteerde *P. floridana* planten (Hoofdstuk 2).

De toepassing van cocktail-ELISA en enzym-amplificatie gaf te zien dat bladluizen die zich gedurende een bepaalde periode gevoed hadden op basisbladeren van *P. floridana* planten geïnfecteerd met PLRV aanmerkelijk minder virus verwierven dan bladluizen op topbladeren van dezelfde plant. De discrepantie tussen de concentratie viraal antigeen gemeten in het bladmateriaal en in de bladluizen die dit als virusbron gebruikt hebben geeft aan dat de beschikbaarheid van het virus voor verwerving door bladluizen niet gelijk is in top- en basisbladeren (Hoofdstuk 3). Veranderingen in de beschikbaarheid van het virus voor verwerving door *M. persicae* werden ook waargenomen bij acht secundair geïnfecteerde aardappelgenotypen met een verschillend niveau van veldresistentie tegen PLRV. Vroeg in het groeiseizoen werd een duidelijke correlatie aangetroffen tussen de concentratie viraal antigeen in de topbladeren van de secundair geïnfecteerde planten en de hoeveelheid virus die daaruit verworven werd door *M. persicae*. Naarmate de symptoomontwikkeling vorderde, kon het virus nog wel aangetoond worden in het plantemateriaal maar niet meer in de bladluizen die zich erop gevoed hadden (Hoofdstuk 4). Uit experimenten waarin bladluizen gedefinieerde hoeveelheden gezuiverd virus in een kunstmatig bladluisdieet aangeboden kregen ter verwerving, bleek duidelijk dat de hoeveelheid viraal antigeen aanwezig in de bladluizen en het percentage virulente bladluizen lineair gerelateerd waren aan de logaritme van de virusconcentratie in het dieet. Deze resultaten wijzen erop dat de concentratie viraal antigeen aanwezig in bladluizen een meer betrouwbare parameter is om de kwaliteit van een bronplant te bepalen dan de concentratie viraal antigeen in deze plant zelf.

Studies met kunstmatig dieet gaven voorts te zien dat de LP_{50} van het virus in *M. persicae* niet beïnvloed werd door de virusconcentratie aangebracht in het dieet, maar door interacties tussen virus en vector. Vier dagen oude nymfen vertoonden een langere LP_{50} dan één dag oude nymfen. PLRV gezuiverd uit topbladeren van geïnfecteerde *P. floridana* planten en via kunstmatig dieet aangeboden aan *M. persicae* werd met een significant kortere LP_{50} overgedragen dan PLRV gezuiverd uit basisbladeren aangeboden in een identieke concentratie. Hiermee werd aangetoond dat de eerder gevonden verschillen in de LP_{50} vanuit top- en basisbladeren van geïnfecteerde *P. floridana* planten niet alleen verklaard kunnen worden door veranderde beschikbaarheid in de bladeren, maar dat ook intrinsieke eigenschappen van het virus de overdraagbaarheid bepalen. Mogelijk dat veranderingen aan het oppervlakte van de eiwitmantel van het virus hierbij een belangrijke rol spelen (Hoofdstuk 3).

Om de relatie te bestuderen tussen de efficiëntie van virusoverdracht en de eiwitstructuur aan het oppervlakte van de eiwitmantel van het virus, werden

monoklonale antilichamen (MAbs) opgewekt tegen PLRV. Na twee fusie-experimenten werden tien MAbs geselecteerd (Hoofdstuk 5). Met behulp van competitieve ELISA werd vastgesteld dat de MAbs gericht waren tegen negen verschillende antigene determinanten op het virale manteleiwit. PLRV isolaten die verschilden in de efficiëntie waarmee ze overgedragen werden door *M. persicae* werden in een 'triple antibody sandwich' ELISA getoetst met deze MAbs. Hieruit bleek dat vier MAbs significant sterker reageerden met virusisolaten die zeer efficiënt overgedragen werden door *M. persicae* dan met minder goed overdraagbare isolaten. Bovendien bleek dat indien de corresponderende epitopen op de virale capside geblokkeerd werden door gezuiverd virus te mengen met deze MAbs, de kans op succesvolle overdracht door bladluizen verkleind werd en de LP_{50} significant verlengd. Twee onderzoekslijnen wijzen er dus op dat de epitopen welke door deze vier MAbs gedetecteerd werden een functionele betrokkenheid hebben in de virusoverdracht (Hoofdstuk 6). Deze vier epitopen bleken afhankelijk te zijn van de tertiaire eiwitstructuur. Competitieve bindingsstudies met de MAbs gaven verder aan dat de bindingsplaatsen van deze vier MAbs op het manteleiwit van PLRV een grote overlap vertoonden (Hoofdstuk 5). Polyklonale antilichamen opgewekt tegen deze vier MAbs werden specifiek herkend door polyklonale antilichamen tegen PLRV, hetgeen erop duidt dat zij beschouwd kunnen worden als blauwdrukken van de epitopen van de PLRV eiwitmantel. Indien deze anti-idiotype antilichamen (AiAbs) voorafgaand aan virusverwerving aangeboden werden via kunstmatig dieet aan bladluizen, werd de overdracht van PLRV belemmerd. Mogelijk dat deze AiAbs bepaalde plaatsen blokkeren in de bladluizen die cruciaal zijn voor het transcellulair transport van het virus in de vector (Hoofdstuk 6).

Toekomstig onderzoek zal erop gericht zijn om een beter begrip te krijgen van de mechanismen die betrokken zijn bij PLRV transport in *M. persicae*, en om na te gaan of de obstructie van virusoverdracht door AiAbs een uitgangspunt vormt voor een alternatieve gewasbeschermingsstrategie.

CURRICULUM VITAE

J.F.J.M. van den Heuvel werd op 24 april 1962 geboren te Aarle-Rixtel. Hij behaalde in 1980 het diploma ongedeeld V.W.O. aan het Sint Carolus Borromeus College te Helmond. In hetzelfde jaar werd een studie aan de Landbouwuniversiteit te Wageningen aangevangen. In 1984 werd het kandidaatsexamen en in januari 1987 het doctoraalexamen in de Planteziektenkunde afgelegd. De ingenieursstudie omvatte de vakken Virologie, Entomologie en Algemene Agrarische Bedrijfseconomie.

Het in dit proefschrift vastgelegde promotieonderzoek werd in december 1986 gestart nadat een door hemzelf geformuleerd projectvoorstel door het College van Bestuur van de Landbouwuniversiteit gehonoreerd werd met een aanstelling als Assistent in Opleiding. Het onderzoek werd onder supervisie van dr ir D. Peters uitgevoerd op de Vakgroep Virologie.

Thans is hij werkzaam als planteviroloog op het Instituut voor Planteziektenkundig Onderzoek te Wageningen.

NAWOORD

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