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A DISTINCTIVE STRAIN OF THE RED CLOVER MOTTLE VIRUS IN THE NETHERLANDS[1]

Een in Nederland voorkomende stam van het vlekkerigheidsvirus van rode klaver

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A virus isolate obtained from red clover in the Netherlands in 1957 has been identified as red clover mottle virus by means of host reaction studies, electron microscopy and serology. This isolate is considered to be a separate strain, however, on the basis of clear differences in severity of symptoms in broad bean, pea and red clover plants and especially of qualitative serological differences between this isolate and the English type strain. The virus produces granular inclusion bodies in artificially infected pea plants. It is especially distinguished from other legume viruses described so far by its reaction in broad bean, pea, and red clover plants. A discussion is given of its distant relationship to cowpea mosaic virus. This is the first report of the occurrence of the red clover mottle virus outside England. At present the virus appears to be of no economic importance in the Netherlands.

INTRODUCTION

In 1957 Dr. J. P. H. van der Want isolated a virus from red clover *(Trifolium pratense)*, which produced a typical top necrosis in artificially infected broad bean plants. The present authors found that it differed from other legume viruses identified at that time. When Sinha (1960) described a seemingly related virus at Rothamsted farm under the name "red clover mottle virus", comparative studies were made to check a possible identity. Since the Dutch virus isolate turned out to be a distinctive strain, our results will be reported in some detail.

MATERIALS AND METHODS

The English isolate of the virus and the original antiserum prepared by Sinha (1960) were obtained from Dr. A. J. Gibbs, Rothamsted, Harpenden. Both the English and the Dutch isolates were maintained in red clover plants in the greenhouse. Standard procedures for studying the virus were those described by Bos *et al.* (1960).

A preliminary antiserum against the Dutch isolate (titre 256) was produced by using partially purified virus. For making antisera of high titre against both isolates the following procedure of virus purification gave good results. Plants of broad bean *(Vicia faba)* showing the first systemic symptoms were homogenized with 0.18 M phosphate citric acid buffer pH7 containing 0.1% thioglycolic acid at 3 °C. The homogenate was squeezed through cheesecloth. After lowering of the pH to 4.5 the sap was centrifuged for 20 min at 8,000 rpm, and then treated by three cycles of differential centrifugation (30,000 rpm for 20 min). Before the last cycle the low-speed supernatant was absorbed with antiserum against healthy plant sap (Oertel, 1960).

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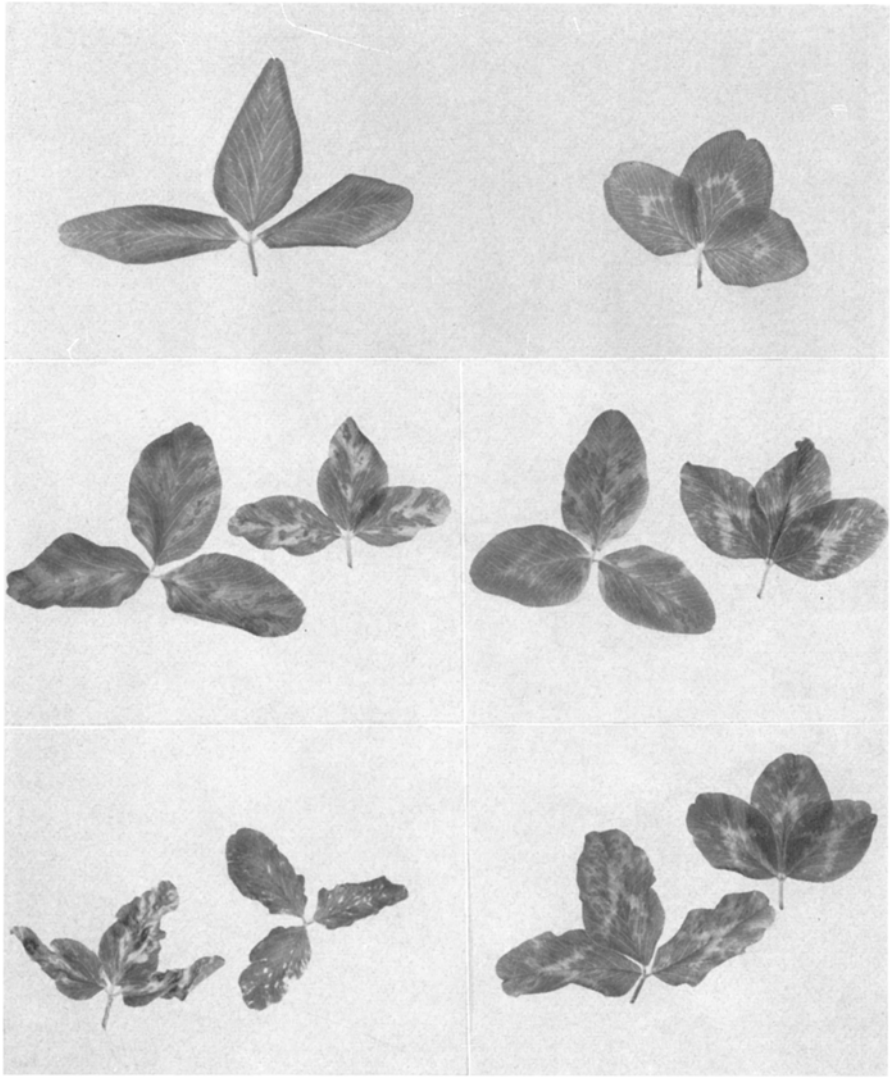

FIG. 1. Leaves of red clover *(Trifolium pratense)* showing systemic symptoms after artificial infection. Upper row: healthy controls; middle: infected with the Dutch isolate; lower row: infected with the English isolate. Left: clone Kyc 71-8; right: local Dutch variety.
Rode-klaverbladeren (Trifolium pratense) *met systemische symptomen na kunstmatige infectie. Boven: gezonde controle; midden: geïnfecteerd met de Nederlandse isolatie; onder: geïnfecteerd met de Engelse isolatie. Links: kloon Kyc 71-8; rechts: Nederlands landras.*

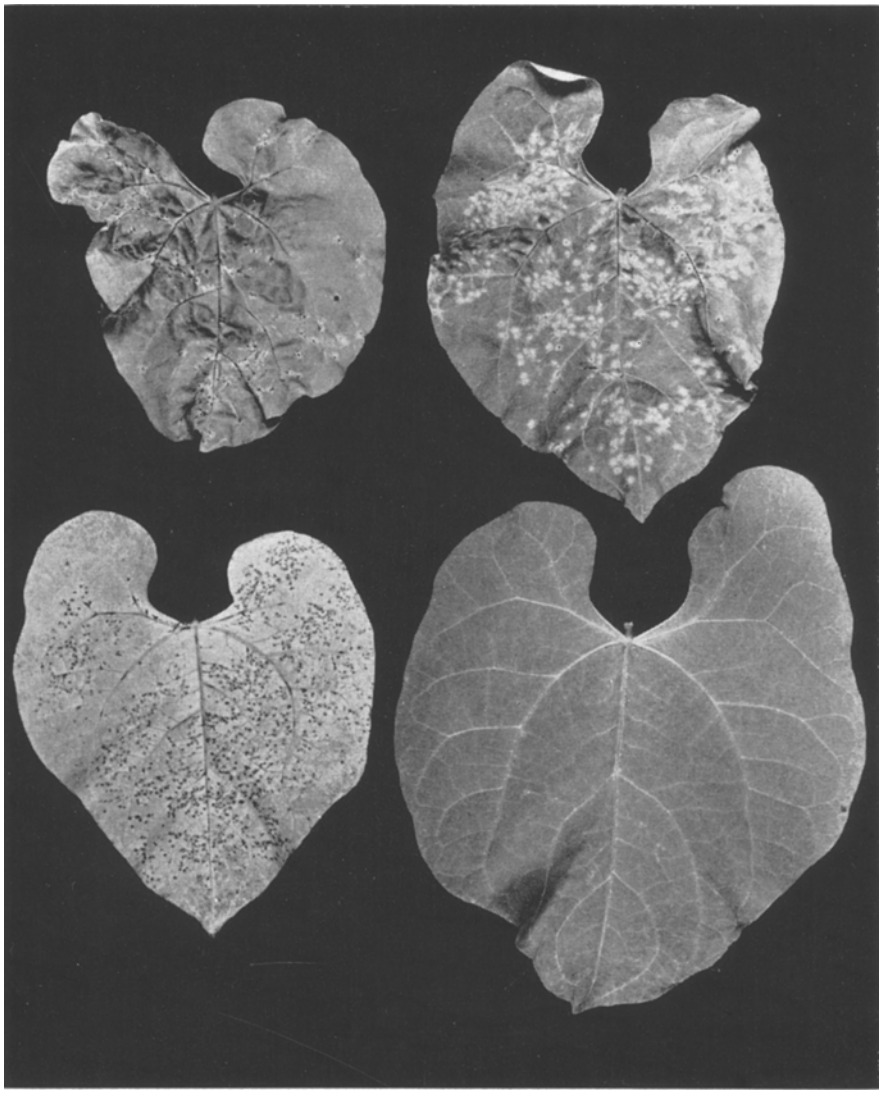

FIG. 2. Local lesions in primary leaves of French bean *(Phaseolus vulgaris)*, cv. 'Beka', inoculated with the Dutch isolate 10 days after sowing (upper row) and 16 days after sowing (lower left). Lower right: non-inoculated leaf. Photograph made 18 days after inoculation.
Lokale lesies in de primaire bladeren van het boneras 'Beka' (Phaseolus vulgaris), *geïnoculeerd met de Nederlandse isolatie 10 dagen (bovenste rij) en 16 dagen (onder links) na het zaaien. Onder rechts: niet geïnoculeerd blad. Foto gemaakt 18 dagen na inoculatie.*

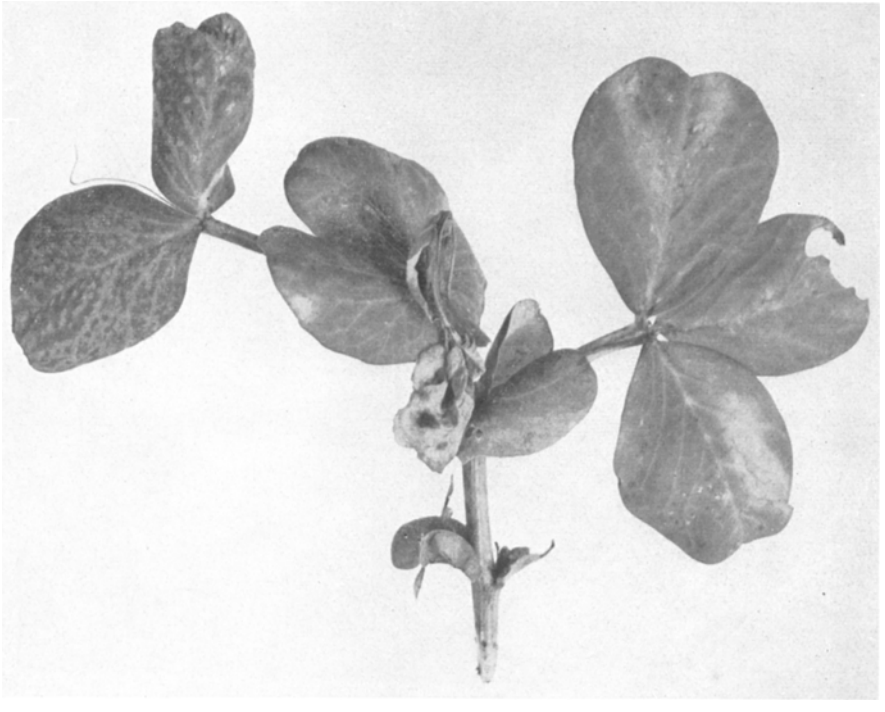

FIG. 3. Pea plant *(Pisum sativum)*, cv. 'Koroza', 21 days after inoculation with the Dutch isolate.
Erwteplant (Pisum sativum), *cv.* '*Koroza*', *21 dagen na inoculatie met de Nederlandse isolatie.*

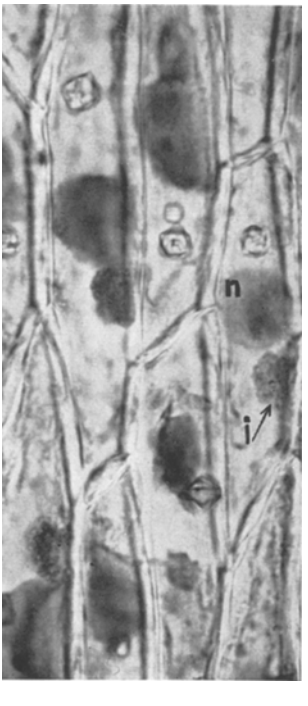

FIG. 4. Granular inclusion bodies (i) in stem epidermis cells of pea caused by the Dutch isolate, after staining with phloxine. The transparent crystals disappeared after treatment with hydrochloric acid. n = nucleus. (Preparation made by Dr. M. RUBIO HUERTOS.)
Granulaire insluitsels (i) *in stengelepidermiscellen van erwt, veroorzaakt door de Nederlandse isolatie, na kleuring met floxine. De doorzichtige kristallen verdwenen na behandeling met zoutzuur.* n = *nucleus. (Preparaat gemaakt door Dr. M. RUBIO HUERTOS.)*

FIG. 5. Severe necrosis in broad bean plants *(Vicia faba)*, cv. 'Driemaal Wit', rapidly proceeding downwards and photographed 13 days after inoculation with the Dutch isolate. Left: healthy control.
Ernstige, zich snel benedenwaarts voortzettende necrose in tuinboneplanten (Vicia faba), *cv.* '*Driemaal Wit*', *gefotografeerd 13 dagen na inoculatie met de Nederlandse isolatie. Links gezonde controle.*

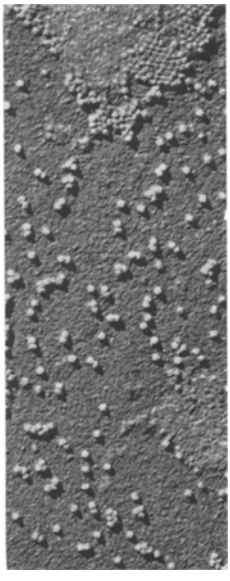

FIG. 6. Electron micrograph of a purified suspension of the Dutch isolate; magnification × 37,000. Preparation made by Dr. H. O. AGRAWAL; photograph made by the Service Institute for Applied Mechanics and Technical Physics in Agriculture, Wageningen.
Elektronenmicroscopische foto van een gezuiverde suspensie van de Nederlandse isolatie; vergroting 37.000 ×. Preparaat Dr. H. O. AGRAWAL; foto Technische en Fysische Dienst voor de Landbouw, Wageningen.

The intramuscular injection of rabbits with purified virus suspension emulsified with FREUND's incomplete adjuvant was followed four weeks later by one intravenous injection. Ten days later titres were found to be over 2048 and the rabbits were bled.

Serological tests were performed by the micro-precipitin test under paraffin-oil according to VAN SLOGTEREN (1955) and results were read after three hours at room temperature.

RESULTS

Host range and symptoms

Trifolium pratense: Plants of the red clover clone Kyc 71–8, obtained from Dr. S. DIACHUN, Lexington, Ky, U.S.A., were very susceptible to both isolates and especially sensitive to the English isolate (Fig. 1, left). About two weeks after inoculation both isolates caused yellow lesions, those of the English isolate having a necrotic centre. Later on in young leaves systemically infected with the Dutch isolate a bright mosaic was produced, whereas the dark green flecks were surrounded by clear yellow lines or irregularly shaped rings. In contrast, the English isolate caused a bright yellow spotting connected with the venation; this often led to severe curling and distortion of leaflets, especially when the spots had necrotic centres.

In a Dutch variety (indigenous breed), however, fewer plants were infected and the symptoms varied greatly presumably due to heterozygosity of the variety, and they could hardly be used for comparative studies (Fig. 1, right). The Dutch isolate often caused a well-defined mosaic, whereas the English isolate showed a tendency to incite chlorotic rings similar to those pictured by SINHA.

Medicago lupulina: In black medic plants symptoms caused by the English isolate were more severe and were produced earlier than those of the Dutch isolate. They consisted of a chlorosis, especially of the veins with the first, and of a diffuse mosaic or mottling with the latter isolate.

Phaseolus vulgaris: In French bean plants, cv. 'Beka', both isolates induced numerous local lesions in the primary leaves approximately four days after inoculation. The size and nature of these lesions depended on the age of the primary leaves at the time of inoculation (Fig. 2). In young leaves the lesions usually were chlorotic. When these had a necrotic centre and veins showed some necrosis, the leaf was slightly deformed. In old leaves many tiny necrotic local lesions occurred and were very conspicuous when the leaves became yellow. No systemic symptoms could be observed, nor could virus be isolated from non-inoculated trifoliate leaves. Similar reactions were observed in the varieties 'Bountiful', 'Citroengele', 'Pinto', 'Topcrop' and 'Walcherse soepboon'. Occasionally a faint systemic mottle occurred in the cv. 'Bataaf' and then virus could be recovered from non-inoculated leaves.

Pisum sativum: All 14 pea varieties (a.o. 'Dippes Foli', 'Eroica', 'Juwel', 'Kelvedon Wonder', 'Koroza', 'Mansholts Pluk', 'Perfected Wales', and 'Wisconsin Perfection') were very susceptible. Both virus isolates caused systemic symptoms of a slight chlorosis of the smaller veins and of some adjacent tissue, followed by internal necrosis of stems and petioles, often leading to top necrosis and distortion. In older leaves yellowing or greenish blotching with a characteristic desiccation or bleaching of tissue along part of the venation

occurred (Fig. 3). These plants generally showed a severe stunting and were often subject to wilting and rotting. In most pea varieties the Dutch isolate gave a more severe necrotic reaction than did the English isolate. Pea plants infected with the Dutch isolate were checked for the presence of inclusion bodies by staining with phloxine. Granular bodies were found located close to the nucleus of stem epidermis cells (Fig. 4).

Vicia faba: In the broad bean variety 'Driemaal Wit' both virus isolates occasionally induced local lesions that were diffuse chlorotic and sometimes necrotic rings of about 3 mm in diameter. About ten days after inoculation systemic symptoms consisted of a severe necrosis, starting as a blueish blackening of top leaves, which rapidly proceeded downwards, killing the plants in a few days (Fig. 5). This necrosis was most severe and rapid with the Dutch isolate.

Vigna unguiculata (= *V. sinensis):* In the cowpea variety 'Giant Black Eye' both isolates induced in about a week numerous etchy ring-like local lesions of about 2–3 mm diameter. The varieties 'Early Red', 'Brabham K 892', 'Brabham Victor', and 'Victor K 798' reacted either with chlorotic or with necrotic local lesions. In no case could virus be recovered by back-inoculation from trifoliate leaves.

Only two non-leguminous plants were found to be susceptible. *Chenopodium amaranticolor* reacted to the Dutch isolate only and showed many whitish pinpoint local lesions. In *Gomphrena globosa* ring-like local lesions were produced in leaves only when these were turning pale while ageing. These lesions were largest with the English isolate. With both isolates virus could be recovered from inoculated *Gomphrena* leaves. No symptoms were observed, nor could virus be isolated by back-inoculation from *Cucumis sativus* (cv. 'Gele Tros'), *Lupinus polyphyllus, Medicago sativa* (cv. 'Du Puits'), *Nicotiana glutinosa, N. rustica, N. tabacum* (cv. 'White Burley'), and *Petunia hybrida.*

Properties in vitro

The isolates have not been compared in detail as far as the persistance of their infectivity in expressed sap is concerned. Both isolates still produced a clear local reaction on 'Beka' bean leaves after 19 days of storage in pea sap at room temperature. After 28 days of storage the British isolate still produced systemic symptoms in pea plants. Broad bean sap containing the Dutch isolate was still infectious to broad bean after 27 days of ageing. After 49 days of storage in pea sap both isolates had lost their infectivity. According to SINHA (1960) the virus lost its infectivity after six days already.

Electron micrographs of purified plant sap (Fig. 6) showed that the particles of the Dutch isolate were polyhedral, approximately 30 mμ in diameter and thus similar to those of the English isolate as published by SINHA.

A serological relationship between both isolates could readily be demonstrated by means of the antiserum against the English isolate (titre 160) prepared by SINHA and our first antiserum against the Dutch isolate (titre 256).

To investigate whether antigenic differences would occur between the two isolates, cross-reaction and cross-absorption tests were performed with antisera of high titre. The results are listed in Table 1. These tests were performed twice: once with clarified sap of infected broad bean plants, and once with purified virus suspensions using the same purification method as applied for the produc-

tion of the antisera. In the cross-absorption tests using the clarified sap as a test antigen the antisera were restored to their original volumes by means of alcohol precipitation (VAN DER VEKEN, 1955) after absorption with the heterologous viruses.

When testing the antisera against clarified preparations lower titres were found than when purified virus suspensions were used. This may be due to the turbidity still left in the clarified sap, impeding the observation of minute amounts of precipitate. Controls with healthy sap and with normal serum yielded negative reactions. They are therefore omitted from the table.

TABLE 1. Antiserum titres obtained in serological cross-reaction and cross-absorption tests with the Dutch and the English isolate of the red clover mottle virus.
Antiserumtiters, verkregen in serologische kruisreactie- en kruisverzadigingsproeven met de Nederlandse en de Engelse isolatie van het vlekkerigheidsvirus van rode klaver.

Cross-reaction tests
Kruisreactieproeven

Antigen	Antiserum	
	Dutch isolate	English isolate
Dutch isolate clarified/*geklaard*	2048	1024
English isolate clarified/*geklaard*	1024	2048
Dutch isolate purified/*gezuiverd*	4096	1024
English isolate purified/*gezuiverd*	2048	2048

Cross-absorption tests
Kruisverzadigingsproeven

	Antiserum	
	Dutch isolate absorbed/*verzadigd*/ with the English isolate	English isolate absorbed/*verzadigd*/ with the Dutch isolate
Dutch isolate clarified/*geklaard*	32	—
English isolate clarified/*geklaard*	—	32
Dutch isolate purified/*gezuiverd*	128	—
English isolate purified/*gezuiverd*	—	128

DISCUSSION

Although the Dutch virus isolate is closely related to the red clover mottle virus in England, there are clear differences in symptomatology, especially in broad bean, pea and red clover plants. The assumption that in this country we are dealing with a separate strain is especially justified, however, by differences as observed in our serological tests. The results obtained in cross-absorption tests point to the fact that these differences are qualitative.

In spite of the fact that mottling is not a constant and characteristic part of the syndrome, partly due to considerable genetic variation of the host, the name red clover mottle virus, as introduced by SINHA (1960), has the advantage that it is distinctive from those of other legume viruses described so far. The Dutch

equivalent proposed here is "vlekkerigheidsvirus van rode klaver". Biologically the virus is easily distinguished from other legume viruses by its characteristic top necrosis proceeding downwards in broad bean, by its reaction in pea and red clover plants, and by its inability to infect cucumber and tobacco species.

Of special interest is the distant serological relationship between the Dutch strain of the red clover mottle virus and the cowpea mosaic virus (the severe strain from Surinam and Trinidad) as found by AGRAWAL & MAAT (1964). This suggests a possible beetle-transmissibility and sheds new light on the inability of SINHA to transmit the clover virus with a series of aphids. Our preliminary data on size and morphology of the red clover mottle virus resemble those of the cowpea mosaic virus as published by AGRAWAL (1964). That author also found granular inclusion bodies in pea plants artificially infected with the cowpea mosaic virus. In host range and symptoms both viruses are quite different, however. We found that the red clover mottle virus does not become systemic in cowpeas, whereas according to AGRAWAL the cowpea mosaic virus does not infect red clover. Similarly we did not succeed in infecting clone Kyc 71–8, which is highly susceptible to the red clover mottle virus, with cowpea mosaic virus.

In extensive surveys for legume viruses in the Netherlands (e.g. VAN DER WANT & BOS, 1958; BOS & VAN DER WANT, 1958), numerous samples of clover and other legumes were tested. So far the red clover mottle virus was isolated only once from red cover. It can be concluded that the virus at present is of no practical and economic importance in this country.

SAMENVATTING

Aangetoond kon worden dat een in 1957 in Nederland uit rode klaver geïsoleerd virus op grond van waardplantreacties (Fig. 1, 2, 3 en 5), elektronenmicroscopie (Fig. 6) en serologie overeenkomt met het in 1960 door SINHA in Engeland beschreven „red clover mottle virus". Hiervoor wordt thans de Nederlandse naam „vlekkerigheidsvirus van rode klaver" ingevoerd. De Nederlandse isolatie moet echter worden beschouwd als een aparte stam van het virus op grond van geconstateerde duidelijke verschillen in hevigheid van de symptomen veroorzaakt in rode klaver (Fig. 1), erwt en tuinboon en vooral op grond van een in kruisreactie- en kruisverzadigingsproeven gevonden kwalitatief serologisch verschil (Tabel 1).

Het virus doet in kunstmatig geïnfecteerde erwteplanten granulaire celinsluitsels ontstaan (fig. 4). Het is gemakkelijk van andere, tot dusver beschreven virussen van vlinderbloemigen te onderscheiden op grond van de in de reeds genoemde plantesoorten opgewekte verschijnselen en het onvermogen om komkommer en tabakssoorten te infecteren. Daar het volgens onderzoek van AGRAWAL & MAAT (1964) een verre verwantschap met het o.a. in Suriname voorkomende mozaïekvirus van *Vigna* („cowpea mosaic virus") vertoont, wordt het vermoedelijk niet door bladluizen, maar door bladhaantjes of aardvlooien overgebracht.

Het virus is op dit moment in Nederland niet van praktische betekenis.

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