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PEA EARLY-BROWNING VIRUS AND TOBACCO RATTLE VIRUS – TWO DIFFERENT, BUT SEROLOGICALLY RELATED VIRUSES[1]

Vroege-verbruiningsvirus van erwt en ratelvirus van tabak, twee verschillende, doch serologisch verwante virussen

BY

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INTRODUCTION

Early browning, a virus disease of peas, was intensively investigated by BOS & VAN DER WANT (1962). They demonstrated a close resemblance between the early-browning virus and tobacco rattle virus, although some distinct differences were noted. Host range, as well as symptoms on many of the hosts, show a great similarity. Both viruses are soil-borne. Their dilution end points, thermal inactivation points and longevities *in vitro* also agree to a considerable extent. The same holds for their morphology, although the particles clearly differ in "normal lengths". In order to examine further the relationship suggested by BOS & VAN DER WANT, the present author made a study of the serological properties of the viruses concerned.

MATERIALS AND METHODS

The isolate of the early-browning virus (EBV) used, was fully comparable to those studied by BOS & VAN DER WANT. The tobacco rattle virus (RV) isolate used was serologically closely related to that used by these authors.

The EBV was propagated in *Pisum sativum* L., var. 'Eroïca', *Nicotiana glutinosa* L. and *Nicotiana rustica* L. The pea plants were harvested one week after inoculation, *N. glutinosa* two weeks after inoculation and *N. rustica* seven to ten days after inoculation. The RV was propagated in *Nicotiana tabacum* L., var. 'White Burley' and *N. rustica*. The former was harvested four and the latter seven to ten days after inoculation.

In all cases the material was ground in a homogenizer (Quisto, Bühler or Waring blendor) in the same quantity (weight/volume) of McIlvaine's phosphate-citric acid buffer solution pH7 (0.18 M), to which was added 0.1% thioglycollic acid, to prevent the formation of brown substances (TOMLINSON et al., 1959). Next the homogenate was pressed through cheesecloth, after which the preparation was treated in different ways. All treatments were performed at about 3 °C.

The first successful attempts to get antisera to the EBV were made in 1960. Sap from infected *N. glutinosa* and *P. sativum* plants, processed in the above-

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mentioned way, was shaken for five minutes with one quarter of its volume of chloroform and centrifuged until clear. This was followed by two cycles of differential centrifugation (90 min. 80,000 g and 20 min. 3,000 g), the pellet from the last high-speed centrifugation being resuspended in about 1/200 of the original sap volume of buffer pH7. Two rabbits were injected with antigen prepared from plants of *N. glutinosa* and one with antigen prepared from plants of *P. sativum*. Three intramuscular injections, given at intervals of one week, and followed three weeks later by one intravenous injection, resulted in antisera with titers of 1/128, ten days after the last injection. An antiserum to the RV was prepared in approximately the same way, *N. tabacum* being used as host plant.

The titers of the antisera obtained were not considered to be satisfactory. In order to get antisera with higher titers, further experiments were carried out in 1962. Both viruses were propagated in plants of *N. rustica*. The sap was treated with diethyl ether and carbon tetrachloride according to the method of WETTER (1960), slightly modified in that only one fourth of the quantities of ether and carbon tetrachloride were used. The sap was mixed with the solvents in a Waring blendor for one to two minutes and centrifuged for 20 minutes at 3,000 g. In this way the use of separation funnels could be avoided. The procedure was followed by four cycles of differential centrifugation (60 min. 80,000 g and 20 min. 3,000 g). After the pellet of the last high-speed centrifugation was resuspended in 1/200 of the original sap volume of buffer pH7 and centrifuged clear, the antigen preparations obtained had precipitation end points of 1/1024 or higher. For injection, virus suspensions with precipitation end points of 1/1024 were used. One rabbit was injected with each virus. To the "RV-rabbit" two intramuscular injections were given simultaneously, using Freund's incomplete adjuvant. For each injection 2 ml of the virus suspension was used. Four weeks later three intravenous injections of 1 ml each were given at intervals of ten days, only the first of which gave a noticeable rise of the antibody titer. Two weeks after the last intravenous injection two further intramuscular injections, equal to the first ones, were given. Three weeks later the antiserum reached its maximum titer. The "EBV-rabbit" was given two intramuscular injections simultaneously, in the same way as the "RV-rabbit". Four weeks later a subcutaneous injection of one ml without adjuvant was given. This was followed two weeks later bij two intramuscular injections, equal to the first ones. Four weeks after these injections the antiserum reached its maximum titer.

All serological experiments were carried out using the microprecipitin test under paraffin oil (VAN SLOGTEREN, 1955). Tests were performed with antigens prepared in the same way as those used in preparing the respective antisera.

RESULTS

Low titer antisera

The first experiments were carried out with the low titer antisera. The antisera were absorbed with sap of healthy plants. Normal serum and sap of healthy plants, treated in the same way as the antigen, were used as control. Because of spontaneous precipitation, results for the RV antiserum were read after 45 min. and for the EBV antisera after 2 hours at room temperature. In all cross-reaction experiments the EBV antisera showed the same reaction. The results are summarized in table 1. They give no support to the idea that the two

TABLE 1. Results of cross-reaction experiments with absorbed EBV and RV antisera of low titers. Tests with the EBV antisera were read after two hours at room temperature. As non-specific precipitation occurred, the tests with the RV antiserum were read after 45 minutes at room temperature.
Overzicht van de kruisreacties met antisera tegen EBV en RV met lage titer. De proeven met de EBV-antisera werden afgelezen na twee uur staan bij kamertemperatuur. De proef met het RV-antiserum werd, in verband met het optreden van spontane vlokking, reeds na 45 minuten afgelezen.

Antigen dilutions (reciprocals)	EBV antiserum dilutions (reciprocals)					Normal serum
	2	8	32	128	512	
EBV 2	+	+	+	+	—	—
RV	—	—	—	—	—	—
Healthy	—	—	—	—	—	—
	RV antiserum dilutions (reciprocals)					
	2	8	32	128	512	
RV 8	+	+	+	+	—	—
EBV	—	—	—	—	—	—
Healthy	—	—	—	—	—	—

+ Positive reaction
— No reaction

viruses are related. The results given in the tables are mainly those from a series of two-fold antigen dilutions, with which the highest antiserum titers were found. When no special dilution is mentioned, none of the dilutions used reacted.

High titer antisera

In the first cross-reaction experiments the high titer antisera were not absorbed. Potato virus X (PVX), also propagated in *N. rustica* and purified in exactly the same way as the EBV and RV, was used as a control. Normal serum was also added as a control. Results were read after $1\frac{1}{2}$ hours at 37 °C followed by $3\frac{1}{2}$ hours at room temperature. They are summarized in table 2. The antisera not only reacted with the homologous and heterologous virus isolates but also with the control. However, this was to a lower extent and with a non-specific type of precipitate, probably due to reaction of normal plant proteins, which were not removed by the purification method used, with their antibodies. The test was repeated with the antisera, absorbed with equal volumes of the purified PVX suspension. The results are given in table 3. EBV and RV still reacted with each other's antisera, but there was no reaction with the control. In this test, normal serum was not included, because it was known from former tests that it would not give a precipitate.

DISCUSSION

BERCKS (1960) found, in his serological experiments with strains of *Phaseolus* virus 2, reciprocal heterologous antiserum titers which were 1/62 and 1/75 of

TABLE 2. Cross-reaction experiments with the unabsorbed EBV and RV antisera of high titers. Tests read after 1½ hours ar 37 °C followed by 3½ hours at room temperature.
Kruisreacties met de onverzadigde EBV- en RV-antisera met hoge titer. Afgelezen na 1½ uur staan bij 37 °C, gevolgd door 3½ uur bij kamertemperatuur.

Antigen dilutions (reciprocals)	RV antiserum dilutions (reciprocals)													Normal serum
	1	2	4	8	16	32	64	128	256	512	1024	2048	4096	8192	
RV 32	+	+	+	+	+	+	+	+	+	+	+	+	+	±	—
RV 64	+	+	+	+	+	+	+	+	+	+	+	+	+	±	—
EBV 32	+	+	+	+	+	±	—	—	—						—
EBV 64	—	—	—	—	±	+	—	—	—						—
PVX 32	×	—	—	—	—	—	—								—
PVX 64	×	—	—	—	—	—	—								—
	EBV antiserum dilutions (reciprocals)														
	1	2	4	8	16	32	64	128	256	512	1024	2048	4096	8192	
EBV 8	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
EBV 16	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
RV 8	—	+	+	+	+	—	—	—	—						—
RV 16	—	—	—	+	+	±	—	—	—						—
PVX 64	×	×	×	×	—	—	—								—
PVX 128	×	×	×	×	—	—	—								—

+ Positive reaction
± Weak reaction
— No reaction
× Non-specific precipitate

TABLE 3. Cross-reaction experiments with the EBV and RV antisera of high titers. Antisera absorbed with X-virus. Readings after 1½ hours at 37 °C followed by 3½ hours at room temperature.
Kruisreacties met de verzadigde EBV- en RV-antisera met hoge titer. Afgelezen na 1½ uur staan bij 37 °C, gevolgd door 3½ uur bij kamertemperatuur.

Antigen dilutions (reciprocals)	RV antiserum dilutions (reciprocals)												
	2	4	8	16	32	64	128	256	512	1024	2048	4096	8192
RV 16	+	+	+	+	+	+	+	+	+	+	+	+	+
RV 32	+	+	+	+	+	+	+	+	+	+	+	+	+
EBV 16	+	+	+	+	—	—	—	—	—				
EBV 32	±	+	+	+	+	—	—	—	—				
PVX	—	—	—	—	—	—	—						
	EBV antiserum dilutions (reciprocals)												
	2	4	8	16	32	64	128	256	512	1024	2048	4096	8192
EBV 8	+	+	+	+	+	+	+	+	+	+	+	—	—
EBV 16	+	+	+	+	+	+	+	+	+	+	+	—	—
RV 8	+	+	+	+	—	—	—	—	—				
RV 16	+	+	+	+	—	—	—	—	—				
PVX	—	—	—	—	—	—	—						

+ Positive reaction
— No reaction

the homologous ones. For this reason it is not possible to conclude from the results of the experiments with the low titer antisera, that EBV and RV are different viruses. Indeed, table 1 shows that the reciprocal heterologous titers are less than 1/64 of the homologous ones. In view of BERCKS's results, this is no reason to reject a relationship between the two viruses. To get a better view of the problem, a test with high titer antisera was necessary. The results of this test, shown in table 2, do not give absolute certainty concerning a possible relationship, as there is still a weak reaction with the control. By absorbing the antisera a reaction with normal plant proteins as well as with PVX was excluded. The EBV and RV isolates were also checked for a contamination with tobacco mosaic virus and PVX, as these viruses are very infectious. There was no such contamination. No other viruses were propagated in their neighbourhood, nor were unspecific symptoms detected on *N. rustica*. Finally the EBV and RV isolates were checked on *P. sativum*, var. 'Eroïca' and *Phaseolus vulgaris* L., var. 'Beka', to exclude possible contamination of one with the other. No contamination was observed. Therefore the results, summarized in table 3, undoubtedly show that EBV and RV are related. It can also be deduced from this table that the reciprocal heterologous titers are 1/256 and 1/128 of the homologous ones.

Serology alone cannot give the answer to the question whether EBV and RV are related viruses or strains of one virus. BOS & VAN DER WANT (1962) mention as "normal lengths" of EBV particles 105 and 210 mμ, and of RV particles 70 and 180 mμ. This not only means a difference in "normal lengths" but also a difference in the ratio between short and long particles. This would indicate that two different viruses are involved. However, taking their serological relationship also into consideration, it is suggested that they be classed as two distinct viruses belonging to one group. Such a relationship was earlier found for a number of other elongated plant viruses, among others by WETTER & QUANTZ (1958), BERCKS (1961), BERCKS & BRANDES (1961) and WETTER, QUANTZ & BRANDES (1962). These authors found a distant serological relationship between viruses of the same shape, but differing in "normal lengths". A survey on this matter is given by BRANDES (1961). The possibility of classifying viruses on the basis of particle morphology is discussed by BRANDES & WETTER (1959). The proposal to regard EBV and RV as separate viruses within one group would correspond closely with what BERCKS & BRANDES (1961) proposed for the white-clover mosaic virus, potato virus X and hydrangea ring spot virus.

SUMMARY

Early-browning virus (EBV) and tobacco rattle virus (RV) show similarities in many of their properties, although there are also some distinct differences (BOS & VAN DER WANT, 1962). Serological experiments carried out by the present author showed that EBV and RV are distantly related. Because of the difference in the "normal lengths" of their particles they have to be considered as separate viruses. It is suggested that they be regarded as members of one group.

SAMENVATTING

Vroege verbruining, een virusziekte bij erwten, is uitvoerig onderzocht door BOS & VAN DER WANT (1962). Uit hun onderzoek is gebleken dat de verwekker van deze ziekte, het vroege-verbruiningsvirus (EBV), veel overeenkomst vertoont met het ratelvirus van tabak (RV). Beide virussen hebben dezelfde deeltjesvorm, maar ze verschillen in lengte (EBV 105 en 210 mμ en RV 70 en 180 mμ). Beide gaan met de grond over en komen overeen wat betreft hun eigenschappen *in vitro*. Ook wat hun uitwerking op waardplanten betreft lijken ze, met uitzondering van enkele karakteristieke verschillen, veel op elkaar. Het duidelijke verschil in deeltjeslengte geeft aan, dat er verschillende virussen in het spel zijn. Het leek echter niet uitgesloten, dat ze toch in zekere mate verwant zouden zijn. Om deze mogelijkheid na te gaan werd uitvoerig serologisch onderzoek verricht. Met behulp van antisera met titers van ca. 1/128 kon geen verwantschap worden aangetoond (tabel 1). Daarom werden sterkere antisera bereid. Hiertoe werden EBV en RV vermeerderd op *Nicotiana rustica* L. en gezuiverd volgens een enigszins gewijzigde methode van WETTER (1960).

Door het toepassen van een combinatie van intraveneuze en intramusculaire injecties werden zeer sterke antisera verkregen. Met behulp daarvan werden beide virussen opnieuw getoetst. Als controle diende aardappel-X-virus (PVX), dat op dezelfde wijze was vermeerderd en gezuiverd als het EBV en het RV. De resultaten van deze proef zijn vermeld in tabel 2. Daar er nog een zwakke reactie met de controle optrad, werden de antisera verzadigd en werd de proef herhaald. De resultaten hiervan zijn weergegeven in tabel 3. Met de controle trad toen geen reactie meer op, terwijl EBV en RV nog duidelijk met elkaars antisera reageerden. Daar zowel een wederzijdse besmetting van EBV en RV als een gemeenschappelijke besmetting met een ander virus was uitgesloten, kunnen we concluderen, dat EBV en RV verwante virussen zijn. Naar analogie van wat door Duitse onderzoekers voor andere virussen is gevonden, kunnen we ook hier spreken van afzonderlijke virussen, die waarschijnlijk in eenzelfde groep ondergebracht kunnen worden.

In het algemeen zijn in de tabellen slechts die antigeenverdunningen weergegeven, waarbij een maximale antiserumtiter werd gevonden. Wanneer geen bepaalde verdunning is aangegeven, betekent dit dat geen enkele verdunning reageerde.

REFERENCES

BERCKS, R., – 1960. Serologische Untersuchungen zur Differenzierung von Isolaten des Phaseolus-Virus 2 und ihrer Verwandtschaft mit Phaseolus-Virus 1. Phytopath. Z. 39: 120–128.

BERCKS, R., – 1961. Serologische Verwandtschaft zwischen Kartoffel-Y-Virus, Rübenmosaik-Virus und Phaseolus-Virus 2. Phytopath. Z. 40: 357–365.

BERCKS, R. & J. BRANDES, – 1961. Vergleichende serologische und elektronenmikroskopische Untersuchung des Weiszkleemosaik-Virus, des Hydrangea ringspot virus und des Kartoffel-X-Virus. Phytopath. Z. 42: 45–56.

BOS, L. & J. P. H. VAN DER WANT, – 1962. Early browning of pea, a disease caused by a soil- and seed-borne virus. Tijdschr. PlZiekt. 68: 368–390.

BRANDES, J., – 1961. Einige Bemerkungen über den Nachweis von Kartoffelviren mit Hilfe des Elektronenmikroskops. Proc. 4th Conf. Pot. Virus Dis., Braunschweig, 1960: 170–175.

BRANDES, J. & C. WETTER, – 1959. Classification of elongated plant viruses on the basis of particle morphology. Virology 8: 99–115.

SLOGTEREN, D. H. M. VAN, – 1955. Serological micro-reactions with plant viruses under paraffin oil. Proc. 2nd Conf. Pot. Virus Dis., Wageningen-Lisse, 1954: 51–54.

TOMLINSON, J. A., R. J. SHEPHERD & J. C. WALKER, – 1959. Purification, properties and serology of cucumber mosaic virus. Phytopathology 49: 293–299.
WETTER, C., – 1960. Partielle Reinigung einiger gestreckter Pflanzenviren und ihre Verwendung als Antigene bei der Immunisierung mittels Freundschem Adjuvans. Arch. Mikrobiol. 37: 278–292.
WETTER, C. & L. QUANTZ, – 1958. Serologische Verwandtschaft zwischen Steinkleevirus, Stauchevirus der Erbse und Wisconsin pea streak-Virus. Phytopath. Z. 33: 430–432.
WETTER, C., L. QUANTZ & J. BRANDES, – 1962. Vergleichende Untersuchungen über das Rotkleeadernmosaik-Virus und das Erbsenstrichel-Virus. Phytopath. Z. 44: 151–169.