Neth. J. Plant Path. 71 (1965): 47–53

SEROLOGICAL DIFFERENCES BETWEEN RED CURRANT SPOON LEAF VIRUS, VIRUS ISOLATES FROM ECKELRADE-DISEASED CHERRY TREES AND THE SCOTTISH RASPBERRY RINGSPOT VIRUS[1]

Serologische verschillen tussen het lepelbladvirus van rode bes, virusisolaties uit kersebomen met Eckelraderziekte en het Schotse „raspberry ringspot"-virus

D. Z. MAAT

Institute of Phytopathological Research (I.P.O.), Wageningen

Antisera were made to red currant spoon leaf virus (SLV), an isolate of the Scottish raspberry ringspot virus (RRV), and two virus isolates from Eckelrade-diseased cherry trees (EV). Different virus isolates, including one from Belgium, were tested against these antisera. The results indicate that we are dealing with a group of virus isolates with different antigenic properties. SLV is very closely related to RRV, being undoubtedly a strain of this virus. Dutch EV isolates differ from SLV and RRV and from each other, the Belgian isolate being closely related to one of the Dutch EV isolates. The serological differences found do not correspond with the geographical distances between the localities where the viruses were collected.

INTRODUCTION

HARRISON (1961) mentioned phytopathological differences between red currant spoon leaf virus (SLV) and an isolate of the Scottish raspberry ringspot virus (RRV). MAAT, VAN DER MEER & PFAELTZER (1962) working on the serological relationship between SLV, a virus (EV) consistently isolated from Eckelrade-diseased cherry trees, and RRV, found that four EV isolates differed clearly from SLV and from RRV, in serological and/or cross-protection tests.

In connection with investigations into the phytopathological properties of SLV (VAN DER MEER, 1965) we carried out some experiments for further characterization of the above-mentioned viruses, particularly of SLV. By testing more isolates, we attempted to confirm results from former serological work (MAAT *et al.*, 1962), and to find out whether phytopathological differences observed would be paralleled by serological differences.

Besides the SLV, RRV and EV isolates from the Netherlands, a Belgian isolate (ROLAND, 1962) from cherry showing symptoms of Eckelrade disease was included in the serological experiments. Since we met with difficulties in the propagation and purification of some of the virus isolates, special attention is paid to these points.

MATERIALS AND METHODS

Virus propagation, virus purification and preparation of antisera

For the preparation of antisera viruses were cultured in greenhouse-grown *Nicotiana rustica*. The plants were potted at the three-leaf stage. From November to March the plants were given additional light from TL tubes during 4–18 hours a day, depending on the natural light conditions. After inoculation they

[1] Accepted for publication 17 September, 1964

were kept in daylight. From April to October direct sunlight was avoided by means of shading with cheese-cloth and whitewash. The temperature was kept between 18 and 25° C. Plants were inoculated for the first time at the five-leaf stage. After about one week a second inoculation was given on the newly formed leaves. One week later the material was harvested. When using virus isolates producing necrotic symptoms, plants were harvested one week after the first inoculation.

Virus purification was carried out at a temperature of about 3 °C. For preparation of antisera 100 g of leaf material was homogenized in a Waring blendor in 100 ml of McIlvaine's phosphate-citric acid buffer solution pH7 (0.18 M), to which was added 0.1 % thioglycollic acid (TOMLINSON *et al.*, 1959). The homogenate was pressed through cheese-cloth and the sap mixed with one quarter of its volume of chloroform for one to two minutes in a Waring blendor. After centrifugation at 6,000 rev. per min. for 20 min., the watery phase was poured off and centrifuged at high speed (two hours at 80,000 *g*). The pellet was resuspended in about 20 ml phosphate-citric acid buffer solution at pH7, to which was added 0.1 % T73 (BRAKKE, 1959) and the suspension was kept overnight. After this the pH was brought back to 5 with 10 % acetic acid and the preparation centrifuged clear. A second high speed centrifugation was then given and the pellet obtained was resuspended in one ml of buffer, kept overnight and centrifuged clear (20 min. at 6,000 rev. per min.). This material, which had higher infectivity than the starting material, was used for injection. The chloroform-butanol method (STEERE, 1956) was not used, as it was found to destroy infectivity of several EV isolates.

Rabbits were given two intramuscular injections simultaneously, each injection containing 2 ml virus suspension emulsified with 2 ml of Freund's incomplete adjuvant. Four weeks later an intravenous booster dose of 4 ml was given. Ten days later the rabbits were bled. As the antisera reacted with healthy material to a low titer, they were absorbed and the γ-globulin fraction was isolated (VAN DER VEKEN, 1955).

Virus isolates and test methods used

Four SLV isolates were tested from red currant plants with typical SLV symptoms. Two of these plants had been infected via the soil under experimental conditions. The antiserum to SLV was prepared against one of the two isolates from naturally infected plants. Eleven isolates were taken from weeds growing in the neighbourhood of diseased red currant plants. They were: one isolate from *Chenopodium* spec., two from *Stellaria media*, two from *Cardamine flexuosa*, two from *Polygonum persicaria*, two from *Veronica agrestis*, and two from *Euphorbia peplus*. Another isolate was taken from gooseberry (VAN DER MEER, 1960, 1965) and one from spinach. The isolates had their origin in "De Bangert", province of N.Holland (VAN DER MEER, 1965).

As to the Eckelrade disease, eight isolates were tested from sweet cherry and three from sour cherry. The three sour cherry isolates, together with one of those from the sweet cherry, came from one orchard. The isolates all originated from the province of Limburg. Antisera were made against one isolate from sweet cherry and one from sour cherry. Another isolate tested was kindly supplied by Dr. G. ROLAND, Belgium. We also made an antiserum to an isolate of the Scottish RRV, kindly supplied by Dr. C. H. CADMAN.

N. rustica was used as a host for the isolates to be tested. In addition, some of the isolates from weeds were propagated in *Petunia* and tomato. Both crude sap and partially purified preparations were used simultaneously in the serological tests. The partial purification was performed as follows. Leaf material was ground in a mortar, pressed through cheese-cloth and the sap centrifuged for 20 min. at 6,000 rev. per min. To the supernatant, acetone was added up to a final concentration of 40% (by volume). The mixture was centrifuged for 10 min. at 6,000 rev. per min., and the pellet resuspended in buffer at pH7 (one fifth of the original volume of sap). The suspension was centrifuged clear and used in serological tests.

As a test method, the Ouchterlony double diffusion test was used (MOORHEAD BALL, 1961). The wells in the agar were cut with a cork borer, 3 mm wide, the distance between the centres of the antigen and antiserum wells being $\frac{1}{2}\sqrt{2}$ cm. Readings were made after two days at room temperature. In addition, in some instances the micro-precipitin test was performed (VAN SLOGTEREN, 1955), using a series of antigen dilutions. Antiserum dilutions were made twofold.

TABLE 1. Results of serological experiments with Dutch virus isolates connected with the spoon leaf disease of red currant (SLV) and with Eckelrade diseased cherry (EV), and antisera to SLV and EV. The bold figures indicate the titers of the homologous antisera.
Resultaten van serologische proeven met Nederlandse virusisolaties, die verband houden met de lepelbladziekte van rode bes (SLV) en met de Eckelraderziekte van kers (EV) en antisera tegen SLV en EV. De vetgedrukte getallen geven de titers van de homologe antisera weer.

Isolates	Number of isolates	Titers of antisera		
		Antiserum to SLV	Antiserum to EV Sour cherry	Antiserum to EV Sweet cherry
SLV red currant	1	**512**	128	64
SLV red currant	3	512		
Gooseberry	1	512		
Spinach	1	512		
Chenopodium spec.	1	512		
Stellaria media	2	512		
Cardamine flexuosa	2	512		
Polygonum persicaria	2	512		
Veronica agrestis	2	512		
Euphorbia peplus	2	512		
EV sweet cherry	1	128	256	**512**
EV sweet cherry	2	128		
EV sweet cherry	1	128		256
EV sweet cherry	2			128
EV sweet cherry	1	256		
EV sweet cherry	1[1]	256	256	
EV sour cherry	1[1]	256	**512**	128
EV sour cherry	1[1]	256	512	
EV sour cherry	1[1]	256	256	

[1] Virus isolates originating from one orchard

All the isolates mentioned except two from sweet cherry were tested against the SLV antiserum. In case the heterologous antiserum titer differed from the homologous one, tests were repeated twice with freshly prepared virus suspensions. Sometimes micro-precipitin tests were carried out with partially purified material in two-fold dilutions. Only some of the isolates were tested against the RRV and EV antisera, each test being repeated twice.

The results of the different test methods used and of the replications agreed very well. The homologous antiserum titers remained constant and the heterologous ones did in most cases. Very rarely were differences of one step in the heterologous antiserum titer observed. In those instances the highest titers are mentioned, the figures in the tables thus representing the closest relationships found between the homologous and the heterologous virus isolates.

Table 1 gives the results of tests performed with the Dutch isolates. Table 2 summarizes the results of cross-reaction experiments with the antisera to SLV, RRV and to EV from sour and sweet cherry and these virus isolates and the Belgian isolate. As the controls with normal serum and with sap of healthy plants were negative, these are not listed in the tables.

TABLE 2. Results of cross-reaction experiments with antisera to red currant spoon leaf virus (SLV), Scottish raspberry ringspot virus (RRV) and to EV isolates from Eckelrade diseased sweet and sour cherry trees (EV) and all these viruses and a Belgian isolate from cherry with Eckelrade disease. The bold figures indicate the titers of the homologous antisera.
Resultaten van kruisreactieproeven met antisera tegen het lepelbladvirus van rode bes (SLV), het Schotse „raspberry ringspot"-virus (RRV) en virusisolaties uit Eckelraderzieke kers (EV) en de desbetreffende virusisolaties en een Belgische isolatie uit kers met Eckelraderziekte. De vetgedrukte getallen geven de titers van de homologe antisera weer.

Antigen	Titers of antisera			
	Antiserum to SLV	Antiserum to RRV	Antiserum to EV Sour cherry	Antiserum to EV Sweet cherry
SLV	**512**	4096	128	64
RRV	512	**4096**	128	32
EV sour cherry	256	2048	**512**	128
EV sweet cherry	128	512	256	**512**
Belgian isolate	256	1024	256	64

Efforts were made to cross-absorb the antisera. Up to 20 ml of crude sap of plants infected with heterologous virus isolates, were added to one ml of antiserum. However, the antisera thus treated still reacted strongly with the virus isolates used.

According to CARPENTER (1958) the titer of an antiserum is the reciprocal of the last dilution showing definite reaction with the appropriate antigen and in this paper the term is used accordingly.

DISCUSSION AND CONCLUSION

In order to obtain a sufficient virus concentration in the plants during summer, it was necessary to shade the plants rather heavily. In crude sap of unshaded plants it was not possible to detect virus serologically, sometimes not even in purified preparations. Virus was directly detectable in crude sap of *N. rustica* when plants were shaded between April and October, and also in the sap of unshaded plants during winter. ROLAND (1962) mentioned that the Belgian isolate, grown in Gembloux, failed to give proper serological reactions. This must be due to low virus concentrations in the plants grown under Belgian greenhouse conditions: cultured under Dutch greenhouse conditions the isolate reacted just as well as the Dutch ones did[2].

The viruses associated with spoon leaf disease and Eckelrade disease, are soil-borne and serologically related to the Scottish RRV (HARRISON, 1961; MAAT *et al.*, 1962). The isolates from the weeds mentioned on page 48, originating from the same region as the other SLV isolates, all reacted up to the same SLV antiserum dilution as the homologous isolate did. This indicates a close relationship. However, herbaceous host species, infected with these isolates, may show a great diversity in their symptom expression, even under identical growing conditions. Nor did some of the isolates produce typical spoon leaf symptoms when inoculated on red currant seedlings (VAN DER MEER, 1965). This means that although they did not show differences in their serological properties, the isolates nevertheless differed in their phytopathological characteristics.

The EV isolates showed a much greater variety in their serological properties than did the SLV isolates. Even isolates from one orchard could be differentiated, as is evident from Table 1. The EV isolates all differed clearly from SLV isolates and this is in agreement with the fact that SLV isolates and EV isolates could be distinguished in cross-protection tests (VAN DER MEER, 1965). Furthermore, VAN DER MEER (1965) has hitherto not succeeded in obtaining typical SLV symptoms in red currant plants inoculated with EV isolates.

HARRISON (1961) has already mentioned that SLV and RRV are not identical. Table 2 shows that SLV is very closely related to RRV and undoubtedly a strain of this virus. Only the reactions of SLV and RRV with the antiserum to the EV isolate from sweet cherry (Table 2, fourth column) reveal a minor difference. SLV and RRV are more closely related to each other than EV is to either of them. RRV and the EV isolate from sweet cherry are furthest apart. The second column of Table 2 shows that the Belgian isolate is intermediate between the EV isolate from sweet cherry and the one from sour cherry as far as their relationship to RRV is concerned. Arranging the five isolates on a straight line would give the following sequence: RRV and SLV, EV from sour cherry, Belgian isolate, EV from sweet cherry. This sequence, however, is contradicted by the data given in the fourth column; here the Belgian isolate, together with SLV, is intermediate between RRV and EV from sour cherry. To elucidate this contradiction, the isolates have to be arranged in a two or even three dimensional system. This would mean that the differences found between the isolates were the result of an evolution in more than one direction. However, nothing

[2] For more extensive data on the Belgian isolate see TAHON's paper (Parasitica 20 (1964): 107–137) which was published when the present article was in print.

more definite can be deduced from the data obtained by the cross-reaction experiments reported in this paper. Better results might have been acquired by means of cross-absorption tests. However, as the concentrations of the viruses concerned were very low in their hosts, it was not feasible to absorb the antisera completely with the heterologous antigens. Summarizing, the results mentioned in Table 2 indicate that the virus isolates studied compose a serological group in which the RRV and an EV isolate from sweet cherry are furthest apart. The Belgian isolate is closely related to the Dutch EV isolate from sour cherry, and SLV is almost identical with RRV.

The present results do not support the idea of geographical variation as suggested by HARRISON (1958, 1960, 1964) and CADMAN (1960): differences between isolates do not correspond with the geographical distance between the localities where they occur. In view of the results of the cross-protection experiments (VAN DER MEER, 1965) it also seems questionable whether EV is to be regarded as a strain of RRV. However, as long as the criteria for differentiating viruses are not fully defined, final conclusions cannot be drawn.

SAMENVATTING

Antisera werden bereid tegen het lepelbladvirus van rode bes (SLV), een isolatie van het Schotse „raspberry ringspot"-virus (RRV) en twee virusisolaties uit kersebomen met Eckelraderziekte (EV). Met behulp van deze antisera werden verschillende virusisolaties getoetst, waaronder één uit België van kers met Eckelraderziekte. De resultaten zijn vermeld in de tabellen 1 en 2. Ze duiden erop, dat we te maken hebben met een groep van virusisolaties met verschillende antigene eigenschappen. SLV is zeer nauw verwant aan RRV en is ongetwijfeld een stam van dit virus. Nederlandse EV-isolaties verschillen zowel van SLV en RRV, als van elkaar. De Belgische isolatie is nauw verwant aan één van de Nederlandse EV-isolaties. De grootte van de gevonden verschillen correspondeert niet met de geografische afstand tussen de plaatsen waar de virusisolaties werden verzameld.

REFERENCES

BRAKKE, M. K., – 1959. Dispersion of aggregated barley stripe mosaic virus by detergents. Virology 9: 506–521.

CADMAN, C. H., – 1960. Studies on the relationship between soil-borne viruses of the ringspot type occurring in Britain and Continental Europe. Virology 11: 653–664.

CARPENTER, PH. L., – 1958. Immunology and serology. W. B. Saunders Company, Philadelphia and London.

HARRISON, B. D., – 1958. Further studies on raspberry ringspot and tomato black ring, soil-borne viruses that affect raspberry. Ann. appl. Biol. 46: 571–584.

HARRISON, B. D., – 1960. The biology of soil-borne plant viruses. Adv. Virus Res. 7: 131–161.

HARRISON, B. D., – 1961. Identity of red currant spoon leaf virus. T. PlZiekt. 67: 562–565.

HARRISON, B. D., – 1964. Specific nematode vectors for serologically distinctive forms of raspberry ringspot and tomato black ring viruses. Virology 22: 544–550.

MAAT, D. Z., F. A. VAN DER MEER & H. J. PFAELTZER, – 1962. Serological identification of some soil-borne viruses causing diseases in fruit crops in the Netherlands. T. PlZiekt. 68: 120–122.

MEER, F. A. VAN DER, – 1960. Onderzoekingen betreffende bessevirussen in Nederland 1. Lepelblad van rode bes. T. PlZiekt. 66: 12–23.

MEER, F. A. VAN DER, – 1965. Investigations of currant viruses in the Netherlands II. Further observations on spoon leaf virus, a soil-borne virus transmitted by the nematode Longidorus elongatus. Neth. J. Plant Path. 71: 33–46.

MOORHEAD BALL, E., – 1961. Serological tests for the identification of plant viruses. Published by The American Phytopathological Society, 1961.
ROLAND, G., – 1962. Étude d'un virus isolé mécaniquement du cerisier. Parasitica 18: 264–285.
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.
STEERE, R. L., – 1956. Purification and properties of tobacco ringspot virus. Phytopathology 46: 60–69.
TOMLINSON, J. A., R. J. SHEPHERD & J. C. WALKER, – 1959. Purification, properties and serology of cucumber mosaic virus. Phytopathology 49: 293–299.
VEKEN, J. A. VAN DER, – 1955. Isolation and preservation of a fraction containing antibodies against plant viruses from the mixture obtained after absorption of antisera by healthy plant extracts. Proc. 2nd Conf. Pot. Virus Dis., Wageningen, Lisse 1954: 40–42.