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VIRUSES OF THE TOBACCO RATTLE VIRUS GROUP IN NORTHERN ITALY: THEIR VECTORS AND SEROLOGICAL RELATIONSHIPS[1]

Het voorkomen van ratelvirus in Noord-Italië, zijn overbrengers en zijn serologische verwantschap met reeds bekende virussen van de ratelvirusgroep

H. A. VAN HOOF, D. Z. MAAT and J. W. SEINHORST
Institute of Phytopathological Research (I.P.O.), Wageningen

Four species of *Trichodorus* have been found in northern Italy, viz. *Trichodorus viruliferus*, *T. teres*, *T. nanus* and an undescribed species. In seven cases tobacco rattle virus (TRV) was isolated from soil samples by means of bait seedlings and in three of these cases the vector was found to be *T. viruliferus*. The seven virus isolates reacted with an antiserum against a Dutch TRV isolate and were divided into two groups according to their reaction with this serum. One isolate from each group was subjected to a more detailed examination. Of these, one was found to be closely related to a Dutch isolate from gladiolus with notched leaf symptoms, while the other showed a distinct relationship to the pea early-browning virus found in Britain. One of the isolates proved to be more closely related to the Dutch TRV than is the Dutch gladiolus isolate.

INTRODUCTION

The first report of the occurrence of tobacco rattle virus in Italy was made by GIGANTE in 1963, who noticed spraing in potato tubers of the 'Majestic' and 'Sirtema' varieties. He succeeded in isolating TRV from the tops of plants cultivated from these tubers. As the spraing-infected tubers were from a mixed batch at the central pota o store at Brunico the field of origin could no longer be traced. The occurrence of possible TRV vectors was not investigated. It would seem from the description of the tuber symptoms that the infection must have taken place in the field and could not have come from the seed piece (VAN HOOF, 1964a).

LOVISOLO (1963) identified TRV in *Ranunculus asiaticus* plants of which the tubers had been imported from the Netherlands. In his view neither *Trichodorus* spec. nor TRV occurred in Italy, so that TRV must have been introduced by the *Ranunculus* seed stock.

Little is known to date on the distribution of *Trichodorus* species in Italy. Specimens found have only been identified to genus (RASKI & AMICI, 1964) and there is no information on the relationship between TRV and *Trichodorus* spec.

During March and April 1965 the first author studied the occurrence of *Trichodorus* and viruses of the TRV group in Italy[2]. The symptoms of a number of TRV isolates were also studied in several host plants. The two co-authors undertook the serological study and the identification of the nematodes respectively.

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MATERIALS AND METHODS

At 120 locations in northern Italy (cf. Fig. 1) a soil sample was taken by removing 2 to 3 kg of soil, preferably cultivated soil, with a shovel to a depth of 30 cm. Nematodes were collected from 500 g of each sample by the "two Erlenmeyer method" (SEINHORST, 1955). The nematodes were fixed as described by SEINHORST (1962). Twenty-nine soil samples, which contained various specimens of *Trichodorus*, were taken back to the Netherlands where plastic pots were filled with about 1 kg of this soil in the greenhouse and three young tobacco plants of the 'White Burley' variety planted in the soil. A month later the roots of these plantlets were washed clean and then macerated in a mortar. The sap was expressed and inoculated on to leaves of 'White Burley' tobacco with the aid of Carborundum. The viruses thus isolated were identified by studying their reactions to different test plants and establishing their serological affinities. For the latter study use was made of isolates of the Dutch tobacco rattle virus (TRV-Lisse) and the Dutch pea early-browning virus (VVV), these being identical with the isolates described by BOS & VAN DER WANT (1962), as well as an isolate (L 20) from gladiolus with notched leaf symptoms (CREMER, in press). The same or very closely related isolates were used for the preparation of antisera against these viruses.

The antiserum against the British strain of the pea early-browning virus (PEBV, isolate Swaffham P5) was supplied in 1963 by Dr. A. J. GIBBS of Rothamsted. The specimen used in our experiments consisted of a combination of dilutions; as a result the titre of this serum is unknown, but it must have been very low.

For purifying the antigens for the tests use was made of the method described by MAAT (1963) but in a modified form. The modification consisted in the application of one or two differential centrifugations, while the time taken for sedimenting in the ultracentrifuge in the second cycle was increased to $1\frac{1}{2}$ hours.

The serological tests were in accordance with the Ouchterlony double diffusion test with 1 % agar (Difco-Bacto, purified) in a physiologic salt solution buffered to a pH of 7. In one instance both agar gel diffusion and the microprecipitation test under paraffin oil were performed.

In the tests of which the results are shown in Fig. 2, the wells in the agar had a diameter of 6 mm and the centres of the antigen and antiserum wells were 1 cm apart. In all other cases these distances were respectively 3 mm and $\frac{1}{2}\sqrt{2}$ cm.

RESULTS

The occurrence of Trichodorus species and TRV

Out of the 120 samples one or more specimens of *Trichodorus* were found in 60; in five samples as many as 100 specimens were found. *Trichodorus viruliferus* was found in 44 cases, *T. teres* in 12, *T. nanus* in three, and an undescribed *Trichodorus* species[3] in one (*T. x* in Fig. 1). It is noteworthy that *T. teres*, which in the Netherlands has hitherto been found only in marine soils, was encountered far inland in Italy. It occurred along the course of the Adige and as far as Brunico, as well as near Sondrio and north of Mantova in the middle of the Po basin. At these places the pH-KCl varied from 5.2 to 7.8. We found pH values between 4.2 and 8.1. in places where *T. viruliferus* occurred. The geographical distribution of the species is shown in Fig. 1.

[3] A description of this nematode by Dr. J. W. SEINHORST will be given later.

Twenty-nine soil samples were examined for the presence of virus with the aid of bait plants (*Nicotiana tabacum* var. 'White Burley'). In addition to tobacco necrosis virus, which was isolated at five places (S. Giorio, Certosa di Pavia, Arenzano, Genova and Massa), there were seven places at which we found TRV. One lot of bait plants showed necrosis on the root neck. In all other cases the virus was detected after inoculating test plants with root sap of bait plants. The soil from which TRV was isolated was taken from: 1. Ivrea – grass and grapes, 2. Soncino (S.E. of Bergamo) – wheat, 3. Rogno (N.E. of Bergamo) – maize between grapes, 4. Mantova – grass, 5. South of Chioggia – maize in a dip in the dunes, 6. Mesola (Po estuary) – wheat, 7. Massa (S. of La Spezia) – grapes.

Apparently TRV is of widespread occurrence in grassland and arable areas. *T. viruliferus* was also found at all these places (at Mantova together with *T. teres*). The results of experiments on virus transmission by the isolated nematodes showed *T. viruliferus* to be the vector of the TRV isolates (cf. Table 1).

TABLE 1. Results of experiments on the transmission of Italian rattle virus isolates by *Trichodorus viruliferus*.

Resultaten van overbrengingsproeven van Italiaanse ratelvirus-isolaties met Trichodorus viruliferus.

Place of origin	Nematodes isolated	Reaction of 'White Burley' inoculated with root sap from bait plants
Ivrea (1)	3 × 10 ♂	+, +, –
	3 × 10 ♀	–, –, –
	3 × 10 larvae	–, –, –
Mesola (6)	1 × 10 ♂	–
	1 × 10 ♀	+
	4 × 10 larvae	+, +, +, –
Massa (7)	2 × 10 ♂	+, +
	2 × 10 ♀	+, +
	3 × 10 larvae	+, +, +
Plaats van herkomst	*Geïsoleerde nematoden*	*Reactie van 'White Burley' na uitwrijven van de wortels van de toetsplanten*

Symptoms of host plants

The systemic symptoms induced by the seven Italian TRV isolates in *Nicotiana tabacum* var. 'White Burley' differed considerably. Isolates 2 and 3 gave rise to necrotic lesions, No. 5 caused chlorotic patches, while the symptoms produced by isolate 6 were of an intermediate character. The lesions gave rise to various secundary malformations of the leaves. Of the three remaining isolates, No. 7 gave only a slight reaction, while Nos. 1 and 4 produced none at all.

Pisum sativum var. 'Koroza' and *Phaseolus vulgaris* var. 'Bataaf' reacted to all isolates except No. 4, the symptoms consisting of localised necrotic patches, identical with the effects of TRV.

Nicotiana rustica reacted systemically to isolates 2, 3, 6 and 7 with severe necrotic lesions, to No. 5 with a somewhat more severe chlorotic response than to the Dutch TRV, while isolates 1 and 4 induced no symptoms. A study was made of the response of the foliage and tuber of the 'Eersteling' potato variety

after inoculation with a number of Italian isolates. Tubers were lifted on 13 July 1965 and inoculated with isolates 2, 5 and 6 with the aid of Carborundum. The Dutch TRV-Lisse was used as control. After being stored in soil, the tubers were assessed on 2 September for the occurrence of internal necrosis. Of the 25 tubers inoculated with TRV-Lisse, 9 exhibited typical spraing symptoms. Of the three Italian isolates, No. 5 also caused spraing in 6 out of 22 tubers, No. 6 produced no symptoms, and No. 2 led to an abnormal response in 3 out of 23 tubers, viz. a small necrotic patch, diameter 6 mm, in the flesh below the skin. One tuber also showed a small, characteristic necrotic ring. In this case spraing was possibly so severe that the affected tuber tissue rapidly became wholly necrotic.

In the leaf inoculations, however, TRV-Lisse was the only virus to cause stem mottle symptoms in the foliage; some tubers of these plants showed malformation with internal necrosis. Plants inoculated with the Italian isolates showed a practically uniform response. Necrotic patches appeared on the inoculated leaf after only three days and spread to the leaf stalk and the stem; the affected leaf then died. Some uninoculated leaves also showed slight necrosis. The plants remained backward in growth and assumed a stiff, stem-mottle habit.

Serological investigation

In a preliminary test all the Italian isolates (Nos. 1 to 7) reacted with the TRV antiserum. Two of these isolates, viz. Nos. 5 and 7, produced two lines in the agar, the others only one. Nos. 5 and 7 also showed a more rapid response in the precipitation test and to a higher antiserum dilution than Nos. 1 to 4 and 6. As a result the seven isolates were split into two groups. One isolate from each group, viz. Nos. 5 and 6, were subjected to further investigation and compared with other isolates of the rattle virus group.

TRV-Lisse, VVV, L 20, 5 and 6 were tested with antisera against TRV-Lisse, VVV and L 20. To this end the antisera were absorbed with three parts of sap from healthy plants. The results are shown in Fig. 2. In tests in which the five antigens were tested against dilutions of the TRV antiserum, titres of 2048 were obtained against TRV-Lisse, 256 against No. 5, 16 against VVV, and 16 against L 20. The Italian isolate No. 6 gave no reaction in these tests.

In a similar test the antiserum against L 20 had the same titre of 256 to L 20, as it also had against the Italian isolate No. 5. The antiserum against PEBV only reacted with the Italian isolate No. 6 (titre 8).

DISCUSSION AND CONCLUSION

The Italian TRV isolates investigated produced typical rattle virus symptoms in the host plants tested; they were transmitted by *Trichodorus viruliferus* and all responded to the TRV antiserum. *T. viruliferus* is also found as the TRV vector in the Netherlands (VAN HOOF, 1964b) and as the PEBV vector in Britain (GIBBS & HARRISON, 1964). In view of this it may be concluded that the Italian isolates are to be classified in the TRV group.

The relationship between the antigens in question can be clearly seen from Fig. 2. Check tests performed with the sap of healthy plants or with preparations of TMV and potato virus X, purified in the same manner as the antigens tested,

showed a negative response. The fact that two lines occur in certain cases is probably due to the participation of particles of varying size in the reaction. Since HARRISON & NIXON (1959) found no antigen differences between long and short particles of the rattle virus it would seem likely that the relationships found represent those existing between the viruses themselves.

Italian isolate No. 5 is closely related to the Dutch isolate L 20. The former differs from L 20 in that it bears a closer relationship to TRV-Lisse than does L 20.

Unlike No. 5, TRV-Lisse, VVV and L 20, the Italian isolate No. 6 showed a marked response to the antiserum against PEBV. The closeness of the relationship cannot, however, be deduced from the tests described because the titre of the antiserum used is not known. According to GIBBS & HARRISON (1964) there is a relationship between the pea early-browning viruses found in Britain and the Netherlands, although the reaction of the PEBV antiserum with VVV was negative in our tests. Hence isolate No. 6 is more closely related to PEBV than is VVV. Since the serum consisted of a combination of dilutions it is possible that a very close relationship exists between No. 6 and PEBV.

HARRISON's (1964) findings with respect to the NEPO viruses (tomato black ring and raspberry ringspot) "that the serologically distinct forms have different vectors and that the geographical distribution of each form reflects that of its vector species" are obviously inapplicable to the NETU viruses since the TRV vectors in Italy are the same as in Britain and the Netherlands.

Moreover the results of the serological tests show that serologically quite distinct isolates may occur close to each other, whereas other more serologically related ones may sometimes occur in widely separated places. Consequently serological affinity should not be related to place of origin. The data at present available are insufficient to permit any closer classification of the virus isolates concerned (TRV-Lisse, PEBV, VVV, L 20, Italian isolates 5 and 6) within the rattle viruses. All we know is that VVV is distinctly different from TRV-Lisse as shown by serological and electron microscopy data and by the symptoms in pea and bean (BOS & VAN DER WANT, 1962; MAAT, 1963). No measurements were made of the seven Italian isolates, nor was a more detailed investigation made of their relationships.

SAMENVATTING

In Noord-Italië werden vier *Trichodorus*-soorten gevonden en wel *T. viruliferus*, *T. teres*, *T. nanus* en een onbeschreven soort (*T. x*) (Fig. 1). In zeven gevallen waar *Trichodorus* voorkwam kon ratelvirus uit de grond worden geïsoleerd met behulp van vangplanten. In drie gevallen kon met geïsoleerde nematoden worden vastgesteld dat *T. viruliferus* de overbrenger van het ratelvirus is (tabel 1).

De zeven virusisolaties reageerden met een antiserum tegen een Nederlandse isolatie van ratelvirus (TRV). Op grond van hun reactie met dit serum werden ze in twee groepen verdeeld. Uit elke groep werd één isolatie aan een nauwkeuriger onderzoek onderworpen: de ene (Nr. 5) bleek serologisch nauw verwant aan een Nederlandse isolatie uit gladiool met kartelbladsymptomen (L 20); de andere (Nr. 6) vertoonde een duidelijke verwantschap met het Engelse vroegeverbruiningsvirus van erwt (PEBV).

Eén van de isolaties (Nr. 5) bleek nauwer verwant met het Nederlandse TRV dan de uit Nederland afkomstige isolatie uit gladiool.

De reacties van de isolaties 5, 6, L 20, een Nederlandse ratelvirus-isolatie (TRV-Lisse) en het Nederlandse vroege-verbruiningsvirus van erwt (VVV) met antisera tegen TRV, VVV en L 20 zijn weergegeven in fig. 2.

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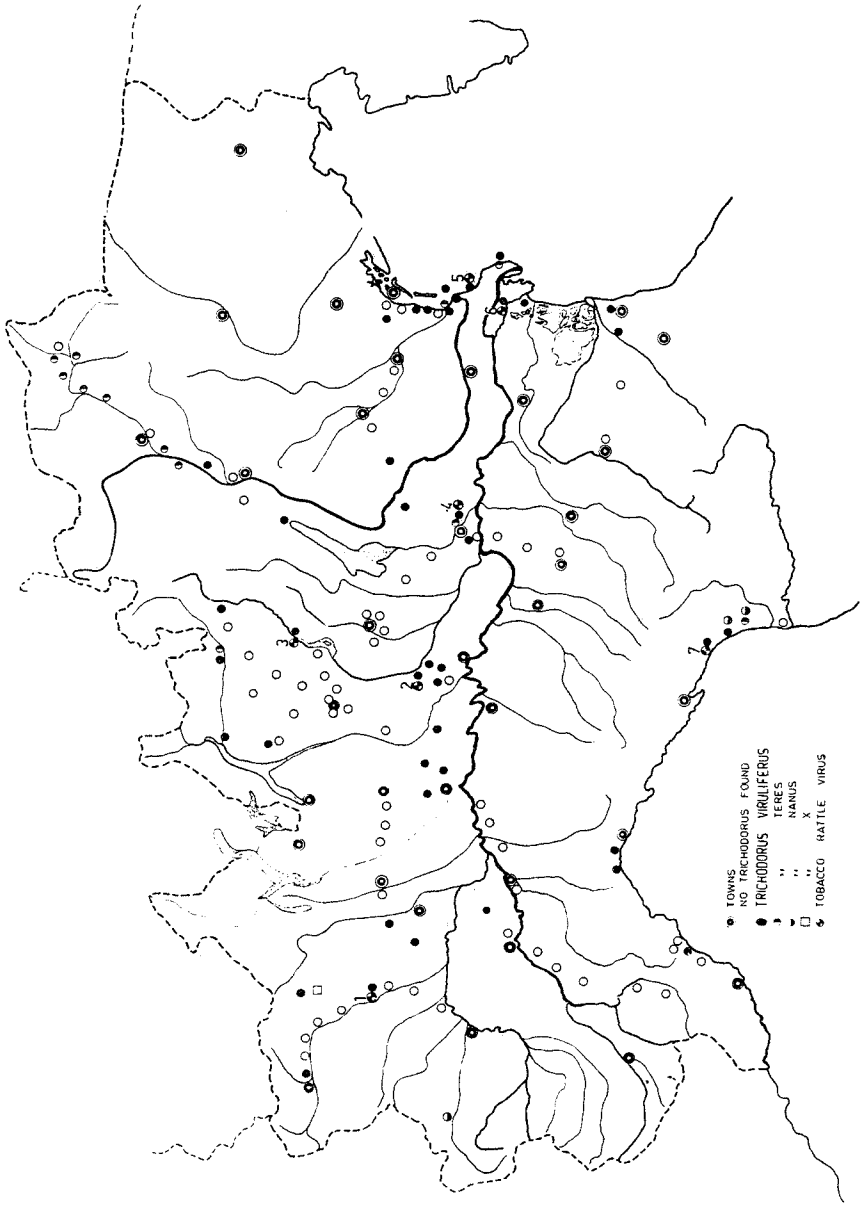

FIG. 1. Distribution of *Trichodorus* species in northern Italy and sites of occurrence of TRV. *Verspreiding van* Trichodorus-*soorten in Noord-Italië en plaatsen waar TRV werd aangetroffen.*

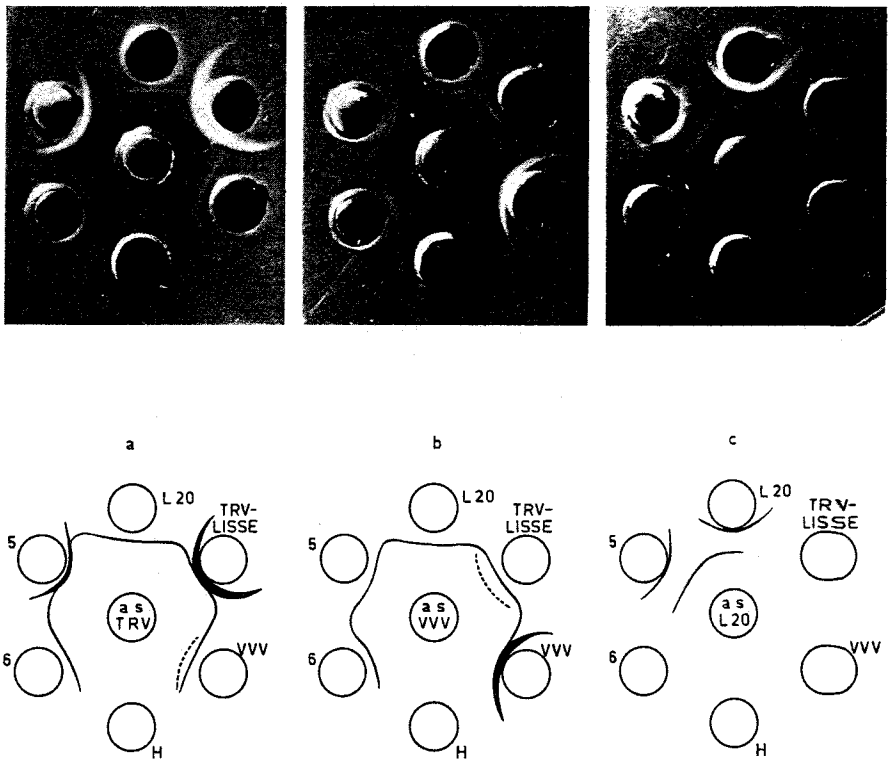

FIG. 2. Reactions of the virus isolates 5, 6, L20, VVV and TRV-Lisse with antisera against (a) TRV, (b) VVV and (c) L20.
Upper row: photograph of the reactions.
Lower row: diagramatic presentation of the reactions, showing also the order in which the antigens are arranged around the antiserum wells.
as = antiserum
H = healthy control

Reacties van de virusisolaties 5, 6, L20, VVV en TRV-Lisse met antisera tegen (a) TRV, (b) VVV en (c) L20.
Bovenste rij: foto van de reacties.
Benedenste rij: schematische weergave van de reacties, tevens vermelding van de plaatsing van de antigenen rondom de antiserumputjes.
as = antiserum
H = gezonde controle