BETWEEN TOBACCO RATTLE VIRUS SEROTYPES AND VECTOR SPECIES OF 'PARATRICHODORUS' AND 'TRICHODORUS' NEMATODES

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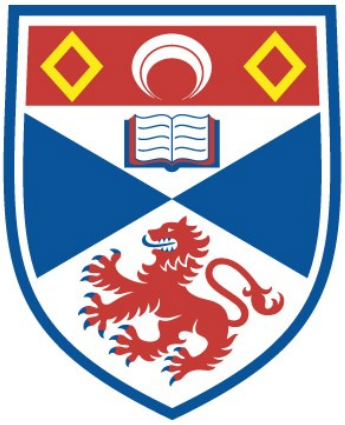

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ASSOCIATIONS BETWEEN TOBACCO RATTLE VIRUS SEROTYPES AND VECTOR SPECIES OF *PARATRICHODORUS* AND *TRICHODORUS* NEMATODES.

by

Antoon Teunis Ploeg

A thesis submitted for the Degree of Doctor of Philosophy in the University of St. Andrews, January 1992.

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ABSTRACT

Tobacco rattle virus (TRV) and trichodorid vector nematodes were found to be widespread in bulb growing areas in The Netherlands. Indications, obtained from pot bait tests, that associations between serotypes of tobacco rattle virus and *Paratrichodorus* and *Trichodorus* vector species occurred were confirmed in bait tests using individual trichodorid nematodes.

Whereas different *Paratrichodorus* vector species each transmitted a serologically distinct TRV-isolate, different *Trichodorus* vector species transmitted serologically indistinguishable TRV-isolates. It remains to be studied whether a particular *Trichodorus* species is able to transmit all isolates serologically indistinguishable from the one with which it was naturally associated.

A TRV-isolate transmitted by *T. cylindricus* from Scryne, Scotland and isolates transmitted by *P. teres* from Julianadorp, The Netherlands were considered anomalous TRV-isolates as they serologically resembled pea early-browning virus but had RNA-1 sequences typical of TRV.

Virus-free *P. pachydermus* from Scotland and from The Netherlands acquired and subsequently transmitted TRV-isolates originally obtained from bait-plants infected by *P. pachydermus* from Scotland or The Netherlands. They failed to transmit a serologically similar isolate obtained from an infected potato tuber.

Virus-free *P. pachydermus* did not acquire and transmit a pseudorecombinant TRV-isolate with the RNA-2 from a non-transmissible isolate, but efficiently transmitted a pseudorecombinant isolate in which the RNA-2 was derived from a efficiently transmitted isolate. This indicated that transmissibility is most likely

determined by the virus coat-protein.

Transgenic tobacco plants, expressing TRV coat-protein were resistant to mechanical inoculation but susceptible to nematode transmission of the virus. A possible low level of coat-protein expression in the roots could not fully explain this result as the nematodes also transmitted TRV directly to detached leaves of such transgenic plants.

CONTENTS

CHAPTER I.

GENERAL BACKGROUND AND SCOPE OF THE THESIS.

A disease in tobacco, later demonstrated to be caused by a virus (Böning, 1931), was observed around the end of the last century in Druten, then a centre of tobacco growing in the Netherlands. The virus was named tobacco rattle virus (TRV) after the rattling sound which could be heard when walking through an infected tobacco crop, caused by the wind moving the virus infected, dried-out leaves (van Hoof, 1972b). Around the same time Behrens (1899) described a similar disease of tobacco, "Mauche", in Germany. The virus causing these diseases was subsequently found to cause diseases in potatoes, ornamental bulbous crops and a range of other crops (Robinson and Harrison, 1989).

At an early stage it was believed that soil factors were important in the ecology of the disease (Behrens, 1899) and van der Want (1959) showed that it was more prevalent in sandy than in clay soils. Also, Sol (1960) reported increased recovery of TRV from bait-plants grown in infected fields after wet periods.

Two years after it was first demonstrated that a nematode transmitted a plant virus (grapevine-fanleaf virus transmitted by *Xiphinema index* ; Hewitt *et al.*, 1958), Sol (1960) reported the involvement of nematodes in the transmission of TRV. Sol *et al.* (1960) subsequently showed that *Trichodorus* (syn. *Paratrichodorus*) *pachydermus*, a nematode belonging to the family Trichodoridae, could transmit TRV. Since then numerous reports have been published describing transmission of TRV and related viruses by a range of trichodorid nematode species (see Chapter IV.3.*a*.).

Until recently most of the research on nematode transmitted viruses has concentrated on the transmission of viruses by *Longidorus* and *Xiphinema* nematodes. In these associations it became evident that there is a high degree of specificity between each vector species and the serological properties of the virus it transmits (Harrison, 1964). The importance of the virus coat-protein as a factor in determining the specificity of transmission was demonstrated by Harrison *et al.* (1974a). Results from tranmission experiments in which non-vector and vector populations of *X. diversicaudatum* were crossed showed that the vectoring ability of *X. diversicaudatum* had an inherited genetic basis (Brown, 1986).

Progress in understanding the relationship between TRV and trichodorid nematodes was comparatively slow and the results obtained were often equivocal. Thus, van Hoof (1962) initially rejected the idea that specific associations also existed between trichodorid vector species and different strains of the virus, but later concluded that "the specificity of nematodes as vectors for different strains of TRV is much greater than of *Longidorus elongatus* for strains of TBRV" (van Hoof, 1968; 1972a).

A study into the specificity of associations between *Paratrichodorus* and *Trichodorus* vector species and the virus isolates they transmit is a prerequisite for understanding the principles involved in the transmission process. Such information is also of practical importance for assessing the risks of virus spread, the opportunities for establishment of newly introduced virus strains, the development of strategies for producing virus-resistant crops and predicting risks when growing particular crop cultivars in trichodorid infested and/or virus infected fields. At present, with the increasing concern regarding the application of considerable

quantities of pesticides for nematode control and restrictions on their use being imposed in several countries, an understanding of the mechanisms basic to TRV-induced diseases in crops is necessary for the development of alternative control techniques.

The objective of this study is to investigate whether and to what extent there is specific association between trichodorid species and tobravirus isolates.

CHAPTER II.

TRICHODORIDAE.

II.1. INTRODUCTION.

The family Trichodoridae is placed in the class NEMATODA, order Dorylaimida, suborder Diphtherophorina, superfamily Trichodoroidea. The Trichodoridae represent a relatively small but agriculturally important group of plant parasitic nematodes. Their identification to species level can be difficult because of the variability of certain diagnostic features (Decraemer, 1988), but is important when studying plant parasitism and more so when determining virus - vector associations.

II.2. THE SYSTEMATICS OF TRICHODORIDAE.

II.2.a.- *Historical review*: Trichodorid nematodes were first mentioned by de Man (de Man, 1880; 1884 ex Loof, 1975) who described *Dorylaimus primitivus*. Cobb (1913) erected the monospecific genus *Trichodorus* with *T. obtusus* as the type member. *T. obtusus* was later synonimized with *Dorylaimus primitivus* and transferred to the genus *Trichodorus* by Micoletzky (Micoletzky, 1922 ex Loof, 1975). Siddiqi (1974) divided *Trichodorus* into two genera: *Trichodorus* and *Paratrichodorus*, with the latter genus containing the subgenera *Paratrichodorus*, *Atlantadorus* and *Nanidorus*. Andrassy (1976) erected the genus *Monotrichodorus* which includes monodelphic species closely related to *Trichodorus* and Rodriguez-M *et al.* (1978) established a fourth genus: *Allotrichodorus*, for monodelphic species

resembling *Paratrichodorus*. Siddiqi (1980) raised the subgenera *Atlantadorus* and *Nanidorus* to genus level but Decraemer (1980b), Decraemer and de Waele (1981) and Sturhan (1985) subsequently considered *Atlantadorus* and *Nanidorus* as junior synonyms of *Paratrichodorus*.

II.2.b.- *Present status*: At present the family Trichodoridae comprises four genera: *Trichodorus*, *Paratrichodorus*, *Allotrichodorus* and *Monotrichodorus*. The genus *Trichodorus* is the biggest genus with 36 valid species (Table 1).

Table 1. List of valid *Trichodorus* species.

Species name	Authority
T. aequalis	Allen, 1957
T. aquitanensis	Baujard, 1980
T. azorensis	Almeida *et al.*, 1989
T. beirensis	Almeida *et al.*, 1989
T. borai	Rahman *et al.*, 1985
T. borneoensis	Hooper, 1962
T. californicus	Allen, 1957
T. cedarus	Yokoo, 1964
T. complexus	Rahman *et al.*, 1985
T. coomansi	de Waele and Carbonell, 1982
T. cottieri	Clark, 1963
T. cylindricus	Hooper, 1962
T. dilatatus	Rodriguez-M and Bell,1978
T. eburneus	de Waele and Carbonell, 1982
T. elegans	Allen, 1957
T. hooperi	Loof, 1973
T. intermedius	Rodriguez-M and Bell, 1978
T. lusitanicus	Siddiqi, 1974
T. obtusus	Cobb, 1913
T. orientalis	de Waele and Hashim, 1984
T. pakistanensis	Siddiqi, 1962
T. persicus	de Waele and Sturhan, 1987
T. petrusalberti	de Waele, 1988
T. philipi	de Waele *et al.*, 1990
T. primitivus	(de Man, 1880) Micoletzky, 1922
T. proximus	Allen, 1957
T. rinae	Vermeulen and Heyns, 1984
T. sanniae	Vermeulen and Heyns, 1984
T. similis	Seinhorst, 1963
T. sparsus	Szcgygiel, 1968
T. taylori	de Waele *et al.*, 1982
T. tricaulatus	Shishida, 1979
T. variopapillatus	Hooper, 1972
T. velatus	Hooper, 1972
T. viruliferus	Hooper,1963
T. yokooi	Eroshenko and Teplyakov, 1975

The genus *Paratrichodorus* at present contains 23 valid species (Table 2).

Table 2. List of valid *Paratrichodorus* species.

Species name	Authority
P. acaudatus	(Siddiqi, 1960) Siddiqi, 1974
P. acutus	(Bird, 1967) Siddiqi, 1974
P. alleni	(Andrassy, 1968) Siddiqi, 1974
P. allius	(Jensen, 1963) Siddiqi, 1974
P. anemones	(Loof, 1965) Siddiqi, 1974
P. anthurii	Baujard and Germani, 1985
P. atlanticus	(Allen, 1957) Siddiqi, 1974
P. catharinae	Vermeulen and Heyns, 1983
P. grandis	Rodriguez-M and Bell, 1978
P. hispanus	Roca and Arias, 1986
P. lobatus	(Colbran, 1965) Siddiqi, 1974
P. macrostylus	Popovici, 1989
P. minor	(Colbran, 1956) Siddiqi, 1974
P. mirzai	(Siddiqi, 1960) Siddiqi, 1974
P. nanus	(Allen, 1957) Siddiqi, 1974
P. pachydermus	(Seinhorst, 1954) Siddiqi, 1974
P. porosus	(Allen, 1957) Siddiqi, 1974
P. renifer	Siddiqi, 1974
P. rhodesiensis	(Siddiqi and Brown, 1965) Siddiqi, 1974
P. sacchari	Vermeulen and Heyns, 1983
P. teres	(Hooper, 1962) Siddiqi, 1974
P. tunisiensis	(Siddiqi, 1963) Siddiqi, 1974
P. weischeri	Sturhan, 1985

The genus *Monotrichodorus* contains three valid species (Table 3).

Table 3. List of valid *Monotrichodorus* species.

Species name	Authority
M. monohystera	(Allen, 1957) Andrassy, 1976
M. campanullatus	Baujard and Germani, 1985
M. vangundyi	Rodriguez-M *et al.*, 1978

Finally the genus *Allotrichodorus* contains seven valid species (Table 4).

Table 4. List of valid *Allotrichodorus* species.

Species name	Authority
A. brasiliensis	Rashid, *et al.*, 1985
A. campanullatus	Rodriguez-M, *et al.*, 1978
A. guttatus	Rodriguez-M, *et al.*, 1978
A. longyspiculis	Rashid, *et al.*, 1985
A. loofi	Rashid, *et al.*, 1985
A. sharmae	Rashid, *et al.*, 1985
A. westindicus	(Rodriguez-M, *et al.*, 1978) Rashid, *et al.*, 1985

The taxonomic position of several species had to be altered as they were identified as being conspecific with earlier described species. Also, some species have been transferred to other, newly erected genera. For example before 1974, when Siddiqi (1974) erected the genus *Paratrichodorus*, the species *P. acaudatus, P. acutus, P. allius, P. anemones, P. atlanticus, P. lobatus, P. minor, P. mirzai, P. nanus, P. pachydermus, P. porosus, P. rhodesiensis, P. teres* and *P. tunisiensis* belonged to the genus *Trichodorus*. The species which have been identified as being identical to previously described species are listed in Table 5.

Table 5. Synonimisation of *Trichodorus* and *Paratrichodorus* species.

Original species description	Present species name	Synonimized by
T. bucrius	*P. porosus*	Siddiqi, 1962
T. castellanensis	*T. primitivus*	Arias and Roca, 1986
T. clarki	*P. lobatus*	Yeates, 1969
T. flevensis	*P. teres*	Hooper *et al.*, 1963
T. kurumeensis	*T. cedarus*	Shishida, 1979
T. litchi	*T. pakistanensis*	Siddiqi, 1974
T. longistylus	*T. cedarus*	Shishida, 1979
T. musambi	*P. mirzai*	Siddiqi, 1974
T. obscurus	*T. primitivus*	Loof, 1975
P. christiei	*P. minor*	Loof, 1975
P. obesus	*P. minor*	Baujard, 1983
P. tansaniensis	*P. allius*	Sturhan, 1989

II.3. MORPHOLOGICAL AND TAXONOMICAL CHARACTERS.

Nematodes belonging to the Trichodoridae are vermiform and all stages occur free living in the soil. They have a plump appearance and range in size from approximately 0.4 to 1.5 mm, which is within the range of most other plant parasitic nematodes (See Fig. 1). Trichodorid nematodes can however be easily distinguished from other free living species by their characteristic curved stylet. This ventrally curved stylet or onchiostyle varies in length in adult specimens from 20 µm to 85 µm depending on the species (Hooper, 1975). The onchiostyle is a solid

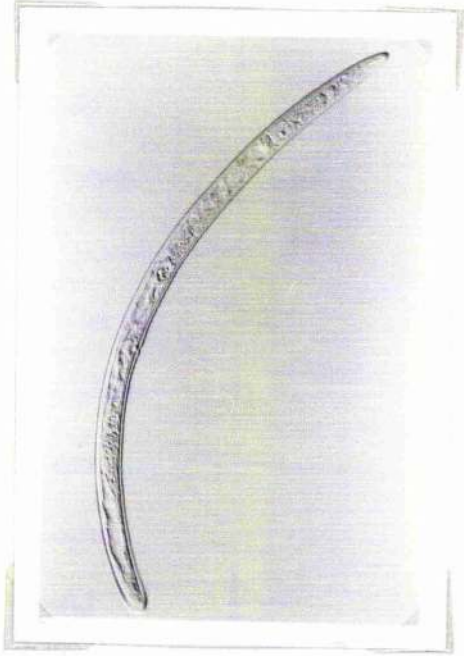

Figure 1. General habitus of a trichodorid nematode (*T. primitivus* female).

cuticular structure with a dorsal groove (Robertson and Taylor, 1975) and is used to penetrate plant root cells during feeding. The posterior part of the onchiostyle is dorsally attached to the pharyngeal protractor muscles. A pharyngeal lumen connects the pharynx directly to the oesophagus which extends into the oesophageal bulb. The intestine may be partly overlapped anteriorly by the oesophageal bulb, the oesophageal bulb itself may be partly overlapped by the intestine, or the junction between the intestine and posterior oesophagus may be offset. An excretory pore is located ventrally, usually opposite the anterior part of the oesophageal bulb. The vulva in females is located approximately halfway along the body and in lateral view sclerotized pieces may be seen which vary between species in size and shape. *Paratrichodorus* and *Trichodorus* females are didelphic, *Allotrichodorus* and *Monotrichodorus* females monodelphic.

Males are common in most species but not in all (e.g. *P. teres*, *P. nanus*). They have a single testis and two well developed spicules, the shape and size of which differs between species. A bursa is present in *Paratrichodorus* males and absent in *Trichodorus* males, although intermediate forms do occur (Decraemer, 1992). Cervical papillae, located ventrally usually anterior to the excretory pore, are present in males of most species. One to four precloacal supplements are present, the number and position depending on the species (Fig. 2).

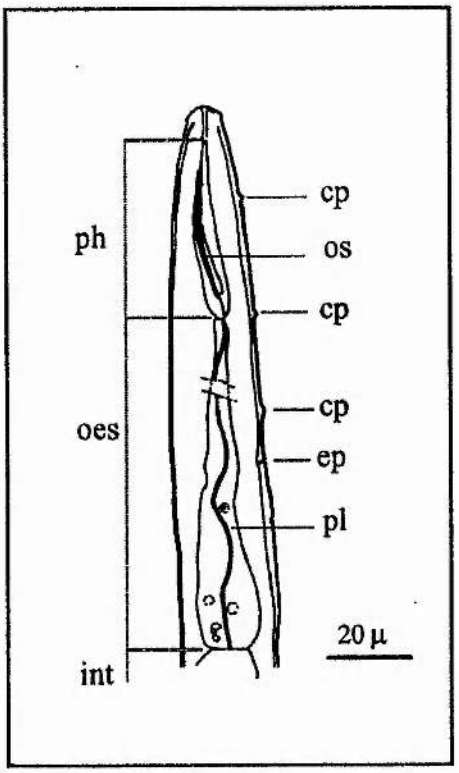

Figure 2. Anterior part of a trichodorid nematode (T.primitivus male). os:onchiostyle, ph:pharynx, oes:oesophagus, pl:pharyngeal lumen, int:intestine,cp:cervical papilla, ep:excretory pore.

Decraemer (1992) lists the diagnostic characters used to distinguish between the four genera of Trichodoridae. A total of 68 characters were described but only a few are of primary importance. *Allotrichodorus* and *Monotrichodorus* species have only been reported from Central- and South-America (Rashid *et al.*, 1985), and the monodelphic females of these species are easily distinguished from the didelphic *Paratrichodorus* and *Trichodorus* females. Separating males of *Trichodorus* from *Monotrichodorus*, and *Paratrichodorus* from *Allotrichodorus* is difficult since no

clear distinguishing characters are available. The characters most readily used to distinguish *Trichodorus* from *Paratrichodorus* are summarized in Table 6, based on Decraemer (1980b), Loof (1975) and Bongers (1988).

Table 6. Characters used to distinguish *Trichodorus* from *Paratrichodorus*.

	Trichodorus	*Paratrichodorus*
Both sexes	Cuticle after fixation not swollen.	Cuticle after fixation swollen.
Females	Vagina up to half body width long.	Vagina less than half body width long.
	Vulval sclerotization strong.	Vulval sclerotization weak.
Males	Bursa present. Copulatory muscles strong. Tail curved ventrally after fixation.	Bursa absent. Copulatory muscles weak. Tail straight after fixation.

T. cylindricus males resemble *Paratrichodorus* males by the presence of a small bursa and the tail being almost straight after fixation. Some swelling of the cuticle may occur with *Trichodorus* species, particularly with females of *T. sparsus*.

During this study 5 *Trichodorus* and 4 *Paratrichodorus* species were recovered from soil samples (See Chapter VI) and identified using keys of Bongers (1988) and Decraemer (1980b, 1992). A dichotomous key adapted from Bongers (1988) and Decraemer (1980b; 1992) for use with species reported on here, can be found in Appendix A.

II.4. BIOLOGY AND ECOLOGY.

II.4.a.- *Life cycle*: The life cycle of trichodorids has been little studied, probably due to the difficulties in culturing the nematodes. *P. christiei* (= *P. minor*), a parthogenetically reproducing species, completed its lifecycle in 17-18 days at 27 °C (Morton and Perry, 1968), and *T. viruliferus* in 45 days at 20 °C (Pitcher and McNamara, 1970). Other sexually reproducing species are thought to have life cycles in temperate regions similar to that of *T. viruliferus* (Hooper and Siddiqi, 1972; Wyss, 1974). Eggs are laid directly into the soil. *T. similis* females produced an average of six eggs per week, with a maximum of 38 eggs at 20 °C on tobacco cv. Samsun (Wyss, 1974). It is widely accepted that first stage juveniles (J1) hatch from the eggs and moult 4 times prior to developing into adults. Kuiper (1977) however, observed only three larval stages with *P. teres* and Decraemer (1979) also found only three larval stages with *P. pachydermus*, *T. similis* and *T. primitivus* from Belgium. This may have been caused by the J1 quickly moulting into the J2, as has been observed with *P. christiei* (= *P. minor*) (Morton and Perry, 1968). The total life span of trichodorid nematodes is unknown. De Pelsmaeker (1987) reported a life cycle of several weeks for trichodorid nematodes but did not provide data to support this. When stored at 4 °C trichodorid nematodes can survive several years in soil in absence of host plants (van Hoof, 1970b).

II.4.b.- *Host range*: Trichodorid nematodes are generally considered to have wide host ranges and have been reported in association with a great diversity of plant species, including weeds and trees. However, detailed information on the

multiplication of individual trichodorid species on different plant species in controlled conditions is scarce. Available information on the host status of some crops for trichodorid species is summarized in Table 7.

Table 7. Host status of four crops for some *Trichodorus* and *Paratrichodorus* species.

Nematode species	Crop			
	Potato	Sugarbeet	Barley	Gladiolus
P. anemones	+		+	
P. pachydermus	+/-	+		
P. teres	+/-	+	+	-
T. primitivus	+	+	-	
T. similis	+/-	+		

+ good host, +/- intermediate host, - non host.
Data from Alphey (1977), Barbez (1983), de Waele and Coomans (1991), Kuiper (1977) and Whitehead *et al.* (1970).

Furthermore, de Pelsmaeker (1987) showed that differences exist between potato cultivars in their host suitability for *T. similis* which indicates the complexity of the interactions between nematode species and host plants.

II.4.c.- *Soil; preferences and distribution*: Several authors (Alphey and Boag, 1976; Barbez, 1983; de Waele and Coomans, 1991) reported a preference by trichodorid nematodes for sandy or light loamy soils, although some differences between trichodorid species occurred. *T. primitivus* prefers heavier (sandy loam) soils (Alphey and Boag, 1976; Barbez, 1983), *T. similis* sandy soils (Barbez, 1983; de Waele and Coomans, 1991) and *P. teres* marine sandy soils (Seinhorst and van Hoof, 1982). Trichodorid nematodes characteristically inhabit deeper soil layers than most other plant parasitic nematodes (de Pelsmaeker, 1987; Hijink and Kuiper, 1966;

Richter, 1969), which may have important implications for their control. Thus, Maas (1974) found that trichodorid nematodes occurring below a depth of 30 cm partly escaped the effects of soil fumigation.

Several authors have reported vertical migration of trichodorid nematodes through the soil profile (Decraemer, 1980a; Kuiper ex Maas, 1974; Maas, 1974; Rössner, 1972), but others (Alphey, 1985; Boag, 1981; Boag and Brown, 1989; Richter, 1969; Szczygiel and Hasior, 1972) did not find any evidence to support this. It is unclear whether fluctuations in population levels at certain depths is due to nematode death or reproduction or due to migration of the nematodes. Pitcher (1967) and Hijink and Kuiper (1966) reported much higher numbers of trichodorid nematodes directly around the host plant roots than short distances away from the roots. According to Pitcher (1967) the nematodes actively migrated towards the roots and therefore it seems likely that the vertical distribution and migration of the nematodes largely reflects the outcome of an interaction between soil characteristics and the distribution and colonization of the soil by newly formed host plant roots. The horizontal distribution of trichodorid nematodes was aggregated (Boag *et al.*, 1987) and irregular (Decraemer, 1980a), and is most likely influenced by soil type, nematode biology, type of crop grown and agricultural practices.

Several trichodorid species are frequently found together (Alphey, 1985; de Waele and Coomans, 1991; Winfield and Cook, 1975). It is assumed that each differs biologically and has its own ecological requirements. However, the occurrence of species mixtures complicates the interpretation of data obtained from field studies or using field soil, especially with regard to transmission of TRV.

CHAPTER III

THE TOBRAVIRUSES.

III.1. DEFINITION OF *ISOLATE*, *STRAIN* AND *SEROTYPE*.

The definition and the use of virus-"isolate", -"strain" and -"serotype" is often confusing and not well defined. To prevent confusion the meaning of these terms in this thesis is defined here:

isolate: Virus of which the identity may be known, but without referring to any further virus characteristics (e.g. a virus-isolate causing necrosis in tobacco, TRV-isolate from tulip, Dutch isolate of PEBV).

strain: Virus of known identity of which certain virus characteristics (e.g. symptomatology, particle length, serology, nucleic acid sequence) are known (e.g. TCM-strain of TRV).

serotype: Group of virus isolates which are characterized by their serological similarity (e.g. PRN-serotype).

III.2. VIRUS MEMBERS.

The tobravirus group comprises three viruses: tobacco rattle virus (TRV), pea early-browning virus (PEBV) and pepper ringspot virus (PRV).

III.2.a.- *Tobacco rattle virus (TRV):* First identified as the cause of a disease of tobacco, "Mauche", it was described by Behrens (1899) in Germany (Böning, 1931; Quanjer, 1943). Later, Rozendaal and van der Want (1948) showed that the same virus also caused the stem mottle disease of potato, and were the first to report symptomatological differences between isolates. Van der Want (1952) noticed the relatedness of the stem mottle disease and the potato-tuber spraing disease and suggested that both diseases were caused by the same virus. Also, Cadman (1958) isolated a virus indistinguishable from TRV from a spraing-affected tuber. Finally, van Hoof (1964) produced spraing symptoms in potato tubers by pricking them with a needle dipped in a TRV suspension. TRV was also identified as the virus causing disease in a number of ornamental bulbous crops e.g. tulip (rattle-disease), hyacinth (malaria), narcissus, crocus, eremurus and gladiolus (notched leaf) (Cremer and Schenk, 1967; van Slogteren, 1958). Other crops in which TRV causes disease include lettuce (Gold *et al.*, 1963; Ragozzino *et al.*, 1978), sweet pepper (Marte *et al.*, 1979; Roca, 1988; Roca *et al.*, 1984), celery (Bos, 1973) and sugarbeet (Gibbs and Harrison, 1964). TRV has, with over 400 plant species including numerous weed species (Noordam, 1956; Schmelzer, 1957), the widest known host range of all plant viruses (Robinson and Harrison, 1989) and has been reported from Europe, the

Soviet-Union, Japan, New Zealand and North America (Harrison and Robinson, 1986).

III.2.b.- *Pea early-browning virus (PEBV):* This virus was isolated from diseased pea in the Netherlands and described by Bos and van der Want (1962). They found strong similarities between PEBV and TRV in morphology, symptomatology and epidemiology but because of differences in serological properties and host range they concluded that PEBV and TRV were related but different viruses. PEBV can be distinguished from TRV by the symptoms they cause in manually inoculated French bean or pea. Whereas PEBV causes local lesions which rapidly enlarge, TRV only causes numerous pinpoint lesions on the inoculated leaves of French bean. Also, PEBV systemically invades French bean and pea whereas with TRV no systemic invasion occurs. PEBV was also isolated from naturally infected broad bean and alfalfa but no disease symptoms were observed (Bos and van der Want, 1962). The virus was subsequently reported from England (Gibbs and Harrison, 1964) and North Africa (Lockhart and Fischer, 1976). Broad bean yellow-band virus, originally thought to be a new tobravirus (Russo *et al.*, 1984) from Italy, was later shown to be PEBV (Robinson and Harrison, 1985), and isolate I6 from Italy, which was initially classified as PEBV (van Hoof *et al.*, 1966; Cooper and Mayo, 1973) was later identified as an anomalous TRV-isolate (Robinson *et al.*, 1987).

III.2.c.- *Pepper ringspot virus (PRV):* This virus was originally found in Brazil, and initially described as the CAM-strain of TRV (Harrison and Woods, 1966). Later, Robinson and Harrison (1985), based on RNA sequences studies, reclassified

this strain as a separate virus. PRV has so far only been reported from Brazil, causing disease in tomato, artichoke and sweet pepper (Harrison and Robinson, 1986).

III.3. CHARACTERISTICS, HOMOLOGIES AND VARIATION.

III.3.a.- *General characteristics of the tobravirus group*: The viruses belonging to the tobravirus group, originally known as the "NETU"-virusgroup (*Ne*matode transmitted *Tu*bular particles) are characterized by a bipartite positive sense single stranded RNA genome. The two RNA genomic parts (RNA-1 and RNA-2) are encapsidated by the virus coat-protein to form rod-shaped particles of two predominant lengths: the L- (long) particles containing the RNA-1 and the S- (short) particles containing the RNA-2 (Harrison and Robinson, 1986). Within each virus the variation between L-particle length (180-197 nm for TRV, 210-212 nm for PEBV, 197 nm for PRV), RNA-1 molecular weight and nucleic acid sequence is small. Indeed, Robinson *et al.* (1987) found that cDNA probes, prepared from TRV-strain SYM RNA-1, together representing the complete RNA-1 molecule, hybridized with all of 5 TRV-strains tested. Therefore, the RNA-1 sequence of different TRV-strains seems highly conserved. The S-particle length (46-114 nm), RNA-2 molecular weight and nucleic acid sequence however is highly variable between isolates within one virus, giving rise to a large number of strains, especially within TRV which has been studied most intensively (Harrison and Robinson, 1978; Robinson, 1989; Robinson and Harrison, 1989).

III.3.b.- RNA-1: genes and function: The total RNA-1 sequence of TRV-strain SYM (spinach yellow mottle) has been analyzed and codes for proteins of 194K, 134K, 29K and 16K (Hamilton *et al.*, 1987). Proteins of similar size were encoded by RNA-1 of TRV strains PLB, PSG, TCM and PRN although with the latter strain the 16K protein was not found (Angenent, 1989; Pelham, 1979). The 134K/194K proteins have been suggested to function in RNA replication and the 29K protein in cell-to-cell transport and symptom induction (Boccara *et al.*, 1986; Hamilton *et al.*, 1987; Ziegler-Graff *et al.*, 1991). It was initially hypothesized that the 16K protein might be involved in the virus transmission by nematodes (Boccara *et al.*, 1986) but this idea was rejected by Guilford *et al.* (1991), who suggested this protein to function in the infection cycle. Liu *et al.* (1991) found the 16K-protein was mainly associated with host plant nuclei although it was also detected in low concentrations in the cytoplasm. Liu *et al.* (1990) were also unable to identify a specific function of this protein but suggested that it might be involved in modulation of host plant nucleic acid metabolism.

III.3.c.- *RNA-2: genes and function*: The RNA-2 genomic part carries the gene for the coat-protein (Harrison and Robinson, 1986) and in some strains for additional proteins (Angenent *et al.*, 1986; 1989). The variability in RNA-2 sequence causes great antigenic diversity (Robinson, 1989; Robinson and Harrison, 1985) and the originally proposed classification of TRV into three serotypes (Harrison and Woods, 1966) now seems unjustified as the variation in coat-protein amino-acid sequence and composition is as extensive within as between these serotypes (Angenent *et al.*, 1986).

III.3.d.- *Anomalous isolates*: A number of "anomalous" TRV-isolates have been found (Angenent *et al.*, 1986; Ploeg, this thesis; Robinson *et al.*, 1987) which are characterized by having RNA-1 sequences homologous to other TRV-isolates but which are serologically similar to PEBV. Their RNA-2's were shown to contain both TRV-like and PEBV-like sequences, and presumably arose by a process involving RNA recombination (Robinson *et al.*, 1987). Recently, Goulden *et al.* (1991) suggested that the RNA-2 of such isolates have 5'-terminal sequences derived from TRV RNA-1, 3'-terminal sequences derived from a TRV RNA-2 and internal sequences derived from PEBV RNA-2. However, considering that TRV-isolates with serological properties similar to those originally thought to be typical for PEBV are not uncommon (see also this thesis) it may be questioned whether coat-proteins, serological behaviour and internal RNA-2 sequences can be designated to be either TRV or PEBV. Complete RNA-2 molecules however can be classified as TRV, PEBV or PRV by the failure or ability to produce pseudorecombinant isolates with foreign RNA-1, as RNA-2 molecules will only produce pseudorecombinant isolates with RNA-1 from isolates of the same virus (Harrison and Robinson, 1986; Robinson *et al.*, 1987). At present no "anomalous" PEBV isolates, being serologically similar to "normal" TRV-isolates, have been reported. This however could be due to the fact that PEBV seems less prevalent than TRV as a result of which fewer isolates have been studied. Also, such isolates would not be recognized as being PEBV unless inoculated unto differential hosts (e.g. French bean) or tested in cDNA hybridization tests using PEBV specific probes.

III.3.e.- *Implications for detection*: The large antigenic diversity makes detection of tobraviruses by serological techniques unreliable. A negative serological response does not necessarily imply the absence of the virus. Isolates may occur (see this thesis) which are serologically different from currently known isolates and which therefor fail to react with any of the available antisera. Another drawback of serological techniques is the fact that "NM-isolates" are not detected. NM- (non multiplying) isolates occur with all three tobraviruses and consist of RNA-1 only. NM-isolates are infective and replicate and spread through infected hosts, but, since the RNA-2 and therefore the coat-protein gene is missing, do not become encapsidated (Harrison and Robinson, 1986). NM-isolates are common in spraing affected potato tubers (Harrison and Robinson, 1982), whereas M- (multiplying) isolates, containing both RNA-1 and RNA-2, are usually found in ornamental bulbous crops (Asjes, pers. comm; Harrison and Robinson, 1986). M-isolates can be easily distinguished from NM-isolates since the former are resistant to repeated freezing and thawing whereas the latter are not (Harrison and Robinson, 1986). cDNA-hybridization techniques however, using specific probes to conserved RNA-1 regions of the viruses, have been proved highly reliable in detection of tobraviruses in plant material (Robinson, 1989).

CHAPTER IV

THE TRV - TRICHODORID COMPLEX

IV.1. FEEDING OF TRICHODORIDS IN RELATION TO VIRUS TRANSMISSION.

IV.1.a.- *Feeding sites*: The feeding process of trichodorid nematodes has been extensively studied by Wyss (1971a; 1971b; 1975) using populations of *T. similis*, *T. primitivus*, *T. sparsus* and *P. pachydermus* feeding on roots of rape, tobacco or strawberry seedlings in sterile agar culture. Also it has been observed that trichodorid nematodes aggregate in the zone directly behind the growing root tip (Pitcher and McNamara, 1970; Wyss, 1971a), which is considered to be the most suitable nutrient source for the nematodes (Högger, 1973; Wyss, 1971a). Feeding was confined to epidermal cells or root hairs (Wyss, 1975) and rarely exceeded 6 min. with *T. similis*. Other trichodorid species appear to have a similar feeding behaviour (Wyss, 1975).

IV.1.b.- *Feeding phases*: Five feeding phases have been observed: exploration, perforation of the cell wall, salivation, ingestion and withdrawal (Wyss, 1975). During exploration the head and lips can be seen making searching movements over the epidermal cell surface. When a suitable feeding site is found the lips are brought into close contact with the cell-wall. The cell wall is perforated by thrusts with the solid stylet. Once the cell wall has been perforated, saliva is exuded into the hole and, as it hardens, is formed into a hollow feeding tube by further thrusts with the stylet. Upon perforation a change occurs in cytoplasmatic streaming which becomes

directed towards the penetration site. The nucleus often migrates towards the perforation site, its contents become disorganized and it starts to swell. Once the feeding tube is formed, ingestion of the accumulated cytoplasm is accomplished by slow stylet thrusts. With each retraction of the stylet a large proportion of cytoplasm, including cell-organelles and sometimes the cell nucleus, are ingested. After ingestion and detachment of the nematode from the cell the feeding tube remains anchored through the cell wall at the site of perforation and, upon detachment, is apparently sealed-off as no leakage of cell contents occurs. After feeding cytoplasmatic streaming usually ceases although occasionally normal cellular activity is resumed. The latter usually occurs when nematode feeding is abruptly aborted after cell puncturing, prior to the ingestion of cytoplasmatic material by the nematode.

The acquisition of virus most likely takes place during the ingestion phase, when a large portion of cytoplasmatic material is being ingested by the nematode. Injection of virus into the plant cell is most likely to occur during the salivation phase , when the feeding tube existing of salivatory material is being formed.

IV.1.c.- *Feeding behaviour and transmission efficiency*: Since a plant virus can only succesfully infect a living cell, it has been suggested that the introduction and establishment of trichodorid transmitted virus in a plant is most likely to be successful when the feeding process is interrupted as described above (Wyss, 1975). How the suitability of a plant species as a host for trichodorid nematodes affects the nematodes feeding behaviour is unknown, but it can be hypothesised that the nematodes are more likely to complete the feeding cycle on good host plants than on non-hosts or intermediate host plants. This would mean that virus transmission is

more likely to be successful with non-host or intermediate host plants, since more cells may be punctured but left relatively undamaged, giving the virus the possibility to become established. Whether this is so, is as yet unknown but observations from tulip, which is generally considered to be a poor host for trichodorids, but which often suffers from a high incidence of TRV infection even at low trichodorid levels, may indicate that such a process is involved.

IV.2. ACQUISITION, RETENTION AND SUBSEQUENT RELEASE OF VIRUS.

IV.2.a.- *Sites of retention*: Most data on the specific sites of retention of virus in vector nematodes refer to longidorids and nepoviruses. Taylor and Robertson (1969) observed raspberry ringspot - and tomato black ring virus particles in *Longidorus elongatus* layered along the inner surface of the stylet guiding sheath. Raspberry ringspot virus particles were observed in ultrathin sections to be associated with the inner wall of the odontostyle and between the odontostyle and the guiding sheath in *L. elongates*, *L. macrosoma* and *L. attenuatus* (Trudgill *et al.*, 1981). In *Xiphinema* spp. virus was found associated with the cuticular lining of the pharyngeal lumen (McGuire *et al.*, 1970; Taylor and Robertson, 1970b; Trudgill *et al.*, 1981). In trichodorids such observations are limited to *P. pachydermus*, in which TRV particles were observed attached to the cuticular lining of the pharyngeal lumen (Taylor and Robertson, 1970a). The difference in virus retention sites in trichodorids and *Xiphinema* spp. as compared to *Longidorus* spp. coincides with long retention periods in *Xiphinema* and trichodorids and short (up to 8 weeks.) retention periods in

Longidorus spp. (Taylor and Robertson, 1975). However, no explanation based on differences in retention mechanisms between these nematode groups is yet available.

IV.2.b.- *The mechanism of retention*: It has been established that specific associations between *Longidorus* vector nematode species and transmitted nepoviruses (Harrison, 1964; Trudgill *et al.*, 1981) probably involve recognition between the virus coat protein and the site of retention in the vector (Harrison *et al.*, 1974a). However, the nature of the retention mechanism is unknown. Harrison *et al.* (1974b) and Taylor and Brown (1981) speculated that the surface charge density of the virus coat protein might be involved in the specific binding between nematode and virus. Later, Robertson and Henry (1986a; 1986b) observed a layer of carbohydrate staining material at the site of virus retention in the pharyngeal lining of *X. diversicaudatum* and *P. pachydermus* vector nematodes. They suggested that virus binding might involve carbohydrate material which recognized specific lectins on the virus coat protein.

IV.2.c.- *Virus release*: It has been suggested that the release of virus particles from the sites of retention could be due to pH changes, changes in ionic strength, or to enzymes associated with the ejection of saliva (Taylor and Robertson, 1975). Each virus release event must however be incomplete since one trichodorid nematode is capable of sequentially infecting a series of plants (van Hoof, 1964d), indicating that only a proportion of the virus particles is discharged during each feed. Furthermore, the release rather than the retention of virus particles may in some instances be the key for transmissibility of a virus isolate. For example, particles of the English and

Scottish strains of raspberry ringspot virus were equally well retained in the odontostyle lumen of *L. macrosoma* but only the English strain was efficiently transmitted by this nematode species (Taylor and Robertson, 1975; Trudgill and Brown, 1978).

IV.2.d.- *Longevity of the virus in the nematode*: As described in IV.2.a., acquisition probably occurs during ingestion. Taylor and Robertson (1970a) envisaged virus acquisition as a mechanical contamination of the stylet followed by specific adsorption at the sites of virus retention. Once trichodorid nematodes have acquired virus, they can retain it in a transmissible and infective form over long periods. Van Hoof (1970b) reported that a *T. similis* population was still able to transmit TRV after a 3 year storage in soil at 4 °C in the dark. There is no evidence to suggest that the virus is retained through the moult or transovarially although very few data are available on this subject (Ayala and Allen, 1968). Furthermore there is no indication that the virus enters the nematode cells or that it replicates within the nematode. Thus, by analogy with aphid-borne viruses one could consider the virus transmission by trichodorids as a non-circulative process, although the long virus retention periods of trichodorids does not follow this analogy.

IV.2.e.- *Direct recovery of virus from the nematode*: The nature of the mechanism involved in the specific retention of TRV by its trichodorid vectors is largely unknown. Direct recovery of the virus from an undescribed *Trichodorus* vector species by inoculation of macerated nematodes to leaves of *Chenopodium amaranticolor* indicator plants was reported by Sänger *et al.* (1962). Similar

inoculations using *Pratylenchus* and *Dorylaimus* spp. gave negative results. Furthermore rod shaped particles similar to those of TRV were observed in preparations of comminuted vector nematodes using the electron microscope (Sänger *et al.* 1962). Van Hoof (1967b) however, found that when nematodes were fed on TRV infected source-plants "rattle virus could not be easily recovered from the vector *Paratrichodorus pachydermus*", by comminuting the nematodes and inoculation of the resulting suspension onto *N. tabacum* cv. White Burley leaves. TRV was demonstrated to be present in other nematodes belonging to species which were not natural vectors of TRV (e.g. *Xiphinema diversicaudatum*, *X. coxi* and *Pratylenchus penetrans*). Inoculation of comminuted anterior body halves of *X. diversicaudatum* onto leaves of *N. tabacum* cv. White Burley produced 11 necrotic spots on the inoculated leaves whereas inoculation of the posterior halves resulted in 127 necrotic spots. This led to the conclusion that TRV directly recovered from nematode bodies largely represented virus present in the gut rather than that retained in the feeding apparatus and that this test is therefore not necessarily indicative of specific virus - vector associations (Taylor and Cadman, 1969; Taylor and Robertson, 1970a; van Hoof, 1967b).

IV.3. VIRUS-VECTOR ASSOCIATIONS.

IV.3.a.- *Reported associations*: After the initial report of virus transmission by a trichodorid nematode species (Sol *et al.*, 1960) much research was initiated to investigate the possible role of other trichodorid species as virus vectors.

Table 8. *Trichodorus* and *Paratrichodorus* species reported as tobravirus vectors.

Species	Virus	Isolate/serotype	Authority
Paratrichodorus			
P. allius	TRV	Californian	Ayala and Allen, 1968
P. allius	TRV	Oregon	Jensen and Allen, 1964
P. anemones	PEBV	English	Harrison, 1967
P. anemones	TRV	Dutch	van Hoof, 1968
P. minor	TRV	Californian	Ayala and Allen, 1966; 1968
P. minor	TRV	Japanese	Komuro *et al.*, 1970
P. minor	TRV	Florida	Walkinshaw *et al.*, 1961
P. minor	PRV	Artichoke yellow band	Salomão, 1973
P. nanus	TRV	Scottish PRN	Cooper and Thomas, 1970
P. nanus	TRV	Dutch	van Hoof, 1968
P. pachydermus	TRV	Dutch	Cremer and Kooistra, 1964
P. pachydermus	TRV	Dutch	Cremer and Schenk, 1967
P. pachydermus	TRV	Dutch	van Hoof, 1964d; 1968
P. pachydermus	TRV	German	Sänger, 1961
P. pachydermus	TRV	Dutch	Sol and Seinhorst, 1961
P. pachydermus	TRV	Dutch	Sol *et al.*, 1960
P. pachydermus	PEBV	Dutch	van Hoof, 1962
P. porosus	TRV	Californian	Ayala and Allen, 1966
P. teres	TRV	Dutch	van Hoof, 1964e
P. teres	TRV	Dutch	van Hoof, 1968
P. teres	TRV	Oregon	Jensen *et al.*, 1974
P. teres	PEBV	Dutch	van Hoof, 1962
P. tunisiensis	TRV	Italian	Roca and Rana, 1981

Continued next page.

Table 8 continued

Species	Virus	Isolate/serotype	Authority
Trichodorus			
T. cylindricus	TRV	Dutch	van Hoof, 1968
T. hooperi	TRV	English	Alphey, 1974
T. primitivus	TRV	English	Harrison, 1961
T. primitivus	TRV	Dutch	van Hoof, 1968
T. primitivus	TRV	English, SYM	Kurppa *et al.*, 1981
T. primitivus	TRV	Scottish	Mowat and Taylor, 1962
T. primitivus	TRV	German	Sänger, 1961
T. primitivus	PEBV	English	Gibbs and Harrison, 1964a
T. similis	TRV	Dutch	Cremer and Schenk, 1967
T. similis	TRV	Dutch, GI	Cremer and Kooistra, 1964
T. similis	TRV	Dutch	van Hoof, 1968
T. sparsus	TRV	Dutch	van Hoof, 1968; 1970a
T. viruliferus	TRV	Dutch	van Hoof, 1964b; 1968
T. viruliferus	TRV	Italian	van Hoof *et al.*, 1966;
T. viruliferus	PEBV	English	Gibbs and Harrison, 1964a

When comparing the number of *Trichodorus* and *Paratrichodorus* species described with the number of species reported to transmit virus it is clear that only a small proportion of the species has at present been shown to be a virus vector.

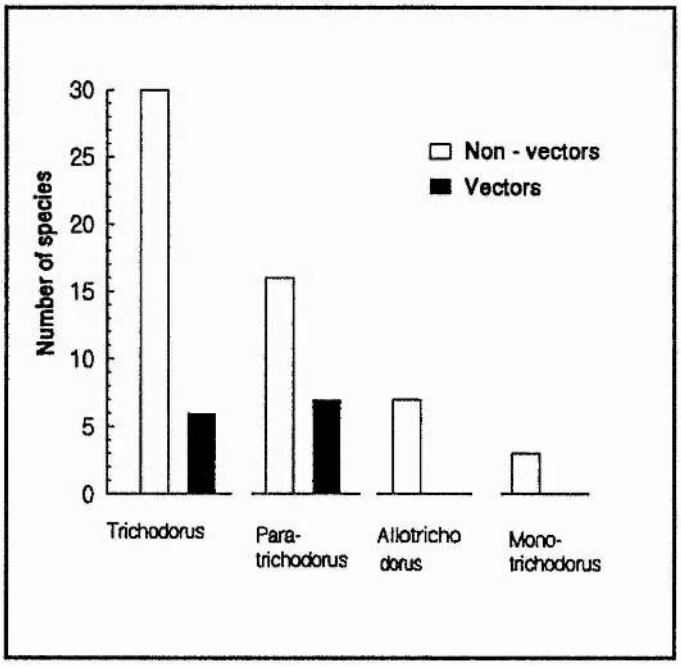

Figure 3. The number of vector and non-vector species of the four trichodorid genera.

IV.3.b.- *Nature of the associations*: It has been suggested that specific associations occur between trichodorid vector species and tobravirus isolates. In 1961 Harrison *et al.* (1961) reported that different strains of a tomato black ring virus were associated with different, but related *Longidorus* species. Later, Harrison (1966; 1967) demonstrated that specific associations also occurred with the transmission of PEBV by trichodorids by allowing virus-free *P. anemones* and *T. primitivus* from England to feed on plants infected with the Dutch and English isolate of PEBV. Both species transmitted the English but failed to transmit the Dutch isolate. Van Hoof (1968) also suggested specific associations to occur on a geographical basis, when he found that TRV was only transmitted by virus-free *P. pachydermus* when the nematodes and virus originated from the same locality. Thus, although there are strong indications suggesting specific associations between trichodorid species and particular virus isolates, the extent and the mechanism of this specificity is unknown.

IV.4. DISTRIBUTION OF *PARATRICHODORUS* AND *TRICHODORUS* SPECIES AND THEIR ASSOCIATED VIRUSES IN EUROPE.

IV.4.a.- *Introduction*: The geographical distribution of trichodorid nematodes in several European countries has been presented as a series of distribution maps in the European atlas of the *Longidoridae* and *Trichodoridae* (Alphey and Taylor, 1986). However, since the publication of the atlas several new species have been described from European countries. Furthermore, no information was presented on the vector status of the species or on the natural associations between the nematodes and the viruses. Therefore, the present information is reviewed here.

IV.4.b.- *Occurrence of trichodorid nematodes and TRV - countries*: Barbez (1983) and Coolen *et al.* (1980) estimated that 17 - 42% of potato fields in Flanders, **Belgium**, were infested with trichodorid nematodes. Subsequent studies by de Pelsmaeker and Coomans (1985; 1986; 1987) and de Pelsmaeker *et al.* (1985) showed that *c.* 15% of samples taken from potato fields in Flanders contained viruliferous trichodorid populations and that a significant correlation existed between the occurrence of TRV and the presence of *T. primitivus*, *T. similis*, *T. cylindricus* and *T. viruliferus*. De Waele and Coomans (1991) reported that 20% of over 2000 samples from a variety of vegetation types contained trichodorids. *T. primitivus* was the most widely distributed trichodorid species in Belgium and occurred in the highest densities although *P. pachydermus* and *T. similis* were also frequently found (Barbez, 1983; de Pelsmaeker and Coomans, 1986; de Waele and Coomans, 1991).

In **Denmark** trichodorids were found in *c.* 75% of samples taken from plant nurseries and tulip-, narcissus- and vegetable fields, with *T. primitivus* being the most prevalent species and *P. pachydermus*, *T. similis* and *P. teres* also frequently occurring (Sønderhousen *et al.*, 1969). Although spraing disease in potato was found in Denmark, the transmission of TRV by trichodorids was not established (Engsbro, 1976).

In **Finland** only *P. pachydermus* has been found, occurring in the south-western and central areas of the country (Kurppa, 1985). TRV, associated with spraing in potato, was first recorded in Finland in 1972 (Seppänen, 1972) and was isolated in the same year from *N. tabacum* cv. Samsun bait-plants grown in soil from Finnish nurseries (Tapio, 1972).

T. primitivus and *P. pachydermus* were the most common trichodorid species in potato fields in **France** (van Hoof, 1967c; van Hoof *et al.*, 1967). Out of 113 samples 52 contained trichodorid nematodes and TRV was isolated from 16 samples (van Hoof 1967c; van Hoof *et al.*, 1967). Spraing disease in potato, locally known as "liege", was observed in several regions in France (Dalmasso *et al.*, 1971; Joubert and Dalmasso, 1974).

In **Germany** *P. pachydermus* was the most widespread trichodorid species, occurring throughout the country, while *T. primitivus* was found mainly in north-western and *T. cylindricus* in the southern part of Germany (Sturhan, 1978). Spraing in potato was widespread in sandy soils in north-west Germany (Reepmeyer, 1973).

In **Great Britain** *T. primitivus* was the most frequently found species, occurring throughout the country, while *P. pachydermus*, a species also often found, was more restricted to eastern parts (Alphey and Boag, 1976; Boag and Alphey, 1977). Cooper (1971), from results obtained in a survey on the occurrence of trichodorid nematodes and TRV in Scotland, estimated that 15-20% of Scottish potato growing was on trichodorid - and 12% on TRV-infected soil.

An unidentified *Trichodorus* species was found in soil samples taken from fallow land in Ilia, **Greece** (Koliopanos and Kalyviotis-Gazelas, 1973).

In **Italy** *T. viruliferus* was the most widespread species and it was found on a range of cultivated plant species (Roca and Lamberti, 1984). TRV caused disease in lettuce (Ragozzino *et al.*, 1978) and sweet pepper (Marte *et al.*, 1979; Roca *et al.*, 1984).

P. pachydermus was the most prevalent trichodorid nematode in the **Netherlands**, and *T. similis* was also widely distributed (Seinhorst and van Hoof, 1982). The

occurrence of *P. teres* was restricted to fine marine sandy soils (Seinhorst and van Hoof, 1982). TRV caused disease in several ornamental bulbous crops and potato in the Netherlands (Maas, 1974), while the early-browning disease of pea, caused by PEBV, was of some importance in north- and south-western parts of the country (Bos and van der Want, 1962).

P. pachydermus as well as an unidentified *Trichodorus* species were common in the southern part of **Norway** . TRV was already in the late 1950's frequently identified from diseased potato plants (Støen and Markussen, 1985).

In **Poland** *P. pachydermus* was the most common trichodorid species while *T. primitivus*, *T. viruliferus* and *T. similis* were also frequently found (Brzeski and Szczygiel, 1974).

In **Portugal** 11 trichodorid species have been recorded (Almeida and Santos, 1992). TRV has not been recorded from Portugal.

Data on the distribution of trichodorids in **Spain** are limited although a number of species have been found (Arias and Andrés, 1989).

In southern **Sweden** *P. pachydermus* and *T. primitivus* are widespread (Eriksson and Banck, 1985) and spraing in potatoes has been observed in many places in south Sweden (Persson, 1968).

Gugerli (1977) estimated over 50% of arable land in **Switzerland** was infected with TRV and spraing disease in potato occurred frequently.

IV.4.c.- *Occurrence of trichodorid nematodes and TRV - species*: ***T. cylindricus*** has been found in Belgium (de Waele and Coomans, 1983), Denmark (Sønderhousen *et al.*, 1969), France (Scotto la Massese, 1985), Germany (Sturhan, 1967), Great

Britain (Alphey and Boag, 1976; Boag and Alphey, 1977), the Netherlands (Seinhorst and van Hoof, 1982; van Hoof, 1967a), Poland (Brzeski and Szczygiel, 1974; Szczygiel and Brzeski, 1985) and Switzerland (Gugerli, 1977). In the Netherlands *T. cylindricus* transmitted an uncharacterized isolate of TRV (van Hoof, 1968) and *T. cylindricus* from Scotland transmitted two serologically distinct isolates of TRV (See Chapter VII).

T. primitivus occurs in Belgium (Coolen *et al.*, 1980; de Waele and Coomans, 1983; 1991), Bulgaria (Choleva, 1988), Denmark (Andersen, 1972; Sønderhousen *et al.*, 1969), France (Scotto la Massese, 1985), Germany (Sturhan, 1967), Great Britain (Alphey and Boag, 1976; Boag and Alphey, 1977), Ireland (Moore 1967), Italy (Coiro *et al.*, 1989), the Netherlands (Seinhorst and van Hoof, 1982; van Hoof, 1967a), Norway (Støen and Markussen, 1985), Poland (Brzeski and Szczygiel, 1974; Szczygiel and Brzeski, 1985), Portugal (Almeida *et al.*, 1989; Craveiro and Santos, 1984), the Soviet Union (Antonova *et al.*, 1982), Spain (Arias and Bello, 1988) and Sweden (Eriksson and Banck, 1985; Persson, 1968). The transmission of TRV by *T. primitivus* was reported in Great Britain initially by Harrison (1961) and Mowat and Taylor (1962). Harrison (1966) later demonstrated that adult and juvenile specimens of *T. primitivus* were able to transmit the British isolate of PEBV (=PEBV-B) to healthy pea bait-plants. The transmitted virus caused typical PEBV symptoms when manually inoculated on *Phaseolus vulgaris* and the identity of the virus was further confirmed in serological tests using an antiserum prepared against PEBV-B. Furthermore, initially virus-free *T. primitivus* acquired PEBV-B from manually inoculated source plants and subsequently transmitted the virus to healthy bait-plants, but failed to do so when given access to PEBV-D, which is only distantly related to

PEBV-B (Harrison, 1966). Kurppa *et al.* (1981) suggested that *T. primitivus* might be the natural vector of TRV strain SYM and Cooper (1972) reported transmission of TRV-isolates ASH1, ASH2, COR, RQ and CA by this species. Isolates ASH1, COR and CA were serologically tested using antiserum prepared against the PRN-strain of TRV resulting in dilution end points of 64, 64 and 128 respectively with the homologous titre being 256. The species has also been reported as a vector of TRV in France, Germany and the Netherlands (Sänger, 1961; van Hoof, 1968; van Hoof *et al.*, 1967), and in Sweden males transmitted TRV to *N. tabacum* cv. White Burley bait-plants (Persson, 1968). *T. primitivus* has also been associated with spraing disease in Belgium in potato cv. Bintje (de Pelsmaeker, 1987; de Pelsmaeker and Coomans, 1987), a cultivar supposedly resistant to TRV (Harrison, 1968), with spraing disease in potato in the Soviet Union (Antonova *et al.*, 1982), in Ireland (Moore, 1967) and in France (Dalmasso *et al.*, 1971; Joubert and Dalmasso, 1974) and with stem mottle of potato in the Netherlands (van Hoof, 1980).

T. similis has been recorded from Belgium (Coolen *et al.*, 1980; de Waele and Coomans, 1983), Bulgaria (Choleva, 1988), Denmark (Sønderhousen *et al.*, 1969), France (Scotto la Massese, 1985), Germany (Sturhan, 1967), Great Britain (Alphey and Boag, 1976; Boag and Alphey, 1977), Italy (Roca and Lamberti, 1984; 1985), the Netherlands (Seinhorst and van Hoof, 1982; van Hoof, 1967a), Norway (Støen and Markussen, 1985), Poland (Brzeski and Szczygiel, 1974; Szczygiel and Brzeski, 1985), the Soviet Union (Antonova *et al.*, 1982) and Sweden (Eriksson and Banck, 1985; Persson, 1968). In the Netherlands *T. similis* transmitted TRV to *N. tabacum* cv. White Burley (van Hoof, 1968) and TRV-isolate "Gl", causing notched leaf symptoms in *Gladiolus* (Cremer and Schenk, 1967). Isolate Gl did not react in

serology with antisera prepared against the Dutch type strain of TRV (= TRV-Lisse ??) nor with an antiserum prepared against PEBV-D (Cremer and Schenk, 1967; Maat, 1967). A mixed trichodorid population of *T. similis* and *P. pachydermus* in the Netherlands was associated with spraing in potato cv. Bintje (van Hoof, 1972a) and a *T. similis* population in Belgium with spraing in potato cv. Kennebec (de Pelsmaeker, 1987; de Pelsmaeker and Coomans, 1987). *T. similis* from "Landes", France was associated with the presence of TRV in potato fields (van Hoof *et al.*, 1967).

T. sparsus has been reported from Belgium (de Waele and Coomans, 1983), Bulgaria (Choleva, 1988), Germany (Sturhan, 1981), Great Britain (Alphey and Boag, 1976; Boag and Alphey, 1977), Italy (Roca and Lamberti, 1984; 1985), The Netherlands (Seinhorst and van Hoof, 1982), Poland (Szczygiel, 1968; Szczygiel and Brzeski, 1985), Romania (I. Popovici, pers. comm.), Sweden (Eriksson, 1974; Eriksson and Banck, 1985) and Switzerland (Loof, 1975). *T. sparsus* , originally referred to as *T.x* by van Hoof (1968) transmitted an uncharacterized isolate of TRV (van Hoof, 1968; 1970a).

T. variopapillatus has been found in Belgium (de Waele and Coomans, 1983; 1991), Germany (Sturhan, 1974), Great Britain (Alphey and Boag, 1976; Boag and Alphey, 1977), Italy (Roca and Lamberti, 1984; 1985), the Netherlands (Seinhorst and van Hoof, 1982) and Poland (Brzeski, 1979; Szczygiel and Brzeski, 1985). *T. variopapillatus* has not been reported to transmit virus.

T. velatus has been recorded from Belgium (de Waele and Coomans, 1983; 1991), Bulgaria (Peneva, 1988), France (Scotto la Massese, 1985), Germany (Sturhan, 1988) and Great Britain (Alphey and Boag, 1976; Boag and Alphey, 1977). *T. velatus* has not been reported to transmit virus.

T. viruliferus has been found in Belgium (de Waele and Coomans, 1983; 1991), Bulgaria (Choleva, 1988), France (Scotto la Massese, 1985), Germany (Sturhan, 1967), Great Britain (Alphey and Boag, 1976; Boag and Alphey, 1977), Italy (Roca and Lamberti; 1984; 1985) the Netherlands (Seinhorst and van Hoof, 1982; van Hoof, 1967a), Poland (Brzeski and Szczygiel, 1974; Szczygiel and Brzeski, 1985) and Sweden (Banck, 1988). *T. viruliferus* extracted from soil from a sweet pepper field in Asti, Italy, with a high incidence of TRV, transmitted TRV to sweet pepper and tomato bait-plants. TRV isolated from infected sweet pepper plants in the field reacted in serology with an antiserum prepared against TRV-strain PV AS-73 (Roca *et al.*, 1977). Also, *T. viruliferus* extracted from soil samples from various sites in northern Italy transmitted TRV. The seven virus isolates obtained from the bait-plants infected by this nematode species were serologically tested using antisera prepared against PEBV-D, PEBV-B, TRV-Lisse and TRV-L20 (=TRV-Gl ?) and all reacted to some extent with the TRV-Lisse antiserum (van Hoof *et al.*, 1966). However one isolate (Italian nr. 6) was closely related to PEBV-B and was later shown to be a natural recombinant having TRV RNA-1 but a coat-protein similar to that of PEBV-B (Robinson *et al.*, 1987), whereas another isolate (Italian nr. 5) was more related to TRV-L20 (van Hoof *et al.*, 1966). In the Netherlands TRV was also transmitted by *T. viruliferus* (van Hoof, 1968) and, in Great Britain, Gibbs and Harrison (1964a) reported transmission of PEBV, serologically and symptomatologically indistinguishable from PEBV-B, by *T. viruliferus* from PEBV infected pea fields in East-Anglia.

P. anemones has been found in France (Scotto la Massese, 1985), Great Britain (Alphey and Boag, 1976; Boag and Alphey, 1977), the Netherlands (Seinhorst and

van Hoof, 1982; van Hoof, 1967a) and Portugal (Almeida and Santos, 1988; 1992). *P. anemones* from the Netherlands transmitted TRV (van Hoof, 1968) and in Britain, where it is relatively widespread in North-East England and East-Anglia (Alphey and Boag, 1976; Boag and Alphey, 1977), it transmitted two British isolates of PEBV but failed to transmit a Dutch isolate of this virus (Harrison, 1967). Recently the transmission of a TRV isolate of unknown serological properties by individual *P. anemones* nematodes from England was reported (See Chapter VII).

P. nanus occurs in Belgium (de Waele and Coomans, 1983; 1991), France (Scotto la Massese, 1985), Germany (Sturhan, 1967), Great Britain (Alphey and Boag, 1976; Boag and Alphey, 1977), Italy (van Hoof *et al.*, 1966), the Netherlands (Seinhorst and van Hoof, 1982; van Hoof, 1967c) and Portugal (Almeida and Santos, 1992). In Britain *P. nanus* is a vector of a TRV-isolate serologically related to the PRN-strain (Cadman and Harrison, 1959; Cooper and Thomas, 1970), and in the Netherlands of an uncharacterized isolate of TRV (van Hoof, 1968). *P. nanus* was found in large numbers (up to 768 nematodes/500 g soil) in samples taken from flower bulb fields in the Netherlands (van Hoof, 1973).

P. pachydermus is widely distributed throughout Europe. In Belgium *P. pachydermus* has been frequently found in potato growing areas in Flanders (de Pelsmaeker and Coomans, 1985; 1986; 1987; de Pelsmaeker *et al.*, 1985). This species has also been found in Bulgaria (Choleva, 1988), Czechoslovakia (Lišková, 1980; Lišková and Valocká, 1977), Denmark (Sønderhousen *et al.*, 1969), Finland (Seppänen, 1972; Tapio, 1972) France (Scotto la Massese, 1985) Germany (Fritzsche *et al.*, 1985; Sturhan, 1967), Great Britain (Alphey and Boag, 1976; Boag and Alphey, 1977), Italy (Ragozzino *et al.*, 1978), the Netherlands (van Hoof, 1967a,

Seinhorst and van Hoof, 1982), Norway (Bjørnstad and Støen, 1967; Støen and Markussen, 1985), Poland (Brzeski and Szczygiel, 1974; Szczygiel and Brzeski, 1985), Portugal, (Almeida and Santos, 1988; 1992), the Soviet Union (Antonova *et al.*, 1982; Gus'kova and Antonova, 1982), Sweden (Eriksson and Banck, 1985; Persson, 1968) and in Switzerland (Gugerli, 1977). In many reports *P. pachydermus* has been associated with tobraviruses. In 1960 Sol *et al.* (1960) established *P. pachydermus* as being a vector of TRV in the Netherlands, the first report of trichodorid nematodes transmitting virus, and subsequently *P. pachydermus* populations from four different locations transmitted uncharacterized isolates of TRV (Sol and Seinhorst, 1961; van Hoof, 1964d). A population from Wanroy, the Netherlands acquired and subsequently transmitted a TRV-isolate from the same location, serologically related to TRV isolate "Lisse" but also reacting slightly with an antiserum prepared against a Dutch isolate of PEBV (=PEBV-D) (van Hoof, 1968). Van Hoof (1963) also obtained uncharacterized TRV-isolates from four of 12 samples in which *P. pachydermus* was the only trichodorid species present. Virus isolates obtained from bait-plants grown in soil containing *P. pachydermus* were tested serologically against an unidentified TRV antiserum. These tests confirmed the presence of TRV and also showed that isolates obtained from different field sites were serologically related (van Hoof, 1963; 1964b). An isolate of TRV causing "notched leaf" symptoms in Gladiolus was also transmitted by *P. pachydermus* (Cremer and Schenk, 1967) and van Hoof (1962) reported *P. pachydermus* to be a vector of an uncharacterized isolate of PEBV. In Great Britain a virus was transmitted to sugar beet seedlings by males and females of *P. pachydermus* extracted from soil taken from a sugar beet field in East-Anglia where beet growth

was poor and plants were stunted. In subsequent serological tests the *P. pachydermus* transmitted virus was identified as TRV (Gibbs and Harrison, 1964b) and it was concluded that TRV and trichodorid nematodes were likely to be an important factor in causing a disease in sugar beet known as "Docking disorder". Transmission of TRV by *P. pachydermus* was also reported in France where the transmitted isolate was serologically related to the Dutch TRV-Lisse isolate (van Hoof *et al.*, 1967), in Germany (Sänger, 1961; Rau, 1975), Finland (Tapio, 1972), Norway (Bjørnstad and Støen, 1967) and the Soviet Union (Leshcheva, 1982). Furthermore, trichodorid populations, consisting mainly of *P. pachydermus*, were associated with the occurrence of spraing disease in potato in Finland (Seppänen, 1972; Tapio, 1972), France (Dalmasso *et al.*, 1971; Joubert and Dalmasso, 1974; Joubert *et al.*, 1971), Germany (Reepmeyer, 1973; Steudel, 1969), Great Britain, (Brown and Sykes, 1973; Cooper, 1971; Cooper and Thomas, 1971; Harrison, 1968; Sykes, 1975), Norway (Bjørnstad and Støen, 1967), the Soviet Union (Antonova *et al.*, 1982), The Netherlands (van Hoof, 1972a) and Sweden (Eriksson and Insunza, 1986; Persson, 1968). In the latter two countries potatoes of cv. Bintje, initially considered to be resistant to spraing (Harrison, 1968), showed severe spraing symptoms (Persson, 1968; van Hoof, 1972a).

P. teres has been found in Belgium (de Waele and Coomans, 1983; 1991), Denmark (Sønderhousen *et al.*, 1969), France (Scotto la Massese, 1985), Germany (Sturhan, 1967), Great Britain (Alphey and Boag, 1976; Boag and Alphey, 1977), Italy (Coiro *et al.*, 1989; van Hoof *et al.*, 1966), the Netherlands (Seinhorst and van Hoof, 1982; van Hoof, 1967a), Poland (Brzeski and Szczygiel, 1974; Szczygiel and Brzeski, 1985) and Sweden (Banck, 1977; Eriksson and Banck, 1985). In the

Netherlands, where this species is confined to very light marine sandy soils, it transmitted TRV to *N. tabacum* cv. White Burley bait-plants (van Hoof, 1964e; 1968) and individual *P. teres* nematodes from a Dutch population transmitted TRV, serologically related to TRV strain ORE (Jensen *et al.*, 1974) to *Petunia hybrida* bait-plants (See Chapter VII).

T. hooperi was found locally in South-West England (Alphey and Boag, 1976; Boag and Alphey, 1977) and Germany (Pietler and Decker, 1981). Alphey (1974) reported that *T. hooperi* acquired TRV from manually inoculated leaves of *N. tabacum* cv. White Burley leaves buried in the soil and subsequently transmitted the virus to healthy leaves.

P. minor has been reported from Germany (Braasch, 1976), Italy (Roca and Lamberti, 1984; 1985), the Netherlands (Bongers, 1988), Portugal (Almeida and Santos, 1988; 1992) and Spain (Arias and Bello, 1988). Although this species has not been reported as a virus vector in Europe it has been shown to transmit tobravirus in Japan (Komuro *et al.*, 1970), Brazil (Salomão, 1973) and the USA (Ayala and Allen, 1966; 1968; Walkinshaw *et al.*, 1961).

P. renifer was found around Boskoop, a centre for forest nurseries in the Netherlands (Bongers, 1988), and in Germany (Braasch, 1976). The distribution of this species is thought to be very restricted and virus transmission by *P. renifer* has not been reported.

T. aequalis was found in Denmark (Sønderhousen *et al.*, 1969), Germany (Rössner, 1969; Sturhan, 1967) and Spain (Santiago *et al.*, 1984).

P. acutus, from the island of Madeira, Portugal, (Almeida and Santos, 1992) was also reported from Spain (Arias and Andrés, 1989). Neither of these latter two species was associated with virus.

P. allius, a vector of TRV in the USA (Ayala and Allen, 1968, Jensen and Allen, 1964) but not associated with virus in Europe, was reported from Italy as *P. tansaniensis* (Roca and Lamberti, 1984; 1985) and has been reported from Portugal (Almeida and Santos, 1992; Craveiro and Santos, 1984).

P. tunisiensis, a vector of an uncharacterized isolate of TRV in Italy (Roca and Rana, 1981), and ***T. taylori*** have been found only in Italy (Roca and Lamberti, 1984; 1985), ***T. azorensis*** has been reported from the Açores islands and other trichodorid species reported only from Portugal are ***T. beirensis***, ***T. lusitanicus***, and ***P. porosus*** (Almeida and Santos, 1992). The latter species is a vector of TRV in the USA (Ayala and Allen, 1966; 1968) but has not been associated with virus in Portugal.

T. cedarus and ***P. hispanus*** have been reported only from Spain (Arias and Andrés, 1989), ***P. weischeri*** only from Germany (Sturhan, 1985) and ***P. macrostylus*** was recently described from Romania (Popovici, 1989). Neither of these four species was associated with virus.

The distribution of trichodorids and associated viruses is shown in Fig. 4.

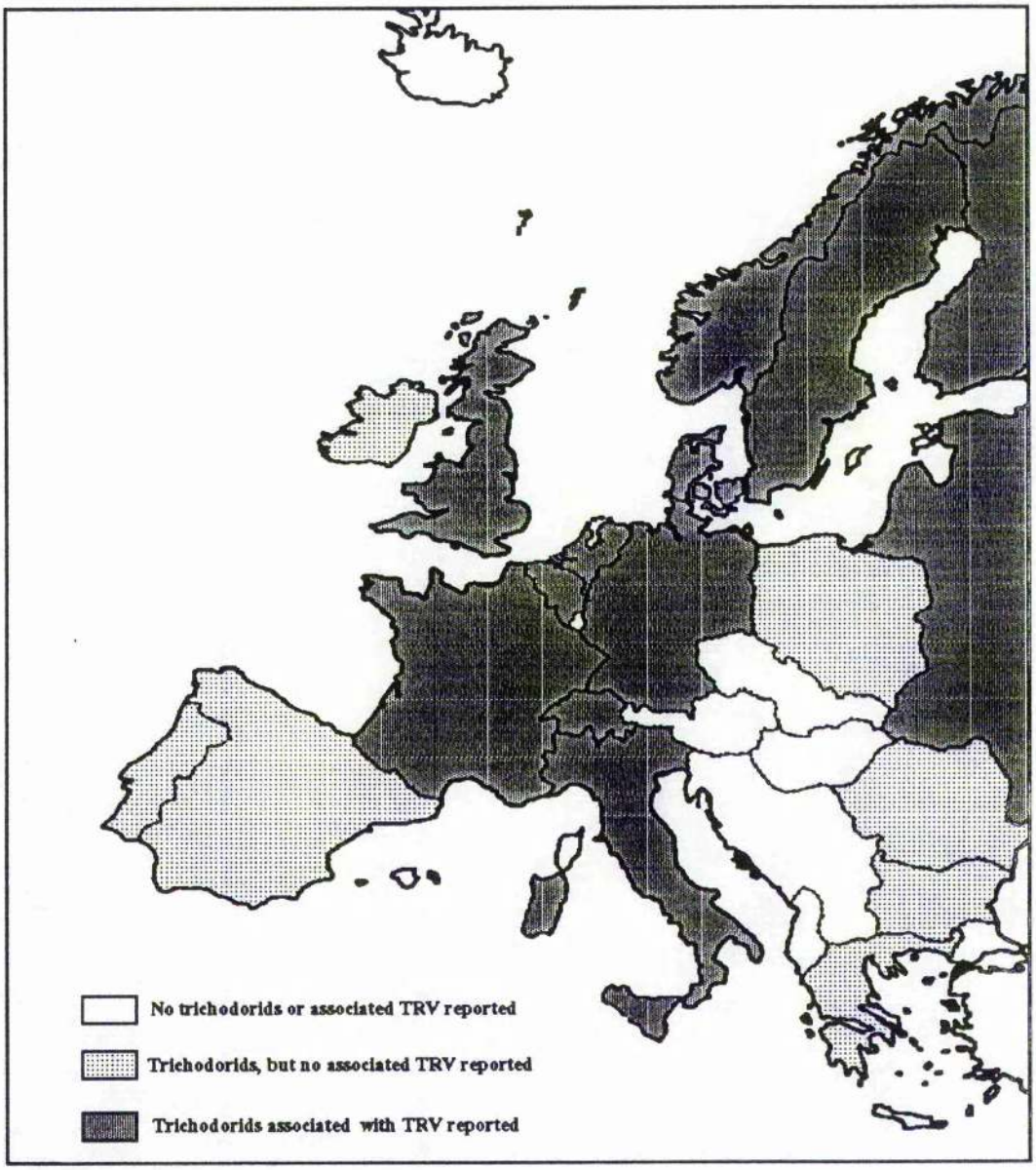

Figure 4. Occurrence of trichodorids and associated viruses in European countries.

IV.5. METHODS AND CRITERIA IN THE STUDY OF VIRUS-VECTOR ASSOCIATIONS

Diverse methods have been used to assess the ability of a trichodorid population to transmit virus. Furthermore, many reports lack a proper description of the methods used or fail to unequivocally identify the virus transmitted and/or to exclude the possibilities of alternative vectors. Also, results based on field observations or on virus transmission to bait-plants using bulk nematode suspensions extracted from field soil, are often difficult to interpret because of the frequent occurrence of mixed-species trichodorid populations. Similar problems were also encountered by workers studying longidorid vector - nepovirus associations. Trudgill *et al.* (1983) therefore proposed a set of criteria which should be fulfilled when claiming a nematode species to be a virus vector. They are:

1. The nematode and virus must be fully identified and characterised.
2. Bait-plant tissue must be shown to be infected with the virus.
3. The nematode must be shown to be the only possible vector of the virus.

Furthermore, a comprehensive virus transmission system for research on nepovirus-longidorid associations was described (Trudgill *et al.*, 1983). Only recently a similar method for studying virus transmission by trichodorids was developed (See V.2.b.), which allows the above mentioned criteria to be fulfilled.

IV.6. FACTORS AFFECTING TRANSMISSION OF AND DISEASES CAUSED BY TOBACCO RATTLE VIRUS.

IV.6.a.- *Soil type*: Several factors are known to affect disease incidence and/or development. Tobravirus problems are almost exclusively encountered on sandy and light loamy soils, which are the preferred habitat of the trichodorid nematodes. However, in some instances the virus can occur in absence of the vector as a result of using vegetatively propagated infected plant material for (e.g. potato tubers, flower bulbs). Primary infection (infection in the year of planting, sowing) in a field can usually be distinguished from secondary infection (infection as a result of planting infected material or sowing infected seed) because the distribution of the former tends to be clumped whereas that of the latter tends to be more random (Asjes, 1989; Cremer and Schenk, 1967).

IV.6.b.- *Soil moisture*: Loevgren and Eriksson (1989) and Richter (1968) found a positive correlation between the amount of rainfall in spring and autumn and the incidence of spraing in potato. During this period tuber initiation starts and it is at this stage that the newly formed are most susceptible to TRV infection (van Hoof, 1964c). Cooke (1973) also found an increase in "Docking disorder", a disease in sugarbeet caused by direct feeding of trichodorid (and longidorid) nematodes, in years with high rainfall in May. These correlations were confirmed by Cooper and Harrison (1973) and van Hoof (1976) who demonstrated an increase in TRV transmission by trichodorids with increasing soil moisture. They attributed this to the increased mobility of the nematodes in the wetter (around soil capacity) soils.

IV.6.c.- *Soil temperature*: With tulip, a crop which is planted in the autumn and in which TRV infection occurs soon after planting, it was found that the proportion of plants showing primary TRV symptoms in the following spring was much higher in those years with long cold spells during the winter (Asjes, 1989). This could be due to the slower growth of the bulbs during such cold periods, giving the virus the opportunity to be translocated from the infected roots to the growing tip, resulting in primary symptoms in the crop the following spring.

IV.6.d.- *Cultivar and crop influence*: Different cultivars of tulip show big differences in susceptibility towards TRV infection. The popular "Darwin hybrid" cultivars are particularly susceptible to TRV (Asjes, 1989). In potatoes differences between cultivars in sensitivity towards spraing also occur (Dale, 1989), with cultivars like "Eigenheimer" and "Pentland Dell" being very sensitive and "Bintje" and "Arran" rarely becoming infected. Especially with tulips however, it is difficult to assess from literature data whether different responses of cultivars towards TRV are based on differences in tolerance/sensitivity or on differences in resistance/susceptibility.

IV.6.f.- *Virus variation*: Maas (1975), van Hoof (1965) and Dale (1989) obtained inconsistent results when testing the susceptibility of new potato cultivars towards spraing in different experimental fields. An explanation for this inconsistency may be that different trichodorid species are associated with different isolates of TRV, resulting in different responses in the potato cultivars. Van Hoof (1964a) demonstrated that different isolates of TRV, when mechanically inoculated (with a

pin), caused different symptoms in tubers from a range of potato cultivars. Differences between TRV-isolates, related to the host plants from which they were isolated, were reported by Linthorst and Bol (1986). They found that TRV-isolates could be characterized by cDNA hybridization as two distinct groups, one naturally infecting potato, the other tulip. The field observations made by Asjes (Asjes, pers. comm.) that tulips do not usually become infected when grown on fields with a history of spraing in potato, is supported by these observations.

IV.7. CONTROL

IV.7.a.- *Chemical*: The control of TRV usually relies on the use of soil fumigants which kill the vector nematodes. Whereas in potatoes spraing can be successfully controlled by the application of such chemicals, control in ornamental bulbous crops is often unsatisfactory.

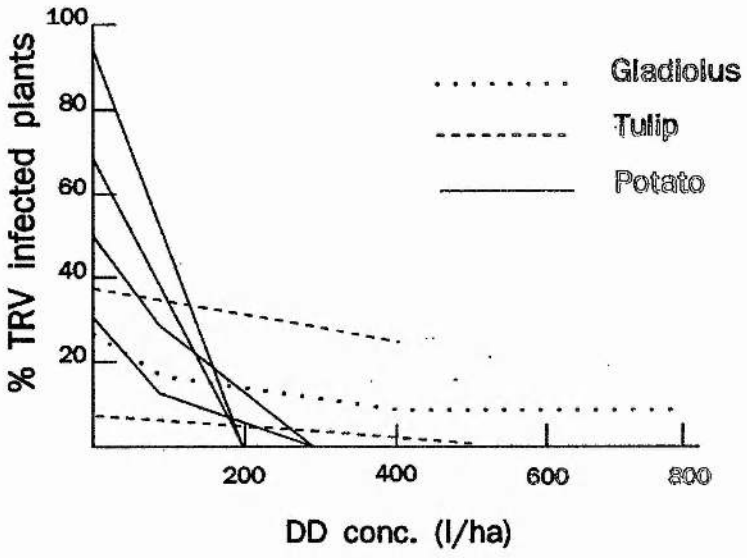

Figure 5. Control of TRV by DD in potato, tulip and *gladiolus*. Data from Cooper and Thomas (1971), Maas (1974; 1975), Seinhorst and van Hoof (1976).

Maas (1974) attributed this to the different ways in which ornamental bulbous crops and potatoes are affected by the virus; bulbous crops generally become systemically infected whereas the virus usually stays localized around the infection site in spraing affected tubers. With ornamental bulbous crops disease incidence is expressed as the number of infected plants (on a yes or no scale), with potatoes spraing incidence can be scored on a graded scale. Thus, with potato the spraing incidence is correlated with the number of virus transmissions by the nematodes to the tubers, as each successful transmission may result in an increase in spraing incidence. With tulip however, transmission of TRV to an already infected plant is not reflected in an increase of the number of TRV-infected plants (disease incidence) in the field, and therefore the correlation between trichodorid numbers and TRV incidence is often not clear. Furthermore, few successful virus transmissions, e.g. due to low numbers of vector trichodorids, may in ornamental bulbous crops still result in a number plants becoming systemically infected with TRV, whereas in potato this situation will usually result in a few arcs or rings on a limited number of tubers.

IV.7.b.- *Crop rotation*: Crop rotation has been studied as a means of controlling the TRV/trichodorid problem. The wide host range of both the trichodorids and the viruses however limits the number of suitable crops which can be used. Several authors (French and Wilson, 1976; Maas, 1975; Symalla, 1972) reported that growing barley decreased the TRV-infectivity of the soil, although barley was found to be a good host for trichodorids (Kegler *et al.*, 1984; Symalla, 1972). Maas (1975) hypothesised that this effect was due to a decrease in the proportion of nematodes carrying virus: the nematodes feeding on barley, which itself does not become

infected by the virus, moult and lose their virus. With no other virus infected plants available to the nematodes this would result in the population gradually losing the virus.

In contrast with these results however are those of Loevgren and Eriksson (1989) and Richter (1968) who found an increased spraing incidence in potato after barley. Weed control is thought to be essential when attempting to control TRV problems by crop rotation since the virus-infected weeds could act as a virus reservoir on which the nematodes feed (Murant, 1981) . Cooper and Harrison (1973) however found that spraing incidence tripled in potato crops following a 18 month weed free period and attributed this to the nematodes not having an alternative (better) plant on which to feed. Although some differences between crops were found regarding TRV infection of the soil in the following season (Kuiper, 1977; Loevgren and Eriksson, 1989; Symalla, 1972), at present there is no crop available which can reduce TRV and or trichodorids to practically acceptable levels.

IV.7.c.- *Other cultural practices*: Other cultural practices e.g. increased soil cultivation, or fertilization have been suggested to decrease trichodorids or TRV (Boag, 1983; Oostenbrink, 1964; van der Want, 1952) but no specific data on these subjects are available. Flooding of flower bulb fields is at present under investigation in the Netherlands as a means of controlling soil borne diseases, but no significant effects on TRV infection were found under field conditions (Asjes, 1989). Late planting of tulips (e.g. November/December instead of October) considerably reduces the incidence of TRV infection in tulips (Asjes, 1974; 1989), most likely due to an inactivation of the nematodes as a result of lower soil temperatures.

IV.7.d- *Resistance*: Breeding for resistance to TRV is being undertaken in potato, but the variability in TRV isolates still poses a problem when screening new cultivars for resistance or insensitivity (Dale, 1989). Genetically engineered resistance to TRV has been achieved in tobacco plants by inserting the virus coat protein gene in the plant genome (Angenent *et al.*, 1990; van Dun and Bol, 1988). However, resistance seems limited only to isolates with coat proteins similar to the one produced by the engineered plant, and results so far are based only on experiments in which plants were manually inoculated with the virus.

CHAPTER V.

GENERAL MATERIALS AND METHODS.

V.1. NEMATODES.

V.1.a.- *Sampling and storage:* Small amounts of soil (max. 2 kg) were sampled with a tulip planting tool - a metal tube with an inner diameter of 15 cm and a length of 30 cm to which a handle is attached. The tool is pushed into the soil, rotated, and withdrawn to provide cores of soil of approximately 2 kg. By sampling twice from the same spot samples of up to 60 cm depth can be obtained.

Larger amounts of soil were sampled with a spade by cutting out a block of soil of 30x30x30 cm. Samples were transferred into plastic bags and transported to the laboratory by car. Upon arrival, samples were stored at 4 °C in the dark, taking care not to store samples on top of one another.

V.1.b.- *Extraction:* Soil samples were extracted according to a modified sieving and decanting method (Brown and Boag, 1988) (see Fig. 6). The soil was spread on a clean table and gently but thoroughly mixed by hand whilst removing large stones and plant debris (1). With a tablespoon a 200 g subsample was taken and transferred into a 5 l plastic bucket. Water from a tap, which had been running for at least several minutes, was added to just cover the soil. This was left for 1 hr. (2). Remaining lumps of soil were gently dispersed by hand and the soil was brought into suspension by filling the bucket with a vigorous jet water (3). After 15 s the supernatant was poured over a bank of sieves of decreasing pore sizes of 2000, 150,

75, 75, and 53 µm (4). The soil debris remaining in the bucket and the catch remaining on the 2000 µm sieve was discarded (5). The catches remaining on the smaller pore size sieves were carefully washed into a 500 ml plastic beaker (6). The suspension was left to settle for 10 sec. and the complete contents of the beaker subsequently washed onto a wet Kleenex tissue (Professional wipes - Kimberley-Clark, London) supported by a plastic extraction sieve (pore size 1 mm) (7). The extraction sieve was immediately placed on a Baermann funnel (Baermann, 1917) filled with enough water to just cover the bottom of the extraction sieve (8). When the objective was to determine total numbers of trichodorid nematodes, 25 ml of the suspension in the bottom of the funnel was collected after 48 hr. in a glass tube. The nematode suspension in the tube was left to settle for 10 min. and subsequently concentrated to 10 ml by removing the top part of the suspension with a Pasteur pipette connected to a vacuum pump. The 10 ml suspension was then examined using a low power microscope at 40 x magnification.

When extracting bulks of soil for nematodes to be used in transmission experiments, samples of up to approximately 400 g were extracted. However, to avoid a decrease in vitality of the nematodes caused by oxygen shortage in the bottom of the Baermann funnel, samples were collected after a 16 hr. extraction period on the Baermann funnel .

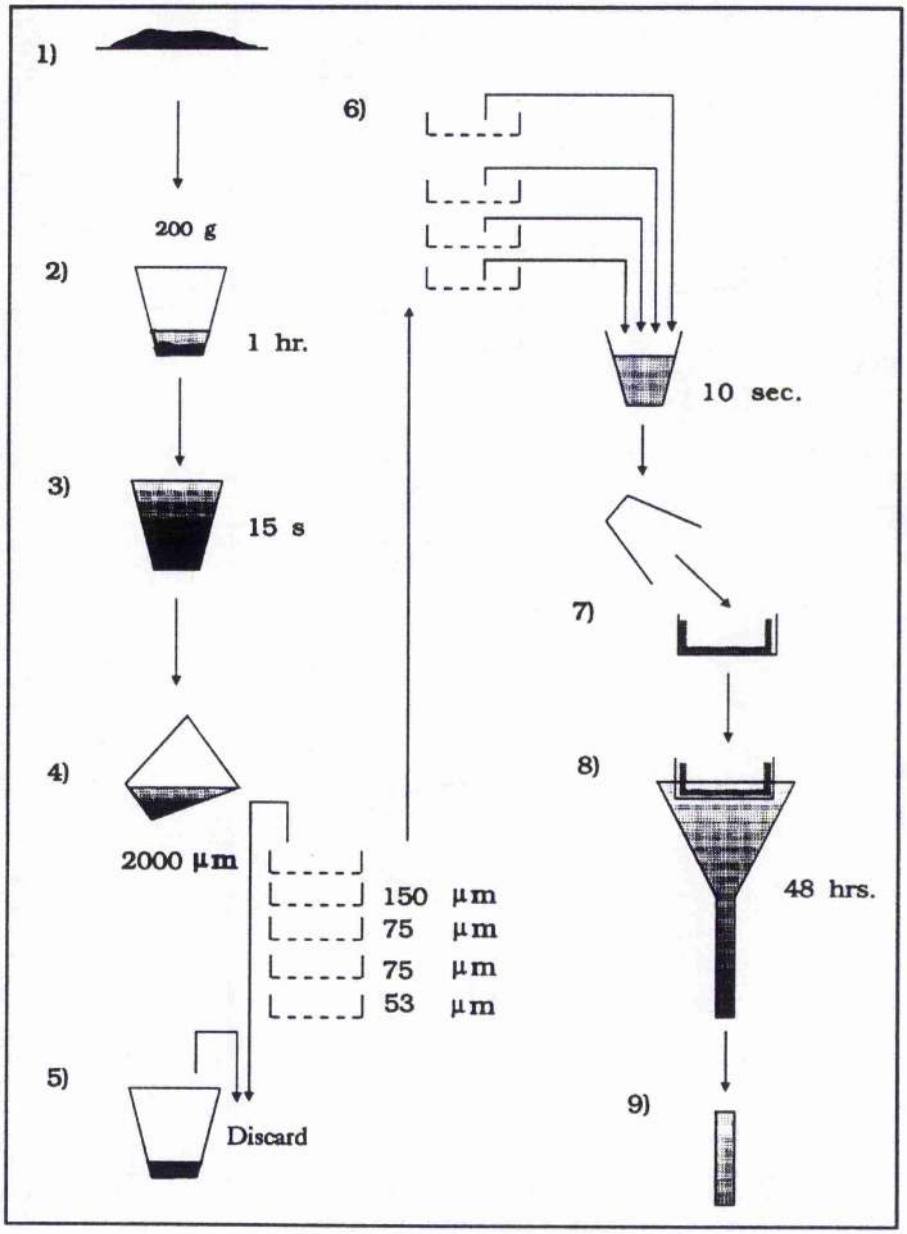

Figure 6. Extraction of trichodorid nematodes from soil.

V.1.c.- *Killing and mounting:* Nematodes were killed according to the method of Seinhorst (1966) and Netscher and Seinhorst (1969); Nematodes were hand-picked with a fine metal needle into a small drop of water on a watchglass. The watchglass was placed on a glass dish (diameter smaller than of the watchglass) filled with water, touching the watchglass. A formaldehyde\propionic-acid solution (10.8 ml formalin + 1 ml propionic acid + 88.2 ml distilled water) was heated to 95 °C in a water bath and poured over the nematodes with a pipette. The nematodes were subsequently fixed and processed into dehydrated glycerol by Seinhorst's rapid glycerol-ethanol method (Seinhorst, 1959). The killed nematodes were transferred into a 1.2 ml PBI-dish containing a 4% formalin solution for fixation. The PBI-dish was covered with a coverslip and placed on a filter paper saturated with 4% formalin in a covered petri dish. The dish was left for at least 14 days. The nematodes were then transferred to solution S-1 (20 ml 96% ethanol + 1 ml glycerol + 79 ml distilled water) in a PBI-dish, which was covered with a coverslip and placed in a closed container over 96% ethanol at 37 °C. After 16 hr. the nematodes were again transferred into another PBI-dish containing solution S-2 (93 ml 96% ethanol + 7 ml glycerol). The PBI-dish was placed on a filter paper saturated with glycerol, covered with a coverslip and left at 37 °C for 3 hr. The coverslip was then removed and three drops of dehydrated glycerol were added to the dish. Four hours later the dish was placed in a desiccator over silicagel and left for *c.* 10 days. The nematodes were mounted in anhydrous glycerol on glass microscope slides (76mm x 26 mm) by the paraffin wax-ring method (de Maeseneer and D'Herde, 1963), and the slides were subsequently sealed (3 times) with Glyceel (Hopkin and Williams, Essex, England).

The nematodes were identified to species using a Zeiss high power microscope with Nemarski interference at magnification 650x.

V.2.- BAIT TESTING.

V.2.a.- *Pot bait test:* A 400 ml, 10 cm diameter plastic pot was filled with a subsample of the soil sample to be tested for the presence of TRV. Two 4 week-old *Petunia hybrida* Vilm. and two 4 week-old *Nicotiana tabacum* cv. White Burley seedlings were planted in the pot. The pots were placed in a temperature-controlled box (Taylor and Brown, 1975) in which a soil temperature of 17 °C was maintained. Tops were grown in daylight glasshouse conditions. After 4 weeks the tops of the bait-plants were visually examined for virus symptoms and the root system of each plant was washed free of soil and tested for the presence of virus (See V.4.a.*i*). Virus isolates were propagated (See V.4.b) and serologically tested in $F(ab')_2$-ELISA (See V.4.c).

V.2.b.- *Individual-nematode bait test:* The technique used for bait testing individual trichodorid nematodes is shown in Fig. 7. Plastic Beem capsules (0.5 ml, size 00, TAAB laboratories, Reading, England) were one-third filled with silver-sand delivered from a 5 ml Gilson pipette-tip from which 3 mm of the tip had been cut of. The silver-sand had been previously washed with tap water, sterilized by boiling for 2 min., air dried and sieved to give particle sizes between 125 and 350 µm.
To each capsule approximately 0.2 ml tap water was added. One trichodorid nematode, hand-picked from a nematode suspension using a fine metal needle under

a low power Leitz microscope at 50x magnification (1), was transferred into each capsule (2). The root systems of two week-old *P. hybrida* seedlings, grown in daylight glasshouse conditions in compost, were washed free of soil and one seedling was added to each capsule. Each capsule was then filled with silversand and water added from a Pasteur pipette to saturate the sand in the capsule (3). The capsules were partially plunged into moist sand in a plastic container which was placed in a covered seedling growth chamber to maintain a high humidity level. The chamber was placed at 20 °C, with an 18 hr. light regime (2000 lux). After 10-14 days the contents of each capsule were washed into a 25 ml glass tube with a Pasteur pipette, using approximately 7 ml of water (4). The suspension was swirled, left for 3 sec. to allow the sand to settle, and poured into a 5x5x1 cm plastic counting dish (5). The suspension in the counting dish was examined under a low power microscope at 40x magnification (6). The nematode, when found, was hand-picked into a drop of water on a glass microscope slide, heat-killed by holding the microscope slide for a few seconds over a flame from a spirit-burner, and identified to species using a high power Zeiss microscope at 650x magnification (7). The corresponding *P. hybrida* plant was transferred into compost and grown for a further 3-4 weeks in a glasshouse at 20 °C under natural daylight conditions. The roots of each *P. hybrida* were then washed free of compost, tested for the presence of virus (See V.4.a.*i*), and virus isolates were propagated (See V.4.b) and serologically tested in $F(ab')_2$-ELISA (See V.4.c).

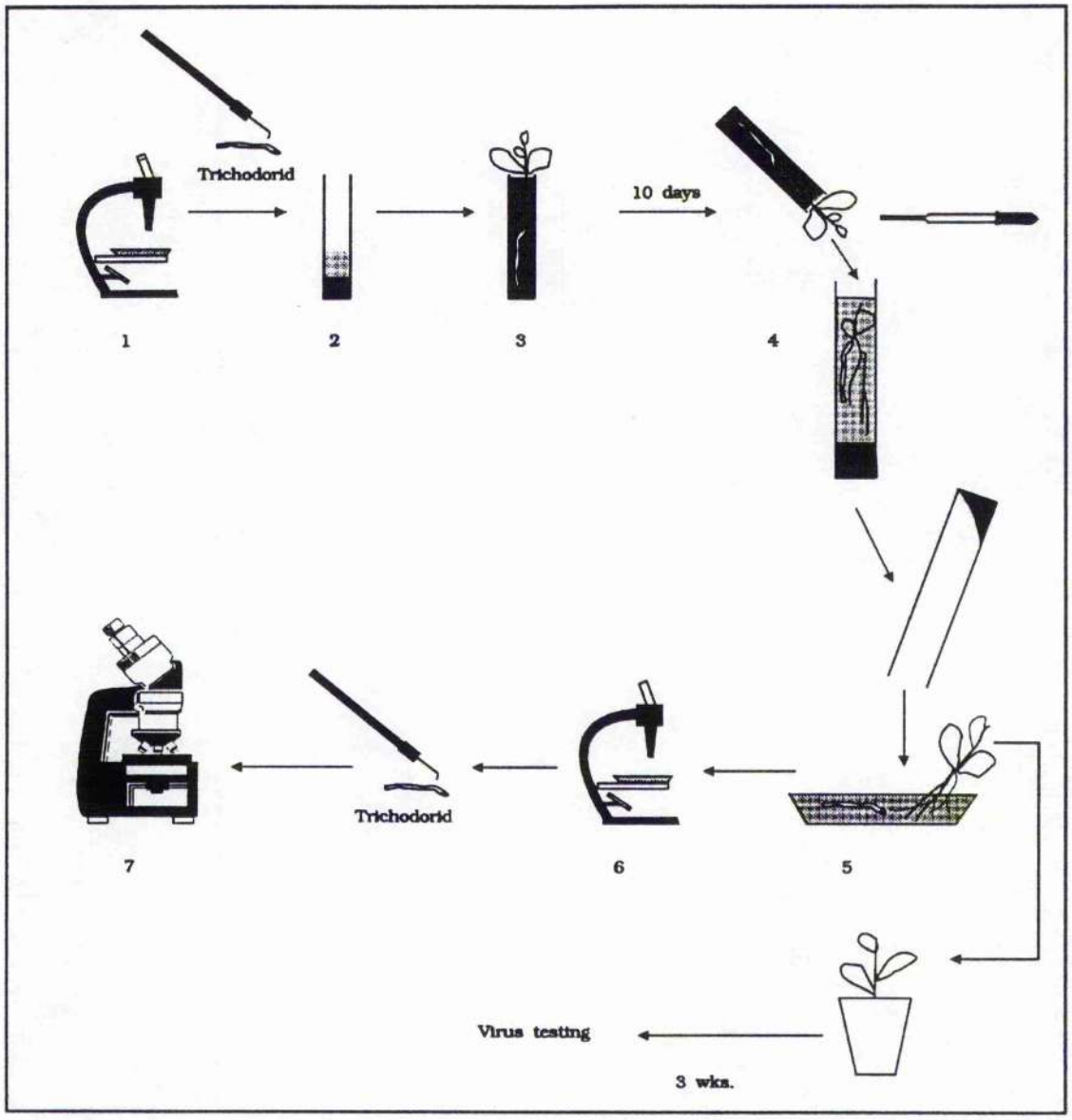

Figure 7. Single-nematode bait test system.

V.3. ACQUISITION AND TRANSMISSION TEST.

V.3.a.- *Virus source-plants:* A 6 week-old *N. clevelandii* plant was inoculated with a TRV-isolate from a frozen stock inoculum by rubbing carborundum dusted leaves with the inoculum. Ten days later two newly formed leaves were harvested, comminuted with a mortar and pestle and inoculated onto carborundum dusted leaves of a 4 week-old *P. hybrida plant.* Presence of virus in the inoculum was tested by inoculation onto a *C. amaranticolor* indicator plant. The inoculated *P. hybrida* plant was planted in compost and left for 1 week at 20 °C in the glasshouse under daylight conditions to allow the virus to systemically invade the root system. The plant was then carefully removed and the root system washed free from adhering compost. A small part of the root system was removed, comminuted with a mortar and pestle, and the resulting suspension inoculated onto carborundum dusted leaves of a *C. amaranticolor* indicator plant. The *P. hybrida* plant was transferred into a 25 ml plastic pot, to which 5 ml water was added. The pot was partially plunged into moist sand in a covered seedling growth chamber to maintain a high humidity level. The chamber was placed at 20 °C, with an 18 hr. light regime (2000 lux). When lesions appeared on the inoculated leaves of the *C. amaranticolor* indicator plant (usually within 3 days) the *P. hybrida* plant was used as a virus-source plant for the nematodes.

V.3.b.- *Acquisition:* Nematodes were extracted from soil as described under V.1.b., and pipetted into a 25 ml plastic pot. A *P. hybrida* virus-source plant was added to the pot, which was then filled with a sterile sand-loam mixture (3 parts

sand, 1 part loam) with particle sizes between 125 and 350 µm. Water was added to the pot to just saturate the soil, and the pot was partially plunged into moist sand in a seedling growth chamber covered, to maintain a high humidity level. The chamber was placed at 20 °C, with an 18 hr. light regime (2000 lux). The pots were watered with fresh tap water to keep the soil just below saturation. Three weeks later nematodes were extracted from the pots by washing the contents into a 500 ml beaker. The contents in the beaker were stirred into suspension, left for 5 sec., and the supernatant fluid subsequently poured onto a Kleenex tissue on an extraction sieve and placed on a Baermann funnel for 24 hr (see V.1.b.).

V.3.c.- *Transmission*: The nematodes extracted from the source-plants were tested for virus transmission using individual nematodes or using bulk nematode suspensions. Individual nematodes were tested in the individual nematode bait test system (See V.2.b). To test bulk nematodes for transmission, they were pipetted into a 25 ml plastic pot to which a 4 week-old virus-free *P. hybrida* bait-plant was added. The pots were filled with a sterile sand-loam mixture (see V.3.b.) and partially plunged into moist sand in a covered seedling growth chamber to maintain a high humidity level. The chamber was placed at 20 °C, with an 18 hr. light regime (2000 lux). After three weeks the *P. hybrida* bait-plants were removed from the pots, washed free of soil and the root system tested for the presence of virus (See V.4.a.*i*). Virus isolates obtained were propagated (See V.4.b.) and tested in $F(ab')_2$-ELISA as described under V.4.c.

V.4. VIRUS TESTS

V.4.a.- *Detection of virus*

V.4.a.*i* - Indicator plants: Plant material was comminuted with a few drops of tap water in a mortar with a pestle and the resulting suspension manually inoculated onto carborundum-dusted leaves of *Chenopodium amaranticolor* Coste and Reyn indicator plants with six fully developed leaves. The development of local lesions on the inoculated leaves, usually within 3-4 days, indicated the presence of virus. When no lesions had appeared within 2 weeks after inoculation, plants were discarded and considered uninfected.

V.4.a.*ii* - Electron microscopy: Virus particles were extracted from infected plants using a micro-mortar (Duncan and Roberts, 1981). To approximately 0.1 g of leaf material in a 1.0 ml Eppendorf tube 30-50 µl 0.07 M phosphate buffer (pH 7) and a small amount of carborundum powder was added. The tissue was then thoroughly ground with a glass rod, while adding further phosphate buffer to a total volume of *c.* 0.5 ml. After centrifugation (2000 g, 1 min.), a drop of the supernatant solution was transferred to a carbon-filmed electron microscope grid, drained briefly and washed with 2-3 drops of 2% phosphotungstate (PTA) stain (pH 6.8). The grid was dried and examined in a Philips CM10 electron microscope operating at 80 kV.

V.4.b. - *Propagation of virus for F(ab')$_2$-ELISA*: Virus isolates to be tested in F(ab')$_2$-ELISA were propagated from infected *C. amaranticolor* indicator plants. One day after the appearance of lesions in inoculated leaves of *C. amaranticolor*, the leaves were comminuted in a mortar with a pestle and the resulting suspension manually inoculated onto carborundum dusted leaves of a *C. quinoa* plant with 6 fully developed leaves. When symptoms appeared on the inoculated leaves, usually within 3-5 days after inoculation, these leaves were comminuted in a mortar with a pestle and the suspension obtained was manually inoculated onto carborundum dusted leaves of a 6 week-old *N. clevelandii* plant. The *C. quinoa* was used as an intermediate host since direct transfer of virus from *C. amaranticolor* to *N. clevelandii* may result in a loss of virus due to virus inhibiting compounds in the *C. amaranticolor* leaf sap (Robinson, 1973). Ten days after inoculation, at least two young *N. clevelandii* leaves were harvested for use in F(ab')$_2$-ELISA (See IV.4.c).

V.4.c.- *F(ab')$_2$-ELISA.*

V.4.c.*i.*- Preparation of IgG and F(ab')$_2$-fragments: IgG was purified from TRV antisera according to Clark and Adams (1977) with minor modifications: 1 ml of sap from a virus-free *N. clevelandii* was added to 1 ml of antiserum and incubated for 1 hr. at 37 °C. Another 1 ml of sap was added and incubated at 37 °C for 1 hr. The suspension was spun in a Baird and Tatlock micro centrifuge at 10.000 rpm for 10 min. Ten ml. neutralized saturated ammonium sulphate plus 7 ml distilled water were added to the supernatant. The suspension was stirred for 2 hr. at room temperature and subsequently spun in a Baird and Tatlock micro centrifuge at 10.000 rpm for 10

min. The pellet was resuspended in 1 ml acetate-buffer, pH 4 (3.43 g $CH_3COONa.3H_2O$ + 4.39 g NaCl + 6 ml CH_3COOH in 1 l H_2O) and dialysed for 16 hr. at room temperature against the acetate-buffer. The solution was spun in a Baird and Tatlock micro centrifuge for 10 min. at 10.000 rpm and the supernatant solution, containing the IgG, collected. The absorbance of the solution was measured at 280 nm in a Philips Unicam spectrophotometer and the concentration adjusted to 1 mg IgG/ml (E_{280}=1.4). The solution was divided into two parts, one of which was again divided in 0.5 ml samples which were stored at -20 °C. The other part of the IgG-solution was used for preparation of $F(ab')_2$-fragments. To 1 ml of the IgG solution 2 µl Pepsin (Sigma) in acetate-buffer, pH 4, (12.5 µg pepsin per µl buffer) was added, resulting in 25 µg pepsin added per mg IgG. The solution was incubated at 37 °C for 16 hr., dialysed against PBS-buffer, pH 7.4 (8.0 g NaCl + 0.2 g KH_2PO_4 + 2.9 g $Na_2HPO_4.12\ H_2O$ + 0.2 g KCl in 1 l H_2O) for 16 hr. at room temperature, divided into 0.5 ml samples and stored at -20 °C.

V.4.c.*ii*.- Antisera and homologous tobravirus-strains: $F(ab')_2$-fragments and IgG of antisera prepared against TRV-strain SYM (Kurppa *et al.*, 1981) and isolate TCB2-8 (See Chapter VII) and against the English PEBV-strain SP5 (Gibbs and Harrison, 1964a) was provided by D.J. Robinson. $F(ab')_2$-fragments and IgG was prepared from antisera against TRV-strains PRN (Cadman and Harrison, 1959), RQ (Robinson and Harrison, 1985b), N5 (Harrison *et al.*, 1983), TSZ34 (See Chapter VII) and TY (TRV from several narcissus plants cv. Hawera; Asjes, pers. comm) as described in IV.4.c.*i*. The PRN- and RQ-antisera were provided by D.J. Robinson, N5-antiserum by W.P. Mowat, TS- (against isolate TSZ34) and TY-antiserum by C.J.

Asjes. Antisera against TRV-strains PRN, ORE (Lister and Bracker, 1969), SYM, and PEBV-strain SP5 used in ISEM studies (See VII.2.g) were provided by I.M. Roberts. TRV-strains PRN, RQ, SYM ORE and N5 and PEBV-strain SP5 were provided by D.J. Robinson. TRV-strain PLB (from potato, Angenent *et al.*, 1989) was provided by J.F. Bol. TRV-isolates TCB2-8 and TSZ34 were obtained in this study (See Chapter VII). The serological affinities of the antisera used in this study with a number of tobravirus-strains is shown in Table 9.

Table 9. Reaction in F(ab')$_2$-ELISA of tobravirus-strains with antisera used in this study.

	Antisera							
Strain	PRN	SYM	N5	TCB2-8	RQ	TY	TS	SP5
PRN	+	-	-	-	-	+	-	-
PLB	+		-			+		-
SYM	-	+	-	-	-	-		-
N5	-	-	+	-	-	-	-	-
TCB2-8	-	-	-	+	-	-	-	-
RQ	-	-	-	-	+			-
TSZ34	-	-	-	-	-	-	+	-
SP5	-	-	-	-	-	-		+
ORE*	-	-	-	-	-	+		-
PEBV-D*	-	-	+	-	-	-		-

+: positive reaction in F(ab')$_2$-ELISA, -: negative reaction in F(ab')$_2$-ELISA, blank space: not tested.
*) tested by D.J. Robinson.

Table 9 shows that TRV-strains PRN and PLB are serologically indistinguishable, both reacting with PRN- and TY-antiserum. TRV-strain N5 is serologically similar to the Dutch strain of PEBV which was also reported by Robinson *et al.* (1987). TRV-isolate TY was not available, neither an antiserum prepared against strain ORE. However, from the reaction of the TY-antiserum with the PRN- and PLB-strain and

with the ORE-strain which did not react with the PRN-antiserum, it is concluded that the TY-antiserum is a mixture of IgG molecules reacting with PRN-serotype isolates and with ORE-serotype isolates.

V.4.c.*iii*.- The $F(ab')_2$-ELISA procedure: Wells of Immuno Maxisorp microtitre plates (Nunc) were coated with 200 µl of the $F(ab')_2$-fragments of the antiserum diluted in coating buffer, pH 9.6 (1.59 g Na_2CO_3 + 2.93 g $NaHCO_3$ in 1 l H_2O) at 37 °C for 3 hr. Concentrations of $F(ab')_2$-fragments used were 1 µg/ml for PRN-, RQ-, N5-, SP5- and SYM-antiserum, 2 µg/ml for TS- and TY-antiserum and 2.5 µg/ml for the TCB2-8-antiserum. Plates were then washed 3 times for 3 min. with PBS-Tween (PBS-buffer + 0.5 ml Tween 20 per litre) and loaded with 200 µl per well of the sample. The samples were made up by grinding leaf material, and diluting the obtained sap 20-fold in PBS-Tween. Plates were left at 4 °C overnight and subsequently washed 3 times, 3 min. with PBS-Tween. IgG, diluted in PBS-Tween, was added (200 µl/well) and left at 37 °C for 3 hr. IgG-concentrations used were 1 µg/ml for PRN-, RQ-, N5-, SP5- and SYM-antiserum, 2 µg/ml for TS- and TY-antiserum and 10 µg/ml for the TCB2-8-antiserum. Plates were washed 3 times, 3 min. with PBS-Tween and Protein-A alkaline phosphatase (Zymed, USA) diluted 1:4000 in PBS-Tween was added (200 µl/well). Plates were left at 37 °C for 3 hr, washed 3 times, 3 min. with PBS-Tween and p-nitrophenyl-phosphate (Boehringer) 1 mg/ml in substrate buffer (97 ml Diethanolamin + 800 ml H_2O, pH to 9.8 with HCl, make up to 1 l with H_2O) was added (200 µl/well). Plates were put at 4 °C overnight and the absorbance at 405 nm was read in a Titertek multiskan Plus Mk II photometer. Absorbance ratio's of "test sample" compared to "healthy control"

bigger than 2 ($A_{405}sample/A_{405}healthy$>2) were considered a positive reaction, ratio's below 2 ($A_{405}sample/A_{405}healthy$<2) were considered a negative reaction.

V.4.d.- *Test for M- or NM-type infections*: Leaf sap from infected *N. clevelandii* plants which failed to react in $F(ab')_2$-ELISA was tested for M- or NM-type infections. Sap was extracted *c.* 10 days after inoculation from systemically infected leaves of a *N. clevelandii* plant with a mortar and pestle, adding a few drops of tap water. The resulting suspension was divided into two approximately equal volumes in a 0.5 ml Eppendorf tube. The suspension in one of the Eppendorf tubes was manually inoculated onto four carborundum dusted leaves of a *C. amaranticolor* indicator plant, whereas the suspension in the other Eppendorf tube was subjected to a threefold cycle of freezing and thawing and subsequently inoculated onto four leaves of a *C. amaranticolor* indicator plant. When lesions developed on the *C. amaranticolor* leaves inoculated with the "fresh" leaf sap, but did not appear on the leaves inoculated with the sap which had been subjected to the freezing and thawing procedure it was concluded that the *N. clevelandii* plant was infected with NM-type virus. No further work was done on NM-type infected plants.

CHAPTER VI.

ASSOCIATIONS BETWEEN TRICHODORID FIELD POPULATIONS AND TOBACCO RATTLE VIRUS SEROTYPES WITH EMPHASIS ON THE DUTCH BULB-GROWING AREAS.

VI.1. INTRODUCTION.

The culture of ornamental flower bulbs is a very important horticultural activity in the Netherlands. A total of 16,400 ha was used for ornamental bulb production in 1990 (Anonymus, 1990). Around 11,000 ha are cultivated with spring flowering crops e.g. tulips (7,000 ha), narcissi (1,600 ha), hyacinths (900 ha), the remaining 5,400 ha with summer and early autumn flowering crops e.g. gladiolus, lily and dahlia. In total *c.* 4,000 bulb growers produce 8.5 million bulbs annually which are sold either directly for use in gardens and landscapes or which are forced into flower and sold as cut flowers. A large proportion of the bulbs and flowers are exported, mainly to Germany, the United States, France and Sweden (de Hertogh *et al.*, 1983).

Bulb culture in the Netherlands historically is located on the dune sand and light fine sandy soils of the provinces North-Holland and South-Holland. However, after the second world war bulb cultivation spread to heavier soil types in the north-western part of the country. More recently bulb growing extended to the marine sandy soils of the North-East-Polder and Flevo-Polder (*c.* 2,200 ha flower bulbs in 1990) and to light soils in the south-western (Zeeland) and south-eastern (Limburg, Brabant) parts of the country. One of the reasons for starting the cultivation of flower bulbs in these areas is that using "new" soils supposedly results in a reduced risk of

soil-borne pathogens. Two such pathogens of economic importance in flower bulb production are tobacco necrosis virus causing Augusta disease in tulip transmitted by the fungus *Olpidium brassicae* and tobacco rattle virus transmitted by trichodorid nematodes (Asjes, 1974). Damage caused by the viruses is difficult to assess since growers normally rogue any plants which they suspect to be virus infected. In some years however, considerable numbers of bulb lots are rejected by the Dutch Flowerbulb Inspection Service (Bloembollenkeuringsdienst) because of high virus incidence. Roguing infected plants provides one means of control of soil-borne viruses, but spread can sometimes be controlled by soil fumigation. Recent emphasis on the environmental aspects of agriculture in the Netherlands, has resulted in legislative restriction on the use of pesticides, including soil fumigants. Consequently, growers as well as the government, have taken a renewed interest in the problem of soil-borne diseases in flower bulbs and other crops and have stimulated research in this area.

Tobacco rattle virus is a well known problem in the flower bulb culture and in potatoes when grown on light soils, but relatively few data are available on the occurrence of the virus and of its trichodorid vectors. Van Hoof (1973) found both the vector nematodes and the virus to be widespread in the Dutch bulb-growing region of North- an South-Holland but did not assess serotypes of TRV recovered in relation to the trichodorid species found. In order to investigate the occurrence of trichodorid nematode species in relation to the occurrence of serotypes of TRV in the bulb growing areas of the Netherlands and a few other sites, soil samples were taken from fields, or from verges adjacent to fields with a history of TRV. Results from this study are presented and discussed in this chapter.

VI.2.- MATERIALS AND METHODS.

VI.2.a.- *Sampling sites:* Soil samples were taken from bulb fields and/or field verges, from a tulip field with a primary TRV infection and from other fields known to contain trichodorids and/or TRV.

VI.2.a.*i*.- Bulb growing areas in the Netherlands: Soil samples from the bulb growing areas in the Netherlands were collected during April 1989 and 1990, the season when most spring flowering bulb crops are blossoming. Samples were taken as described in V.1.a. The samples were collected throughout the bulb region. This was not a random survey as sampling was concentrated on fields with a history of TRV problems. A considerable number of samples was also taken from road- and field-verges directly adjacent to the bulb fields to increase the likelihood of finding trichodorid nematodes, since agricultural practices such as soil fumigation and cultivation may strongly reduce populations in the fields. The locations of the samples are shown in Fig. 8.

VI.2.a.*ii*.- Tulip field showing primary infection: Ten samples around the roots of TRV affected tulips were taken randomly from an experimental tulip (cv. Apeldoorn) plot at Julianadorp in which primary TRV infection was evident. Five of these were taken from a part of the field in which rape (*Brassica rapa*) had been grown before planting the tulips, the other five samples were taken from a part of the field which had only been covered with straw before planting the tulips.

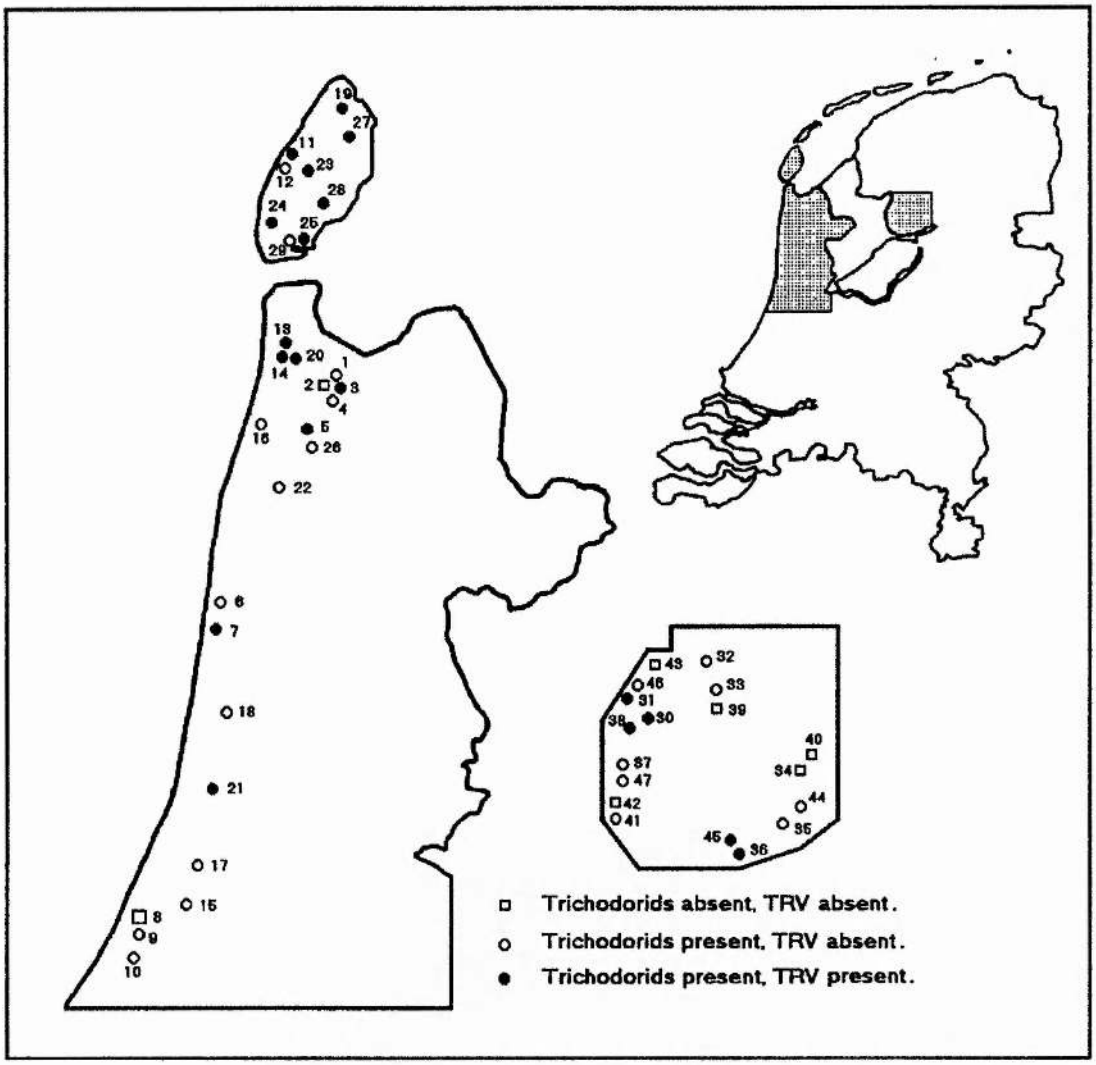

Figure 8. Location of soil samples collected in Dutch bulb-growing areas.

VI.2.a.*iii*.- Other sites: Samples were collected from fields in several other parts of the Netherlands and from fields in Scotland, England and from a field in Sweden where either TRV or high trichodorid populations were known to occur.

VI.2.b.- *Bait tests, virus detection and serology:* The samples were bait tested for virus presence as described in V.2.a. and nematodes were extracted from the samples as described in V.1.b., counted and, if available, at least 20 trichodorid specimens were fixed and mounted in anhydrous glycerol as described in V.2.c.

Virus was detected as described in V.4.a and propagated for testing in $F(ab')_2$-ELISA as described in V.4.b.

$F(ab')_2$-ELISA was done as described in V.4.c.*ii.* using antisera prepared against TRV-strains PRN (Cadman and Harrison, 1959), RQ (Robinson and Harrison, 1985b), N5 (Harrison *et al.*, 1983) and TS (See VII). $F(ab')_2$-fractions were used in concentrations of 1, 1, 1, and 2 µg/ml respectively and IgG in concentrations of 1, 1, 1 and 2 µg/ml respectively.

VI.3.-RESULTS.

VI.3.a.- *Bulb growing areas in the Netherlands:* The data obtained from samples taken in the bulb-growing region of the provinces North- and South-Holland are shown in Table 10.

Table 10. Occurrence of trichodorid nematodes and tobacco rattle virus serotypes in soil samples from the coastal bulb-growing area of the provinces of North- and South-Holland.

Sample	Location	Crop	Number of trichodorid nematodes /250g soil	Species*	TRV serotype**
1	Breezand	grass^{v}	30	Pp	-
2	Breezand	grass^{v}	0	-	-
3	Breezand	grass^{v}	74	Pp Ts	TS
4	Breezand	grass^{v}	11	Pp	-
5	'tZand	grass^{v}	200	Ts	TS
6	Egmond	grass^{v}	48	Pp Tc	-
7	Egmond	grass^{v}	201	Pp Tc Ts	TS
8	Noordwijkerhout	grass^{v}	0	-	-
9	Noordwijk	grass^{v}	27	Pp	-
10	Noordwijk	grass^{v}	57	Pa	-
11	De Koog	grass^{v}	147	Pp Tv	PRN
12	De Koog	grass^{v}	185	Pn Pp Tv	-
13	Julianadorp	grass^{v}	150	Pp Tv	PRN
14	Julianadorp	grass^{v}	81	Pt Ts	N5
15	De Zilk	grass^{v}	13	Pp	-
16	Callantsoog	grass^{v}	57	Pp Tc	-
17	Vogelenzang	grass^{v}	45	Pp Ts	-
18	Heemskerk	grass^{v}	7	Pn Pp	-
19	Cocksdorp	grass^{v}	51	Pn Pp Tv	PRN
20	Julianadorp	tulip	21	Pp	PRN
21	Santpoort	tulip	246	Pp Tsp	PRN
22	Schagerbrug	tulip	4	juv.	-
23	De Koog	narcissus	25	Pn Pp	PRN
24	Den Hoorn	narcissus	190	Pp Ts	PRN
25	Horntje	narcissus	44	Pp Ts	PRN
26	'tZand	crocus	12	Ts	-
27	Eierland	crocus	11	Pn Pp Tv	PRN
28	Den Burg	freesia	40	Pp	PRN
29	Horntje	wheat	35	Pp	-

* Pa: *P. anemones*, Pn: *P. nanus*, Pp: *P. pachydermus*, Pt: *P. teres*, Tc: *T. cylindricus*, Ts: *T. similis*, Tsp: *T. sparsus*, Tv: *T. viruliferus*.

** TRV isolates were tested in F(ab')$_2$-Elisa with antisera against TRV strains PRN, RQ, N5 and TS. - = no virus detected.

^{v}: tulip field margin.

In 27 of the 29 samples (93%) from these areas trichodorid nematodes were found. *P. pachydermus* occurred in 22 of the samples and was the most widely distributed species. *T. similis*, *T. viruliferus*, *P. nanus* and *T. cylindricus* were less widely distributed, occurring in eight, five, five and three samples respectively. The remaining species, *P. anemones*, *P. teres* and *T. sparsus* were only found in one sample each. TRV was recovered from bait-plants from 14 samples. In each of these samples trichodorid nematodes were also detected. This means 52% of the trichodorid populations transmitted virus. All virus isolates obtained could be classified as reacting to antisera prepared against TRV-strains PRN, TS or N5. No isolates were found reacting with the RQ-antiserum. In 10 samples monospecies trichodorid populations were found, 12 trichodorid populations consisted of a mixture of two species and three populations of three species.

Data obtained from samples taken in the bulb-growing area of the North-East Polder are shown in Table 11.

Table 11. Occurrence of trichodorid nematodes and tobacco rattle virus serotypes in soil samples from the bulb-growing area of the North-East Polder.

Sample	Location	Crop	Number of trichodorid nematodes /250g soil	Species*	TRV serotype**
30	Creil	grass^{v}	41	Pt	N5
31	Rutten	grass^{v}	84	Pt	N5
32	Bant	grass^{v}	4	Pt	-
33	Bant	grass^{v}	1	Pt	-
34	Blokzijl	grass^{v}	0	-	-
35	Kraggenburg	grass^{v}	5	Ts	-
36	Ens	grass^{v}	19	Pt	N5
37	Espel	grass^{v}	3	Pt	-
38	Creil	fallow	66	Pt	N5
39	Bant	potato	0	-	-
40	Blokzijl	potato	0	-	-
41	Tollebeek	potato	3	Pt	-
42	Tollebeek	potato	0	-	-
43	Rutten	tulip	0	-	-
44	Kraggenburg	tulip	134	Tp	-
45	Ens	strawberry	5	Pt	N5
46	Rutten	carrot	4	Pt	-
47	Espel	eremurus***	1	Pt	-

* Pt: *P. teres*, no males found, Tp: *T. primitivus*, Ts: *T. similis*.
** TRV isolates were tested in F(ab')$_2$-Elisa with antisera against TRV strains PRN, RQ, N5 and TS. - = no virus detected.
*** eremurus is a flower bulb crop.
^{v}: verge of tulip field.

In the North-East-Polder trichodorid nematodes were found in 13 of the 18 samples (72%). *P. teres*, found in 11 samples, was the most common species in this area. The other species, *T. primitivus* and *T. similis*, were only found in one sample each. TRV was isolated from bait-plant roots from five samples with *P. teres* as the only trichodorid species found, of 11 samples with trichodorid nematodes (39%). These virus-isolates all reacted with the N5-antiserum only. Trichodorid nematode numbers were significantly higher in samples from North- and South-Holland than in samples from the North-East-Polder (Wilcoxon-test, $\underline{W}$=281; significant at $P \leq 0.01$).

Overall, 85% of samples taken in the bulb-growing areas contained trichodorid populations and 48% of these populations transmitted virus.

VI.3.b.- *Tulip field showing primary infection:* The data obtained from 10 tulip cv. Apeldoorn plants showing symptoms of TRV are shown in Table 12.

Table 12. Occurrence of trichodorid nematodes and tobacco rattle virus serotypes in 10 soil samples taken from around 10 tulips showing TRV symptoms at an experimental plot at Julianadorp.

Sample	Field cover before tulip	Number of trichodorid nematodes /250g soil	Species*	TRV serotype**
66a	rape	680	Pn	-
66b	rape	527	Pn	N5
66c	rape	587	Pn Psp	N5
66d	rape	269	Pn	N5
66e	rape	194	Pn	N5
66f	straw	28	Pn	-
66g	straw	15	Pn	N5
66h	straw	29	Pn	-
66i	straw	24	Pn	-
66j	straw	143	Pn Psp	N5

* Pn: *P. nanus*, no males found, Psp: juvenile Paratrichodorus spp. other than *P. nanus*.
** TRV isolates were tested in $F(ab')_2$-Elisa with antisera against TRV strains PRN, RQ, N5 and TS. - = no virus detected.

Apart from *P. nanus* another *Paratrichodorus* species was found in two samples. It could be distinguished from *P. nanus* because of its greater stylet length (see Appendix A), but not be identified to species level since all specimens found were juveniles. Total trichodorid numbers in samples 66a-e were significantly greater than those in samples 66f-j (Wilcoxon-test, $\underline{W}=15$; significant at $P \leq 0.01$).

VI.3.c.- *Other sites:* Data obtained from samples taken from fields outside of the Dutch bulb-growing areas are shown in Table 13.

Table 13. Occurrence of trichodorid nematodes and tobacco rattle virus serotypes in soil samples from fields outside the Dutch bulb-growing area.

Sample	Location	Crop	Number of trichodorid nematodes /250g soil	Species*	TRV serotype**
48	Kinshaldy (SC)	grass	330	Pp Ts	PRN
49	Barry (SC)	grass	105	Pp Tc	PRN
50	Morton (SC)	potato	57	Pp	PRN
51	Woodhill (SC)	barley	375	Pp Tc Tp	-
52	Edinburgh (SC)	grass	35	Tc Tp	RQ
53	Edinburgh (SC)	grass	674	Tc	-
54	Scryne (SC)	barley	96	Pp Tc Tp	RQ
55	York (E)	wheat	34	Pa	n.r.
56	Oxford (E)	potato	12	Tp	RQ
57	Overloon (NL)	maize	6	Ts	-
58	Groningen (NL)	potato	274	Tc Tp Tv	RQ
59	Wehe (NL)	potato	430	Tp	RQ
60	Peel (NL)	maize	51	Ts	-
61	Peel (NL)	maize	148	Ts Pp	-
62	Peel (NL)	potato	85	Pp	PRN
63	Wageningen (NL)	grass	37	Pp Tv	PRN
64	Wieringen	beech	62	Pt Tp	n.r
65	Sweden	potato	75	Pp Tsp	PRN

* Pa: *P. anemones*, Pp: *P. pachydermus*, Pt: *P. teres*, Tc: *T. cylindricus*, Tp: *T. primitivus*, Ts: *T. similis*, Tsp: *T. sparsus*, Tv: *T. viruliferus*.

** TRV isolates were tested in $F(ab')_2$-Elisa with antisera against TRV strains PRN, RQ, N5 and TS. - = no virus detected, n.r = virus detected but no reaction with the 4 antisera used.

All samples taken from fields in Scotland, England, the Netherlands and Sweden contained trichodorid nematodes, some in high numbers. Five virus isolates obtained from the roots of the bait-plants (samples nrs. 52, 54, 56, 58 and 59) reacted with the RQ-antiserum, which was not isolated from bait-plants grown in soil from the Dutch bulb regions. Furthermore, two virus isolates (samples 55 and 64) failed to react with

any of the four antisera used. These isolates were subsequently studied in more detail (see Chapter VII).

VI.3.d.- *Trichodorid numbers and presence of virus:* The presence or absence of transmitted virus in the soil samples was found to be associated with trichodorid numbers (Chi-test, $X_2^2 = 12.22$ significant at $P \leq 0.01$) (See Table 14).

Table 14. The association between presence or absence of virus in soil samples and trichodorid numbers (log x) in those samples.

	log (nr. trichodorids/250g soil)			
	0-1	1-2	2-3	total
Virus absent	10*	14	6	30
Virus present	1	22	15	38
total	11	36	21	68

*) Number of samples; samples with no trichodorids not included.

VI.3.e.- *Trichodorid species and serotypes of TRV:* The associations between individual trichodorid species and serotypes of recovered virus is shown in Table 15.

Table 15. Trichodorid species in soil samples and serotypes of TRV recovered from those samples.

trichodorid spp.	Virus serotype PRN	RQ	N5	TS	other
P. anemones	0*	0	0	0	50
P. nanus	19	0	50	0	0
P. pachydermus	100	20	0	67	0
P. teres	0	0	50	0	50
T. cylindricus	6	60	0	33	0
T. primitivus	0	100	0	0	50
T. similis	19	0	8	100	0
T. sparsus	13	0	0	0	0
T. viruliferus	31	20	0	0	0
Total nr. of samples recovered per serotype	16	5	12	3	2

*) percentage of samples per serotype with particular trichodorid species present.

Table 15 shows that whenever PRN-serotype TRV was recovered from bait-plants, *P. pachydermus* was present in the corresponding soil sample. Similarly, recovery of RQ-serotype TRV coincided with the presence of *T. primitivus* and recovery of TS-serotype TRV with the presence of *T. similis*. No clear association exists between the other TRV-serotypes and trichodorid species.

VI.3.f.- *Symptoms in bait-plants:* Systemic symptoms characteristic for TRV were observed in leaves of *N. tabacum* cv. White Burley bait-plants, which had been grown in soil from samples 11, 13 and 23 (PRN-serotype isolates recovered, See Table 10). No symptoms were observed in *P. hybrida* bait-plants. No differences were found between *P. hybrida* and *N. tabacum* cv. White Burley bait-plants regarding the presence or absence of virus in the roots of those plants, although

number of lesions developing in *C. amaranticolor* leaves inoculated with *P. hybrida* root extracts were generally higher than in leaves inoculated with *N. tabacum* cv. White Burley root extracts.

VI.4. DISCUSSION AND CONCLUSIONS.

Although the locations of the samples were not chosen randomly, but selected because of their history of TRV and/or trichodorid occurrence, the results justify some conclusions to be made. All nine species found in this study are known vectors of TRV (see IV.3.a.). *P. pachydermus* and *T. similis* were widespread in the bulb growing areas of North and South-Holland, whereas *P. teres* was the predominant species in the light marine sandy soils of the North-East-Polder. This corresponds with the trichodorid distribution maps by Seinhorst and van Hoof (1982). Both TRV and trichodorid nematodes were widespread in the coastal bulb growing areas in the Netherlands, which confirms results by van Hoof (1973) who found trichodorids in 77% and TRV in 30% of samples from these areas. The greater species diversity of trichodorid populations in the coastal provinces of the Netherlands as compared to the north-East-Polder was reflected in the serological variation of TRV-isolates recovered from those regions. The observation that trichodorid numbers in samples from North- and South-Holland were significantly higher than in samples from the North-East-Polder may be the result of a complex of factors e.g. cultivation practices, crop influences, cropping history etc. This study however was not primarily designed to assess absolute numbers of trichodorids in soil and therefore from the data available it is impossible to identify the cause(s) of the observed difference. It should

be noted however, that all but two trichodorid populations from the North-East-Polder consisted of *P. teres*, whereas in the coastal provinces this species was only found once. *P. teres* in particular has been reported to be highly sensitive towards mechanical injury (Bor and Kuiper, 1966). Transportation of samples, unless done very carefully, considerably reduced numbers of *P. teres* recovered (Bor and Kuiper, 1966). Whether this sensitivity is a characteristic of the species itself or results from the fact that the marine sandy soils in which *P. teres* naturally occurs are very easily disrupted is unknown. However, the transport of samples from the Netherlands to Scotland may have decreased numbers of *P. teres* more strongly than the other trichodorid species, which could have been one of the factors responsible for the lower population densities recovered from the North-East-Polder.

Similarly, the reason for the significant difference between trichodorid numbers in soil around tulip after rape and after field cover with straw (See Table 12) cannot be identified with absolute certainty. It is however very likely that the high numbers of *P. nanus* after rape are a direct result of rape being a good host for this species, since no other obvious variables were present in the field from which the samples were taken. Table 12 also shows that within the field there is no correlation between the total numbers of trichodorids found and the recovery of virus from the soil. When all samples from which trichodorid nematodes were recovered were considered however, the presence or absence of virus in the soil was associated with total trichodorid numbers (See Table 14). Thus, the probability of recovering virus from bait-plants increases with an increasing number of trichodorid nematodes found in the corresponding soil sample. Table 14 however, also shows that from one out of 11 (9%) samples in which between one and 10 trichodorids per 250 g of soil were

found, virus was recovered and, vice versa, that from six out of 21 (29%) samples with trichodorid numbers ranging from 100 to 1000 no virus was recovered. Similar results were obtained by Gugerli (1977) and Maas (1974) who also found low correlations between trichodorid population densities and the presence or absence of TRV as determined by infection of *N. tabacum* cv. White Burley bait-plants or of tulip. Both Gugerli (1977) and Maas (1974) mention the recovery of TRV from soil samples with very low to "almost undetectable" numbers of trichodorids. Higher correlations with the number of trichodorid nematodes present in the soil were found when assessing spraing damage in potato tubers as an indicator of TRV infectivity of the soil (Cooper and Harrison, 1973; Cooper and Thomas, 1971). Maas (1974) explains the higher correlation found with potato as compared with tulip or tobacco because of the differences in which TRV invades these crops after infection. Whereas with tulip and tobacco each successful TRV transmission by a single nematode may result in a systemically infected plant, with potato each successful virus transmission causes only one necrotic arc or ring to appear in one tuber. Therefore, in potato a high percentage of heavily infected tubers must be the result of numerous successful transmissions of the virus by the nematodes, whereas in tulip or tobacco only relatively few successful transmissions may result in a considerable number of plants becoming systemically infected with the virus.

The generally obtained low correlation between trichodorid numbers and TRV incidence in systemically infected crops may, according to Gugerli (1977), be caused by differences between populations in the proportion of viruliferous trichodorid nematodes and by the fact that not every trichodorid species in a mixed species population acts as a vector.

Thus, it can be concluded from data obtained in this study, which are similar to those obtained by Gugerli (1977) and Maas (1974), that only assessing trichodorid numbers in soil, without referring to the species present and without information on the vectoring ability of the species found, is of limited value for the prediction of the presence or absence of transmissible TRV in that soil.

The consistent recovery of isolates from bait-plants which react with one antiserum only, was also found when bait testing single trichodorid nematodes (See Chapter VII) and possible explanations for this are discussed in Chapter VII.

Several authors (see IV.3.a. and IV.3.b) have reported specific associations between trichodorid vector species and transmitted TRV strains. From our data it can be concluded that 3 trichodorid species, *P. pachydermus*, *T. primitivus* and *T. similis*, appear to be associated with PRN-, RQ- and TS-serotype isolates respectively. N5-serotype isolates were at some sites associated with *P. teres* and at other sites with *P. nanus*. Consequently, it has to be concluded that isolates within the N5-serotype appear to be transmitted by both species. One of the isolates which failed to react with the four antisera used was recovered from soil in which the only trichodorid species detected was *P. anemones* (sample 55, Table 13). *P. anemones* is therefore most likely the vector of this isolate. The other isolate which failed to react in ELISA was however obtained from a sample in which both *T. primitivus* and *P. teres* were found (sample 64, Table 13). From my data it is not possible to determine which species transmitted this virus. As noted above, *T. primitivus* was associated with the RQ- and *P. teres* with the N5-serotype indicating that one species probably transmitted at least two serologically distinct TRV-isolates. Results from earlier work also indicate that one species may transmit serologically distinct isolates of TRV (see

IV.3.a., Table 8). From the data on the associations between TRV-serotypes and trichodorid species it may be concluded that whenever PRN-serotype isolates are recovered from soil *P. pachydermus* is likely to be present, when RQ-serotype isolates are recovered *T. primitivus* is likely to be present and whenever TS-serotype isolates are recovered *T. similis* is likely to be present. Similarly, recovery of N5-serotype isolates is likely to coincide with the presence of either *P. nanus* or *P. teres*. Extrapolation of these conclusions to field circumstances would imply that the co-occurrence of particular trichodorid species in field soil with the presence of the associated TRV-serotype isolates in the field, e.g. in weeds, planting material etc, poses an increased risk of TRV infection when susceptible crops are grown or of the virus becoming established in the field. Prediction of TRV-damage in crops by assessing both the presence of trichodorid species and TRV serotypes however is probably not feasible considering the effort and time which such an assessment would require.

Finally, it can be concluded that recording systemic symptoms in *N. tabacum* cv. White Burley bait-plants is not recommended as a method for assessing the presence or absence of transmitted TRV in soil as has been suggested by Dale and Solomon (1988). In this study the majority of *N. tabacum* cv. White Burley bait-plants with TRV infected roots failed to produce aerial symptoms. Sol (1960; 1962) also frequently recovered TRV from roots of symptomless *N. tabacum* cv. White Burley bait-plants.

N. tabacum cv. White Burley and *P. hybrida* were found to be equally suitable as bait-plants for the detection of TRV. Generally however, more lesions developed on *C. amaranticolor* leaves inoculated with *P. hybrida* root extract than with *N. tabacum*

cv. White Burley root extract. This observation was however not pursued and it is therefore not possible to attribute this to e.g. higher TRV concentrations in the *P. hybrida* root systems.

CHAPTER VII.

ASSOCIATIONS BETWEEN TRICHODORID SPECIES AND SEROTYPES OF TRV IN SINGLE NEMATODE BAIT TESTS AND SOME CHARACTERISTICS OF TRANSMITTED ISOLATES.

VII.1. INTRODUCTION.

From results obtained by earlier workers and from studies on associations between Longidorid vector species and nepoviruses (See IV.3) one could hypothesize that different trichodorid vector species transmit different serotypes of TRV. However the data and the discussion in Chapter VI show that analysis of viruses and nematodes derived from particular soil samples does not always elucidate the possible specificity of associations between the vector trichodorid species and TRV-isolates. This is particularly so where trichodorid populations occur as a mixture of species which makes it difficult to identify the specific vector of any viruses that are transmitted. Furthermore, the criteria of Trudgill *et al.* (1983) (See IV.5) cannot be met since the possibility of alternative vectors transmitting virus cannot be eliminated. Some earlier workers also recognized the shortcomings of virus bait tests on soil samples and van Hoof (1964d) developed a bait test system using a single trichodorid nematode feeding on the roots of a *Nicotiana tabacum* cv. White Burley seedling in a watchglass containing sterilized silver-sand. From this system the technique for bait testing single trichodorid nematodes for virus transmission described in V.2.b was developed. Using this technique, each transmitted virus-isolate corresponds with a single trichodorid nematode, thereby circumventing the problem of mixed species

populations. Also, the possibility of alternative vectors transmitting virus and the danger of virus contamination is greatly reduced. Furthermore, the technique allows an estimation of transmission efficiency of vector populations, of species within populations and of males, females and juveniles within species. Another advantage is that virus-isolates obtained are less likely to consist of mixtures of different TRV-serotypes, as may happen when isolating TRV from naturally infected plant material. However, this depends on the assumption that a single trichodorid nematode usually transmits only one TRV-serotype isolate at the time.

The objectives of this part of the work were: to unequivocally identify the transmission of serotypes of transmitted TRV-isolates by individual trichodorid nematodes, and to assess whether an association between vector species and the serotypes of the isolates transmitted by these species exists. Also, to compare transmission efficiencies of males, females and juveniles within species and to assess whether TRV-isolates could be related to particular vector species on the basis of symptomatology and/or length distribution.

VII.2. MATERIAL AND METHODS.

VII.2.a.- *General:* The nematodes used in the single nematode bait tests were extracted from a number of the soil samples used in the pot bait tests as described in Chapter VI. Those samples were selected from which virus was recovered and in which trichodorid numbers were not low. Sampling procedures, extraction of nematodes, single nematodes bait testing, detection of virus, propagation of

transmitted isolates, origin and serological characteristics of the antisera and $F(ab')_2$-ELISA are described in Chapter V.

VII.2.b.- *Location of samples:* Samples used in this study are described in Chapter VI and were collected from:

Kinshaldy, Fife, Scotland - permanent pasture (48)*) **)

Barry Stables, Tayside, Scotland - permanent pasture(49)**)

Morton, Fife, Scotland - potato (50)

Edinburgh, Scotland - grass (52)

Scryne, Tayside, Scotland - barley (54)**)

York, England - wheat (55)

Oxford, England - potato (56)

Wageningen, the Netherlands - grass (63)

Wieringen, the Netherlands - alder (64)

'tZand, the Netherlands - grass (5)

Julianadorp, the Netherlands - grass (14)

Cocksdorp, the Netherlands - grass (19)

Santpoort, the Netherlands - tulip (21)

Julianadorp, the Netherlands - tulip (66c)

Sweden - potato (65)

*) Numbers between brackets refer to sample nrs. in Table 10, Table 12 and Table 13.

**) Sampled more than once.

VII.2.c.- *Nomenclature of transmitted isolates:* Isolates obtained in single nematode bait tests were as far as possible given a code consisting of three letters

followed by a number. The first two letters refer to the vector species, the third to the location of the sampling site, the numbers to the number of the single trichodorid nematode in the bait test. Isolates transmitted by juveniles were given a code starting with "JV". Thus, isolate PPK20 was transmitted by a single *P. pachydermus* specimen, from soil from Kinshaldy, nr. 20 in the bait test. In the isolate code PPS49 (*P. pachydermus*, Sweden, nr.49) the "S" refers to Sweden as only one sample from this country was available. Soil from some sites were sampled and bait tested more than once and the codes for the isolates obtained from these sites received an additional number indicating the number of the bait test (e.g. TCB2-8: *T. cylindricus*, Barry site, bait test 2, nematode number 8).

VII.2.d.- *Symptomatology of transmitted isolates:* For a number of transmitted isolates symptom development was recorded in *C. amaranticolor*, *C. quinoa*, *N. clevelandii*, *N. tabacum* cv. White Burley, *N. glutinosa*, and/or *Phaseolus vulgaris* after manual inoculation of leaves with sap extracted from a 10 days earlier manually inoculated *N. clevelandii*.

VII.2.e.- *Virus detection by electron microscopy:* Sap of *N. clevelandii* plants shown to be infected with virus when tested on *C. amaranticolor* indicator plants (See V.4.a.*i*) and which were confirmed not to be infected with NM-type virus (See V.4.d), but which failed to react in $F(ab')_2$-ELISA was examined in the electron microscope as described in V.4.a.*ii*.

VII.2.f.- *Immunosorbent electron microscopy (ISEM).*

VII.2.f.*i.*- Trapping of virus: Carbon coated electron microscope grids were coated with antiserum (PRN, ORE, or SP5) by floating the grids on 10 µl drops of diluted antiserum (1:1000 in 0.06 M phosphate buffer, pH 6.5). After incubation (1 hr., 37 °C) grids were removed from the drops, drained, washed with phosphate buffer (2x10 min.) and drained again. Grids were then transferred onto 10 µl drops of sample (PPK20, PTW1 or TCS44), prepared as described in V.4.a.*i.* After 1 hr. at 4 °C grids were stained by washing them with 2 drops of 2% potassium phosphotungstate acid (PTA) and examined in a Philips EM301 electron microscope.

VII.2.f.*ii.*- Trapping and decoration of virus: Particles of TRV-isolate PPK20 (See this Chapter) were trapped onto grids (See VII.2.f.*i*) with a 1:1 mixture of antisera SYM and PRN (final dilutions 1:1000). Grids were then floated on 10 µl drops of antiserum PRN (1:50 in 0.06 M phosphate buffer, pH 6.5) or antiserum SYM (1:50 in 0.06 M phosphate buffer, pH 6.5) for 1 hr at 4 °C, stained with 2% PTA and examined in a Philips EM301 electron microscope. Particles of TRV-isolate PTW1 (See this Chapter) were trapped onto grids using ORE- or PRN-antisera (1:1000) as described above.

VII.2.g.- *Particle length distribution:* Particles of TRV-isolates PPK20, PTW1 and PAY68 and TCS44 (See this Chapter) were measured in the electron microscope. Particles of isolates PPK20 ,PTW1 and TCS44 were trapped onto grids using PRN-, ORE- and SP5-antiserum respectively (See VII.2.e.*i*). Samples of TRV-isolate

PAY68 were prepared as described in V.4.a.*ii* except that sap extract was clarified by adding 2 drops of chloroform, after which it was spun for 2 min at 2000 rpm in an Eppendorf table centrifuge. Grids were loaded with a drop of the supernatant solution and stained with 2% PTA as described in V.4.a.*ii*. From a number of area's on the grids EM-photographs were made. Before and after each series of photographs a catalase-grid (periodicity 8.6 nm) was also photographed at the same magnification to serve as a calibration for the magnification. Photographic negatives were then projected (*c*. 8 times magnified) onto paper and particles were traced and measured.

VII.3. RESULTS.

VII.3.a.- *Transmission of TRV by single trichodorid nematodes:* Transmission results are given for males and females for the species recovered in the bait tests. Although juveniles were initially identified to genus-level, transmission results for juveniles have been amalgamated per bait test, since unequivocal identification of juveniles is almost impossible.

VII.3.a.*i*.- Kinshaldy: Soil of the Kinshaldy site was collected on three occasions, at *c*. 6 months intervals, the first sample being taken in April 1989, the second in October and the third again in April the following year. At all sampling dates the field was under permanent pasture.

Table 16. Transmission of TRV by single trichodorid nematodes from Kinshaldy .

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
April 1989				
125	Pp	m	2/48	PPK20, PPK96
	Pp	f	2/39	PPK39, PPK92
		Total	4/87 (5%)	
	Ts	m	0/8	
	Ts	f	0/20	
		Total	0/28 (<4%)	
		j	0/0	
		Overall	4/115 (3%)	
October 1989				
125	Pp	m	4/12	PPK2-12, PPK2-15, PPK2-55, PPK2-71
	Pp	f	6/17	PPK2-22, PPK2-44, PPK2-65, PPK2-77, PPK2-83, PPK2-125
		Total	10/29 (35%)	
	Ts	m	1/15	TSK2-78
	Ts	f	1/47	TSK2-72
		Total	2/62 (3%)	
		j	0/0	
		Overall	12/91 (13%)	
April 1990				
120	Pp	m	3/18	PPK3-27, PPK3-92, PPK3-106
	Pp	f	5/25	PPK3-9, PPK3-15, PPK3-29, PPK3-39, PPK3-80
		Total	8/43 (19%)	
	Ts	m	0/19	
	Ts	f	0/27	
		Total	0/46 (<2%)	
		j	0/4	
		Overall	8/93 (9%)	

*) Pp: *P. pachydermus*, Ts: *T. similis*.
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

Isolates PPK2-12, PPK2-15, PPK2-22, PPK2-44, PPK2-55, PPK2-71, PPK2-83, PPK2-125, TSK2-72 and TSK2-78 were found to be NM-forms. The transmission frequency of *P. pachydermus* adults ranged between 5% and 35%, although the higher transmission coincided with the recovery of a large number of NM-type isolates. Transmission by *P. pachydermus* males was not significantly different from that by females at any of the sampling dates (April 1989 χ^2=0.05, October 1989 χ^2=0.01, April 1990 χ^2=0.08), nor was the transmission in October 1989 by *T. similis* males different from that by females (χ^2=0.75) . Although the transmission frequency of *P. pachydermus* was at all sampling dates greater that of *T. similis*, the 5% transmission efficiency of *P. pachydermus* in April 1989 was not significantly greater than the non-transmission of *T. similis* (χ^2=1.33). In October 1989 and in April 1990 however, the transmission by *P. pachydermus* adults was significantly greater than that by *T. similis* adults (χ^2=16.86 P≤0.001 and χ^2=9.40 P≤0.01 respectively). The overall transmission frequency was associated with sampling date (χ^2_2=6.3 P≤0.05). This was caused by the differences in transmission by *P. pachydermus* at the three dates as the 2% transmission by *T. similis* in October 1989 was not significantly different from the lack of transmission by this species at the other two dates (χ^2=2.42). The 5% transmission of *P. pachydermus* in October 1989 was significantly lower than the 35% and 19% transmission at the other two dates (χ^2=18.30 P≤0.001 and χ^2=6.74 P≤0.01 respectively).

VII.3.a.*ii*.- Barry Stables: Soil of the Barry Stables site was collected three times, with intervals of *c.* 6 months, the first sample being taken in April 1989, the second

in October 1989 and the third again in April, the following year. At all sampling dates the field was under permanent pasture.

Table 17. Transmission of TRV by single trichodorid nematodes from Barry Stables.

April 1989

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
100	Pp	m	4/40	PPB10, PPB71, PPB79, PPB96
	Pp	f	6/20	PPB18, PPB27, PPB34, PPB49, PPB63, PPB65
		Total	10/60 (15%)	
	Tc	m	0/5	
	Tc	f	1/25	TCB4
		Total	1/30 (3%)	
		j	0/0	
		Overall	11/90 (12%)	
October 1990				
100	Pp	m	1/26	PPB2-93
	Pp	f	0/49	
		Total	1/75 (1%)	
	Tc	m	4/7	TCB2-7, TCB2-8, TCB2-86, TCB2-87
	Tc	f	2/10	TCB2-9, TCB2-54
		Total	6/17 (35%)	
		j	0/0	
		Overall	7/92 (8%)	

Continued next page.

Table 17 continued.

Nr. set up	Spp.*	Sex**	Transmission***		Isolate
April 1990					
110	Pp	m	0/11		
	Pp	f	1/17		PPB3-103
		Total	1/28	(4%)	
	Tc	m	1/24		TCB3-82
	Tc	f	0/26		
		Total	1/50	(2%)	
		j	0/0		
		Overall	2/78	(3%)	

*) Pp: *P. pachydermus*, Tc: *T. cylindricus*.
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

Isolate PPB2-93 was found to be an NM-form. The transmission frequency of *P. pachydermus* adults ranged from 1% to 15% and that of *T. cylindricus* adults from 2% to 35%. Transmission by *P. pachydermus* males was not significantly different from that by females at any of the sampling dates (April 1989 χ^2=3.84, October 1989 χ^2=1.91, April 1990 χ^2=0.67). The same was true for *T. cylindricus* (χ^2=0.21, 1.11 and 1.11 respectively). In October 1989 the transmission frequency of *T. cylindricus* adults was significantly greater than that of *P. pachydermus* adults (χ^2=22.74 P≤0.001) but at the other two sampling dates the transmission frequencies of the two species were not significantly different (April 1989 χ^2=0.67, April 1990 χ^2=0.18). The overall transmission frequency was not associated with sampling date (χ^2_2=5.49). The transmission frequency of *P. pachydermus* however, was associated with sampling date (χ^2_2=12.20 P≤0.01) caused by the significantly greater transmission at

the first sampling date compared to that at the second sampling date (χ^2=10.47 P≤0.01). Similarly, the transmission by *T. cylindricus* was associated with sampling date (χ^2_2=19.97 P≤0.001). The transmission by this species in April 1989 was significantly greater than in October 1989 or April 1990 (χ^2=8.75 P≤0.01 and χ^2=15.03 P≤0.001 respectively).

VII.3.a.*iii*.- Morton: Soil from Morton was sampled in November 1989 after potatoes.

Table 18. Transmission of TRV by single trichodorid nematodes from Morton.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
100	Pp	m	0/27	
	Pp	f	3/37	PPM1, PPM10, PPM66
		Total	3/64 (5%)	
		j	0/6	
		Overall	3/70 (4%)	

*) Pp: *P. pachydermus*.
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

Isolate PPM1 was found to be an NM-form.

Assuming that all trichodorid juveniles found were *P. pachydermus*, there was no significant difference between transmission by males, females or juveniles (χ^2_2=2.80).

VII.3.a.*iv*.- Edinburgh: Soil from a grass field at East-Craigs (close to Edinburgh), was sampled in May 1990.

Table 19. Transmission of TRV by single trichodorid nematodes from Edinburgh.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
40	Tp	m	3/9	TPE11, TPE20, TPE35
	Tp	f	2/10	TPE5, TPE9
		Total	5/19 (26%)	
	Tc	m	0/4	
	Tc	f	0/5	
		Total	0/9 (<11%)	
		j	0/7	
		Overall	5/35 (14%)	

*) Tp: *T. primitivus*, Tc: *T. cylindricus*
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

All juveniles were identified as *Trichodorus*, but it was impossible to identify them to species.

There was no significant difference between transmission rates of *T. primitivus* males and females (χ^2=0.43) or between *T. primitivus* and *T. cylindricus* adults (χ^2=2.88).

VII.3.a.*v*.- Scryne: Soil from Scryne was collected twice, the first time in November 1989 from underneath grass (See Table 20), the second time in May 1990 from underneath barley (See Table 21). The results from the two sampling dates were not presented in one table as the crops at the two sampling dates were different.

Table 20. Transmission of TRV by single trichodorid nematodes from Scryne sampled November 1989 from a grass field.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
50	Pp	m	0/2	
	Pp	f	0/2	
		Total	0/4 (<25%)	
	Tc	m	0/15	
	Tc	f	2/25	TCS26, TCS44
		Total	2/40 (5%)	
		j	0/3	
		Overall	2/47 (4%)	

*) Pp: *P. pachydermus*, Tc: *T. cylindricus*
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

T. cylindricus females did not significantly transmit more efficiently than males (χ^2=1.26), neither did *T. cylindricus* adults significantly transmit more efficiently than *P. pachydermus* adults (χ^2=0.20).

Table 21. Transmission of TRV by single trichodorid nematodes from Scryne sampled May 1990 from a barley field.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
100	Tp	m	1/4	TPS2-14
	Tp	f	1/2	TPS2-17
		Total	2/6 (33%)	
	Tc	m	3/46	TCS2-1, TCS2-30, TCS2-67
	Tc	f	2/40	TCS2-7, TCS2-10
		Total	5/86 (6%)	
		j	0/2	
		Overall	7/94 (8%)	

*) Tp: *T. primitivus*, Tc: *T. cylindricus*
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

Both juveniles were identified as *Trichodorus* but could not be identified any further. Isolates TCS2-7 and TCS2-67 were NM-forms.

The transmission of *T. primitivus* males was not significantly different from that of females (χ^2=0.38). The same was true for *T. cylindricus* males and females (χ^2=0.09). The transmission by *T. primitivus* adults was significantly higher than that by *T. cylindricus* adults (χ^2=6.04 P≤0.05).

The population composition differed between the two sampling dates, with *P. pachydermus* only found at the November sampling and *T. primitivus* only at the May sampling.

VII.3.a.*vi*.- York: Soil from a wheat field at Helperby (close to York), was sampled in March 1990.

Table 22. Transmission of TRV by single trichodorid nematodes from York.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
75	Pa	m	2/15	PAY27, PAY69
	Pa	f	7/29	PAY39, PAY41, PAY48, PAY51, PAY68, PAY73, PAY75
		Total	9/44 (21%)	
		j	4/16	JVY2, JVY24, JVY44, JVY59
		Overall	13/60 (22%)	

*) Pa: *P. anemones.*
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

All juveniles were identified as being *Paratrichodorus*. Assuming that all juveniles were *P. anemones*, there was no significant difference in transmission rates of *P. anemones* males, females and juveniles (χ^2_2=0.82)

VII.3.a.*vii.*- Oxford: Soil from Oxford was sampled in October 1990 from a field from which the potato crop had just been harvested.

Table 23. Transmission of TRV by single trichodorid nematodes from Oxford.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
28	Tp	m	0/8	
	Tp	f	3/12	TPO23, TPO28, TPO37
		Total	3/20 (15%)	
		j	1/5	JVO38
		Overall	4/25 (16%)	

*) Tp: *T. primitivus*.
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

All 5 juveniles found were identified as *Trichodorus* spp.

Assuming that all juveniles were *T. primitivus*, there was no significant difference between transmission rates of males, females and juveniles (χ^2_2=2.31).

VII.3.a.*viii*.- Wageningen-Hoog: Soil from Wageningen-Hoog was sampled in April 1990 from permanent pasture.

Table 24. Transmission of TRV by single trichodorid nematodes from Wageningen-Hoog.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
50	Pp	m	2/7	PPW10, PPW40
	Pp	f	4/17	PPW9, PPW24, PPW48, PPW49
		Total	6/24 (25%)	
	Tv	m	0/1	
	Tv	f	1/9	TVW56
		Total	1/10 (10%)	
		j	1/11	JVW13
		Overall	8/45 (18%)	

*) Pp: *P. pachydermus*, Tv: *T. viruliferus*.
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

Isolates PPW24 and PPW40 were found to be NM-forms. The one juvenile specimen transmitting TRV was identified as a *Paratrichodorus* juvenile.

There was no significant difference between transmission rates of *P. pachydermus* males and females (χ^2=0.07), between *T. viruliferus* males and females (χ^2=0.12) or between *P. pachydermus* adults and *T. viruliferus* adults (χ^2=0.97).

VII.3.a.*ix*.- Wieringermeer: Soil from the Wieringermeer was sampled from underneath an alderhedge and kindly provided by P.A.A. Loof.

Table 25. Transmission of TRV by single trichodorid nematodes from Wieringermeer.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
91	Pt	m	7/9	PTW1, PTW25, PTW45, PTW67, PTW80, PTW81, PTW82
	Pt	f	11/14	PTW7, PTW8, PTW11, PTW14, PTW19, PTW26, PTW34,PTW39, PTW47, PTW61, PTW90
		Total	18/23 (78%)	
	Tp	m	0/4	
	Tp	f	0/16	
		Total	0/20 (<5%)	
		j	13/22	JVW3, JVW13, JVW18, JVW23, JVW37, JVW38, JVW46, JVW49, JVW52, JVW68, JVW72, JVW74, JVW91
		Overall	31/65 (48%)	

*) Pt: *P. teres*, Tp: *T. primitivus*
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

From the 22 juveniles 19 were identified as *Paratrichodorus*, of which 13 transmitted virus. Three juveniles were identified as *Trichodorus*, which failed to transmit virus. Transmission rates of *P. teres* males and females were not significantly different (χ^2=0.00), but transmission by *P. teres* adults was significantly higher than that by *T. primitivus* adults (χ^2=26.92 P≤0.001).

VII.3.a.x.- 'tZand: Soil from 'tZand was sampled in April 1990 from the grass boundary of a tulip field.

Table 26. Transmission of TRV by single trichodorid nematodes from 'tZand.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
70	Ts	m	7/46	TSZ17, TSZ19, TSZ34, TSZ41, TSZ47, TSZ51, TSZ57
	Ts	f	1/19	TSZ7
		Total	8/65 (12%)	
		j	0/0	
		Overall	8/65 (12%)	

*) Ts: *T. similis*.
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

Isolates TSZ17 and TSZ47 were found to be NM-forms.

There was no significant difference between transmission rates of *T. similis* males and females (χ^2=1.23).

VII.3.a.*xi.*- Julianadorp: Soil from Julianadorp was sampled in April 1990 from the grass boundary of a tulip field.

Table 27. Transmission of TRV by single trichodorid nematodes from Julianadorp

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
65	Pt	m	0/0	
	Pt	f	11/40	PTJ3, PTJ8, PTJ21, PTJ22, PTJ23, PTJ28, PTJ50, PTJ53, PTJ57, PTJ59, PTJ63
		Total	11/40 (23%)	
	Ts	m	0/1	
	Ts	f	0/2	
		Total	0/3 (<33%)	
		j	3/8	JVJ47, JVJ60, JVJ65
		Overall	14/51 (28%)	

*) Pt: *P. teres*, Ts: *T. similis*
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

All juveniles were identified as *Paratrichodorus*. Isolates PTJ8 and PTJ23 were NM-forms. The non-transmission by *T. similis* adults was not significantly lower than the 23% transmission by *P. teres* females (χ^2=1.11).

VII.3.a.*xii*.- Cocksdorp: Soil from Cocksdorp was sampled in April 1990 from a grass boundary of a narcissus field.

Table 28. Transmission of TRV by single trichodorid nematodes from Cocksdorp.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
95	Pp	m	3/7	PPC12, PPC17, PPC93
	Pp	f	5/7	PPC16, PPC29, PPC30, PPC49, PPC73
		Total	8/14 (57%)	
	Tv	m	2/8	TVC31, TVC47
	Tv	f	4/18	TVC33, TCV37, TVC53, TVC68
		Total	6/26 (23%)	
	Pn	m	0/0	
		f	0/39	
		Total	0/39 (<3%)	
		j	0/0	
		Overall	14/79 (18%)	

*) Pp: *P. pachydermus*, Tv: *T. viruliferus*, Pn: *P. nanus*
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

Isolates TVC31 and TVC33 were NM-forms.

There was no significant difference between transmission rates of *P. pachydermus* males and females (χ^2=1.17) or of *T. viruliferus* males and females (χ^2=0.02). There was a significant association between transmission rate and trichodorid species (χ^2_2=23.83 P≤0.001). When comparing transmission rates of species pairwise, the transmission by *P. pachydermus* adults was significantly greater than that by *T. viruliferus* adults (χ^2=4.64 P≤0.05) and than the non-transmission by *P. nanus*

(χ^2=26.25 P≤0.001). Also the transmission by *T. viruliferus* adults was significantly greater than the non-transmission by *P. nanus* (χ^2=9.92 P≤0.01).

VII.3.a.*xiii*.- Santpoort: Soil from Santpoort was sampled in April 1990 from a tulip field.

Table 29. Transmission of TRV by single trichodorid nematodes from Santpoort.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
50	Pp	m	1/2	PPSa56
	Pp	f	3/4	PPSa11, PPSa31, PPSa36
		Total	4/6 (67%)	
	Tsp	m	0/15	
	Tsp	f	0/17	
		Total	0/32 (<3%)	
		j	1/3	JVSa8
		Overall	5/41 (12%)	

*) Pp: *P. pachydermus*, Tsp: *T. sparsus*
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

The 3 juveniles were identified as *Paratrichodorus*.

The transmission rate of *P. pachydermus* males was not significantly different from that of females (χ^2=0.38), but the transmission rate of *P. pachydermus* adults was significantly greater than that of *T. sparsus* adults (χ^2=23.84 P≤0.001).

VII.3.a.*xiv*.- Julianadorp: Soil from Julianadorp was sampled from a tulip field in April 1990.

Table 30. Transmission of TRV by single trichodorid nematodes from Julianadorp.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
135	Pn	m	0/0	
	Pn	f	0/105	
		Total	0/105 (<1%)	
		j	1/2	JVJ6
		Overall	1/107 (1%)	

*) Pn: *P. nanus*
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

The 2 juvenile specimens were identified as a *Paratrichodorus* species different from *P. nanus* because of the length of the stylet being greater than that of adult *P. nanus* specimens.

VII.3.a.*xv*.- Sweden: Soil from Sweden was sampled from a potato field in May 1990 and kindly provided by B. Eriksson.

Table 31. Transmission of TRV by single trichodorid nematodes from Sweden.

Nr. set up	Spp.*	Sex**	Transmission***	Isolate
50	Pp	m	0/6	
	Pp	f	1/11	PPS49
		Total	1/17 (6%)	
	Tsp	m	0/1	
	Tsp	f	0/2	
		Total	0/3 (<33%)	
		j	0/22	
		Overall	1/42 (2%)	

*) Pp: *P. pachydermus*, Tsp: *T. sparsus*
**) m: male, f: female, j: juvenile.
***) Numerator is the number of nematodes transmitting, denominator is the number tested.

From the 22 juvenile specimens, 18 were identified as *Paratrichodorus*, 4 as *Trichodorus*.

No significant differences existed between transmission rates of *P. pachydermus* males and females (χ^2=0.58) or of *P. pachydermus* adults and *T. sparsus* adults (χ^2=0.19).

VII.3.b.- *Overall transmission efficiencies:* An analysis of the transmission frequencies based on the cumulative data of all bait tests, showed that there was an association between the transmission of virus and the sex (male, female or juvenile) of the trichodorid vector (χ^2_2=10.83 P≤0.01). A pairwise comparison of the

transmission frequencies (males with females: χ^2=0.00, males with juveniles: χ^2=8.86 P≤0.01, and females with juveniles: χ^2=9.94 P≤0.01) showed that juveniles were significantly more likely to transmit virus than males or females (See Table 32).

Table 32. Overall transmission rates of trichodorid males, females and juveniles recovered in single-bait tests.

Sex	Nr. of transmissions	Nr. of non transmissions	Transmission frequency (%)
Males	52	419	11
Females	85	683	11
Juveniles	24	87	22

The significantly higher transmission of juveniles however is mainly due to the high number of juveniles (13 out of 22) transmitting in the bait test on soil from Wieringermeer (See Table 24).

When comparing the overall transmission rates per species the transmission rate was clearly associated with species (χ^2_8=172.99 P≤0.001).

Table 33. Overall transmission rates per trichodorid species.

Species	Nr. of transmissions	Nr. tested	Transmission frequency (%)
P. teres	29	63	46
P. anemones	9	44	21
T. viruliferus	7	36	19
T. primitivus	10	65	15
P. pachydermus	56	451	12
T. cylindricus	15	232	7
T. similis	10	206	5
T. sparsus	0	35	<3
P. nanus	0	144	<1

A pairwise comparison (χ^2-test) of the transmission rates of the species showed that *P. teres* was a more efficient vector of TRV than the other species.

Table 34. Results of pairwise comparisons of transmission rates of trichodorid species (χ^2-test; *** significant at P≤0.001, ** significant at P≤0.01, * significant at P≤0.05.)

Species[a]	PT	PA	TV	TP	PP	TC	TS	TSP
PA	**							
TV	**	ns						
TP	***	ns	ns					
PP	***	ns	ns	ns				
TC	***	**	**	*	*			
TS	***	***	**	**	**	ns		
TSP	***	**	**	*	*	ns	ns	
PN	***	***	***	***	***	*	**	ns

[a] PT: *P. teres*, PA: *P. anemones*, TV: *T. viruliferus*, TP: *T. primitivus*, PP: *P. pachydermus*, TC: *T. cylindricus*, TS: *T. similis*, TSP: *T. sparsus*.

VII.3.c.- *Serology of TRV-isolates transmitted by single trichodorid nematodes:*

The virus isolates which were recovered from the single nematode bait tests and of which it was confirmed that they were M-type isolates, were tested in $F(ab')_2$-ELISA as described in V.4.c.*iii*. From each site, with the exception of the Santpoort site, at least one isolate was picked as a "type-isolate" which was tested against a range of available antisera. From the 31 isolates obtained in the bait test from the Wieringermeer sample (See Table 24) 12 samples were randomly picked and used in $F(ab')_2$-ELISA. For the origins of the antisera and their reaction with a number of tobravirus-strains see V.4.c.*ii*.

Table 35. F(ab')$_2$-ELISA results of TRV-isolates transmitted by single trichodorid nematodes.

TRV-isolate	PRN	SYM	N5	Antisera TCB2-8	RQ	TY	TS	SP5
Kinshaldy								
PPK20*	+	+/-	-	-	-	+	-	-
PPK39	+	-	-					
PPK92	-	+	-					
PPK96	-	+	-					
PPK2-65	+	+/-	-					
PPK2-77	+	+/-	-					
PPK3-9	+	-	-					
PPK3-15	+	-	-					
PPK3-27	+	-	-					
PPK3-29	+	-	-					
PPK3-39	+	-	-					
PPK3-80	+	-	-					
PPK3-92	+	-	-					
PPK3-106	+	-	-					
Barry Stables								
PPB10	+	+/-	-	-	-	+	-	-
PPB18	+	-	-					
PPB27	+	-	-					
PPB34	+	-	-					
PPB49	+	-	-					
PPB63	+	-	-					
PPB65	+	-	-					
PPB71	+	-	-					
PPB79	+	-	-					
PPB96	+	-	-					
TCB4	-	-	-	-				
TCB2-7	-	-	-	+				
TCB2-8	-	+/-	-	+	-	-	-	-
TCB2-9	-	-	-					
TCB2-54	-	-	-					
TCB2-86	-	-	-					
TCB2-87	-	-	-					
PPB3-103	+	-	-					
TCB3-82	-	-	-	+	-			

Continued next page.

Table 35 continued

TRV-isolate	Antisera PRN	SYM	N5	TCB2-8	RQ	TY	TS	SP5
Morton								
PPM10	+	+/-	-	-	-	+	-	-
PPM66	+	+/-	-					
Edinburgh								
TPE5	-				+			-
TPE9	-				+			-
TPE11	-		-		+	-	-	-
TPE20	-				+			-
TPE35	-				+			-
Scryne								
TCS26	-	-	-	-	+	-		-
TCS44	-	-	-	-	-	-		+
TCS2-1	-				-			-
TS2-10	-				+			-
TCS2-30	-				+			-
TPS2-14	-				+			-
TPS2-17	-				+			-
York								
PAY27	-	-	-	-	-			-
PAY39								-
PAY41								-
PAY48								-
PAY51								-
PAY68	-	-	-	-	+/-	-	-	-
PAY69								-
PAY73	-	-	-	-	+/-			-
PAY75								-
JVY2								-
JVY24								-
JVY44								-
JVY59								-
Oxford								
TPO23	-	-	-	-	+			
TPO28	-	-	-		+			
TPO37	-	+/-	-	-	+	-	-	-
JVO38	-	-	-		+			
Wageningen-Hoog								
PPW9	+				-			
PPW10	+	+/-	-	-	-	+	-	-
PPW48	+				-			
PPW49	+				-			
TVW56	-	+/-		-	+	-		-
JVW13	-	-		-	+	-		-

Continued next page.

Table 35 continued

TRV-isolate	Antisera PRN	SYM	N5	TCB2-8	RQ	TY	TS	SP5
Wieringermeer								
PTW1	-	+/-	-	-	-	+	-	-
PTW7	-	+/-	-	-	-	+		
PTW19	-					+		
PTW25	-					+		
PTW26	-					+		
PTW47	-					+		
PTW61	-					+		
PTW80	-					+		
JVW52	-					+		
JVW72	-					+		
JVW74	-					+		
JVW91	-					+		
'tZand								
TSZ7		-					+	-
TSZ19		+/-					+	-
TSZ34	-	-	-	-	-	-	+	-
TSZ41	-	+/-	-	-	-		+	-
TSZ51		+/-					+	-
TSZ57		+/-					+	-
Julianadorp								
PTJ3			+					
PTJ21	-		+		-	-	-	
PTJ22			+					
PTJ28			+					
PTJ50			+					
PTJ53			+					
PTJ57			+					
PTJ59			+					
PTJ63			+					
JVJ47			+					
JVJ60			+					
JVJ65			+					

Continued next page.

Table 35 continued

TRV-isolate	Antisera PRN	SYM	N5	TCB2-8	RQ	TY	TS	SP5
Cocksdorp								
PPC12	+				-			
PPC16	+				-			
PPC17	+		-		-	+	-	
PPC29	+				-			
PPC30	+				-			
PPC49	+				-			
PPC73	+				-			
PPC93	+				-			
TVC37	+/-				+/-			
TVC47	-		-		+	-	-	
TVC53	-				+			
TVC68	-				+			
Santpoort								
PPSa11	+							
PPSa31	+							
PPSa36	+							
PPSa56	+							
JVSa8	+							
Julianadorp								
JVJ6	-		+		-			
Sweden								
PPS49	+	+/-	-	-	-	+	-	-

*) Isolates in bold typeface were picked as "typical" isolates and tested against a larger range of antisera. Isolates starting with "PA" transmitted by *P. anemones*, with "PP" by *P. pachydermus*, with "PT" by *P. teres*, with "TC" by *T. cylindricus*, with "TP" by *T. primitivus*, with "TS" by *T. similis*, with "TV" by *T. viruliferus* and with "JV" by juvenile trichodorids.
+: positive reaction, +/-: inconsistent cross reaction; A_{405} readings *c.* half the value of the homologous isolate, -: negative reaction, blank space: not tested.

Table 35 shows that the PRN-antiserum reacted positively with isolates transmitted by *P. pachydermus*. The SYM-antiserum only reacted positively with two isolates but cross-reacted with a large number of isolates. The N5-antiserum recognized isolates transmitted by *P. teres* from Julianadorp and an isolate transmitted by a *Paratrichodorus* juvenile from Julianadorp. Antiserum TCB2-8 only reacted with its homologous isolate TCB2-8 and two more isolates also transmitted by *T. cylindricus*

from the same site. The RQ-antiserum reacted with a number of isolates transmitted by different *Trichodorus* spp. (*T. cylindricus*, *T. primitivus* and *T. viruliferus*), but not with those transmitted by *Paratrichodorus* spp., although in some tests a cross reaction with isolates transmitted by *P. anemones* was observed. The TY-antiserum reacted with all isolates reacting with the PRN-antiserum, but also with the isolates transmitted by *P. teres* from Wieringermeer, which did not react with the PRN-antiserum. The TS-antiserum only reacted with *T. similis* transmitted isolates and the SP5-antiserum only with isolate TCS44.

Results of an EM decoration test using isolate PPK20 trapped onto a grid with a mixture of PRN- and SYM-antiserum showed that all particles were heavily decorated by PRN-antiserum but that no decoration could be observed using SYM-antiserum.

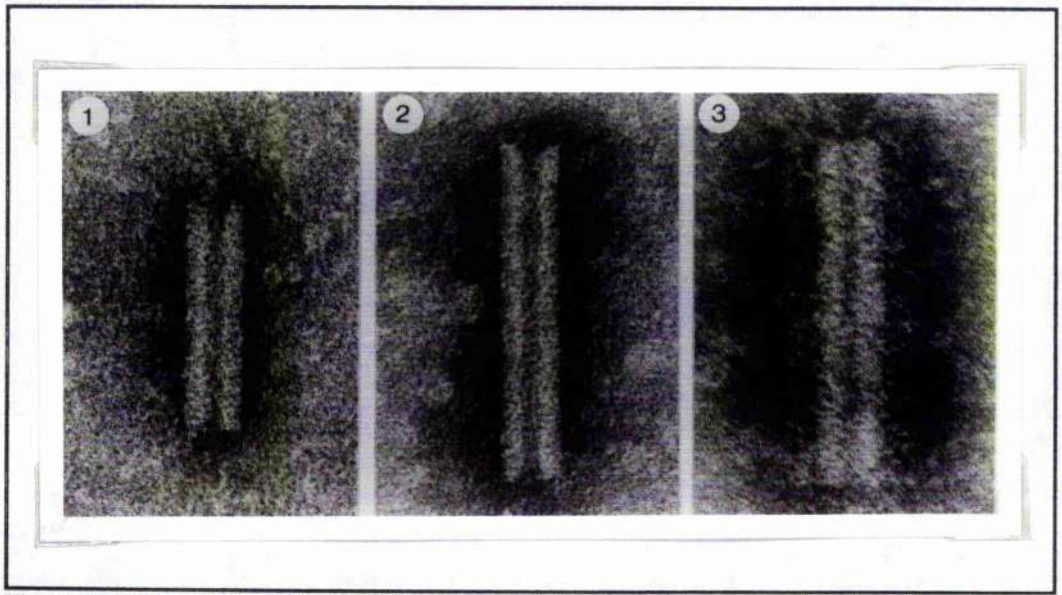

Figure 9. TRV particles of isolate PPK20. 1: Not decorated, 2: Decorated with SYM-antiserum, 3: Decorated with PRN-antiserum

Trapping of isolate PTW1 with ORE-antiserum onto an EM-grid resulted in a high increase of the number of particles compared to no trapping and to trapping with PRN-antiserum.

Table 36. Number of TRV particles of isolates PTW1 on EM-grids without trapping and when trapped with ORE- or PRN-antiserum. EM magnification x13000. Relative area factor (RAF) 389 at standard area (SA) 1000 μm^2 (Roberts, 1980). Four gridholes examined for each treatment.

	Average nr. of particles per field of view	Nr. particles per standard area	Trapping efficiency index
No trapping*	0.10	38.90	1
Trapped with PRN-A\S*	0.06	23.34	0.6
Trapped with ORE A\S**	22.17	8624.13	221.7

*) 120 fields of view examined
**) 12 fields of view examined

VII.3.d.- *Symptomatology:* Since most isolates caused very similar symptoms in *C. amaranticolor*, *C. quinoa* and *N. clevelandii* plants, only those isolates which caused consistent anomalous symptoms are discussed in detail. Furthermore, results are presented of symptoms in other test plant species inoculated with selected TRV-isolates.

All isolates caused necrotic lesions in inoculated leaves of *C. amaranticolor* within 3-4 days after inoculation. Lesions sometimes tended to spread but no consistent differences between isolates were observed.

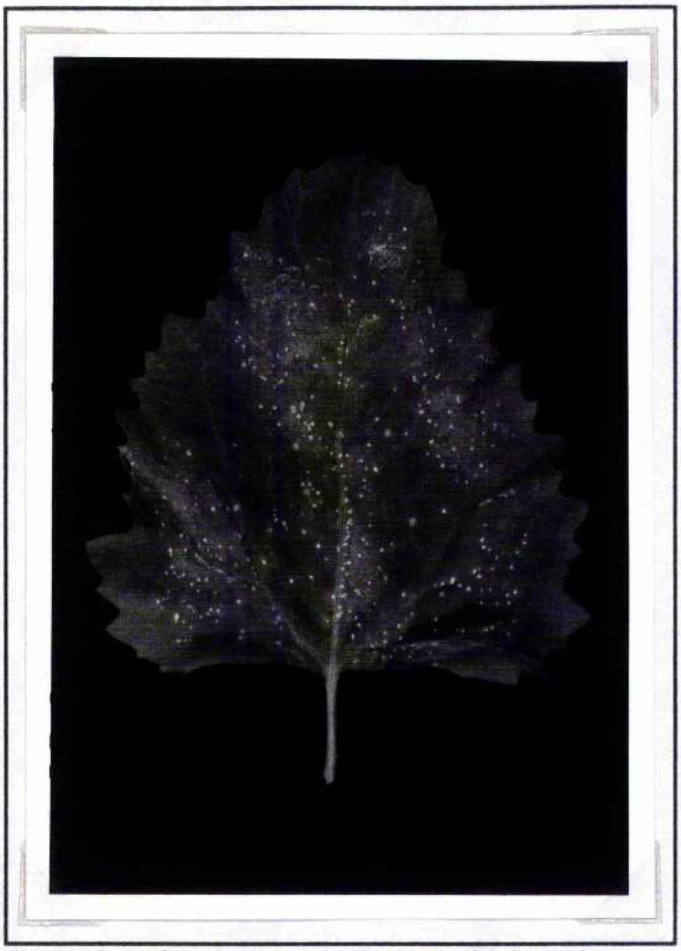

Figure 10. Lesions in *C. amaranticolor* leaf 3 days after manual inoculation with TRV.

Isolates PPK92 and PPK96 caused systemic necrosis of the tops usually within 10 days after inoculation. None of the other isolates caused systemic symptoms.

Symptoms in *C. quinoa* consisted of the appearance of necrotic lesions or specks in the inoculated leaves usually within 3-4 days after inoculation, often followed by a collapse of these leaves. Isolates PPK92, PPK96, all isolates transmitted by *P. anemones* (See Table 22) and all isolates transmitted by *T. similis* from 'tZand (See Table 26) caused systemic necrosis of the tops, usually within 10 days after inoculation.

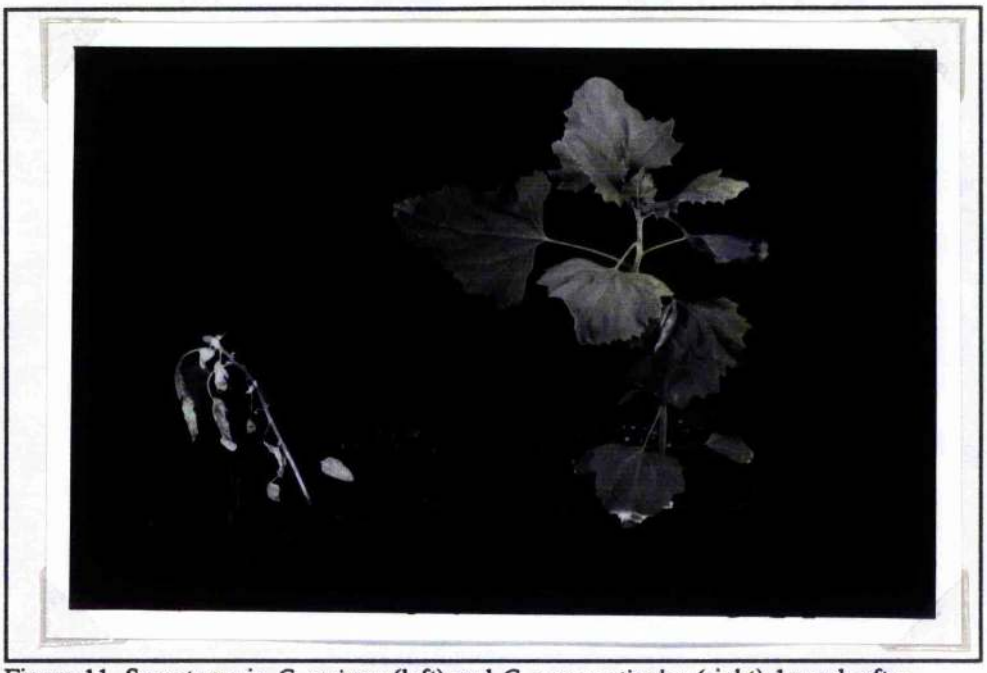

Figure 11. Symptoms in *C. quinoa* (left) and *C. amaranticolor* (right) 1 week after manual inoculation with TRV-isolate PAY68.

An NM-type PAY68 isolate, obtained by inoculation of diluted infected sap of *N. clevelandii* (1 part sap in 1000 parts tap water) onto *C. quinoa* failed to cause systemic symptoms in *C. quinoa.*

General symptoms caused by TRV-isolates in *N. clevelandii* consisted of the appearance of necrotic rings or arcs in the inoculated leaves *c.* 5 days after inoculation. Systemic symptoms consisting of a light mottling, often accompanied by an increase in the number of leaves developing, which subsequently showed slight malformation, could usually be observed from *c.* 2 weeks after inoculation.

Figure 12. *N. clevelandii* 3 weeks after manual inoculation with TRV-isolate PPK20 (left) and water (right).

Isolate PPK92 could be distinguished from the other isolates obtained from the Kinshaldy soil because it failed to produce any symptoms in *N. clevelandii*. Isolate TCS44 usually caused severe necrosis of inoculated leaves, causing these leaves, and sometimes the complete plant, to collapse. Usually, however, the collapse of inoculated leaves was followed by an increased number of developing new leaves showing typical mottling and slight malformation symptoms.

Isolates PAY68, PAY27, TCS44, PTJ21 PTJ28, PTJ59 and PTJ63 were inoculated onto primary leaves of 2 *P. vulgaris* cv. The Prince plants. TRV-strain PRN and PEBV-strain SP5 were also inoculated onto 2 *P.vulgaris* plants. The seven isolates obtained in this study and the PRN-strain of TRV caused numerous pinpoint lesions in the inoculated leaves of *P. vulgaris* within 2-3 days after inoculation. The SP5-strain of PEBV however caused large, spreading lesions in *P. vulgaris*.

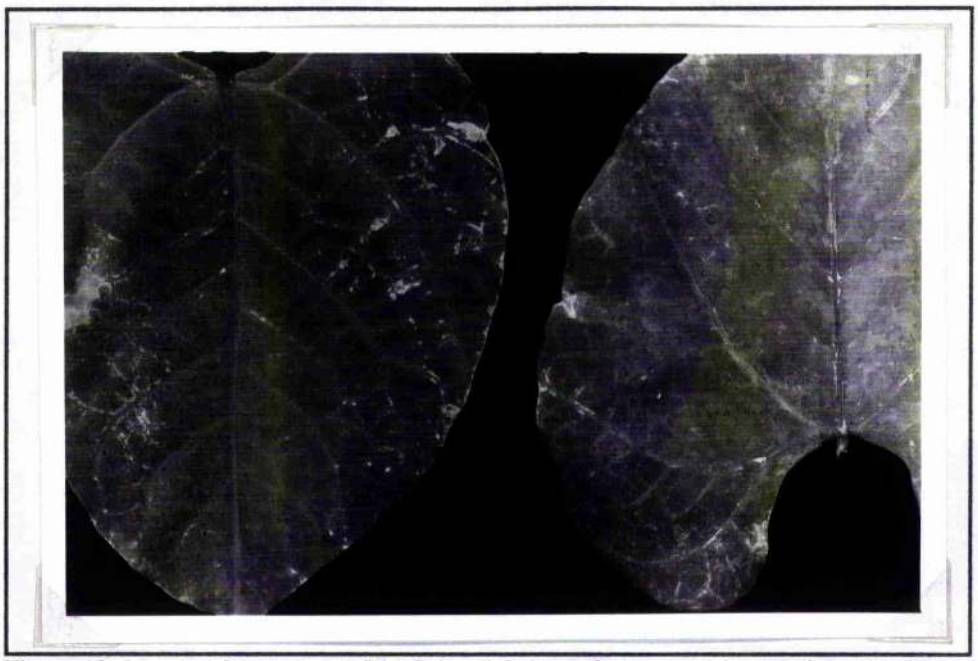

Figure 13. Leaves of *P. vulgaris* "the Prince" 3 days after manual inoculation with TRV (left) or PEBV (right).

Isolates obtained from Barry Stables (See Table 17) were each inoculated onto 3 *N. tabacum* cv. White Burley and 2 *C amaranticolor* plants. They could be clearly categorized into two groups with isolates transmitted by *P. pachydermus* causing severe systemic symptoms consisting of necrotic arcs and rings, and isolates transmitted by *T. cylindricus* causing only mild systemic mottling. Numbers of lesions developing in inoculated leaves of *C. amaranticolor* were not clearly different between isolates indicating that differences in virus concentration between isolates was unlikely to be the cause of the observed differences in systemic symptoms.

Isolates PTW1, PTW7, PTW25, PTW26, JW72 and JW91 (See Table 25) were each inoculated onto 2 *N. glutinosa* and 2 *C. amaranticolor* plants. Systemic symptoms in *N. glutinosa* ranged from numerous necrotic flecks all over the systemically infected leaves (isolates PTW1, JW72) to sporadic necrotic and chlorotic lesions (PTW26, JW91) to very few systemic symptoms (PTW7, PTW25). All

inoculated leaves of *C. amaranticolor* developed numerous lesions indicating that differences in virus concentration between isolates was unlikely to be the cause of the observed differences in systemic symptoms.

Figure 14. *N. glutinosa* 2 weeks after manual inoculation with TRV-isolates PTW1 (left), PTW26 (middle) or PTW7 (right).

VII.3.e.- *Particle length distribution:* Particle lengths of TRV-isolate PPK20 showed two clear peaks, the first at 100-105 nm, the second at 195-200 nm. A possible third peak can be observed at 65-70 nm.

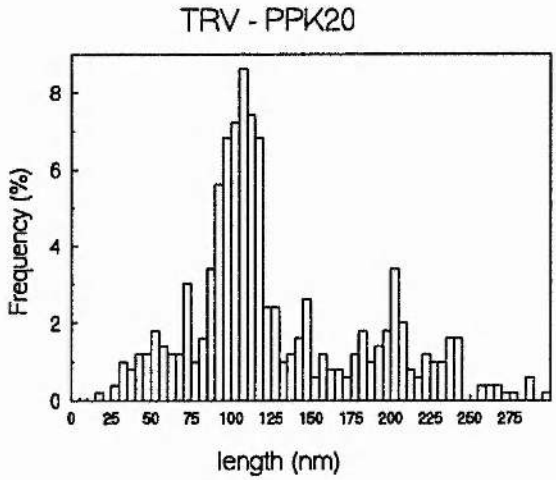

Figure 15. Particle length distribution of TRV-isolate PPK20. (500 particles measured).

Particle measurement of TRV-isolate TCS44 revealed higher number of particles with lengths between 90 and 95 nm and between 185 and 190 nm.

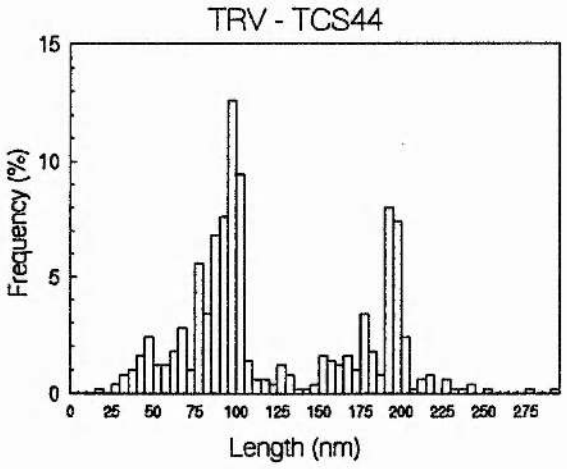

Figure 16. Particle length distribution of TRV-isolate TCS44. (500 particles measured).

Particle measurement of TRV-isolate PAY68 showed particle length peaks between 105 and 110 nm and between 185 and 190 nm.

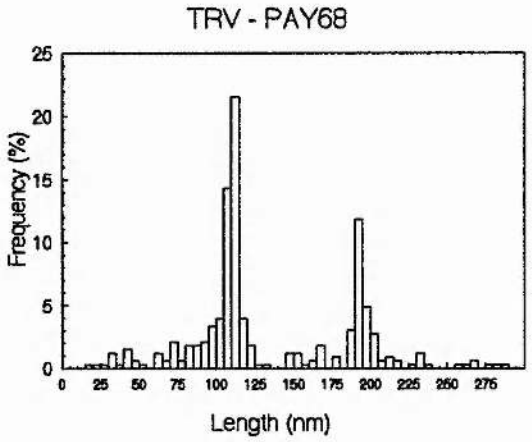

Figure 17. Particle length distribution of TRV-isolate PAY68. (330 particles measured).

Finally, particle length measurement of TRV-isolate PTW1 showed particle length peaks at 95-100 nm and at 195-200 nm with a possible third small peak at 40-45 nm.

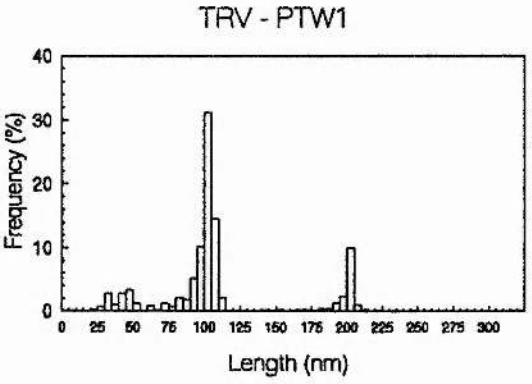

Figure 18. Particle length distribution of TRV-isolate PTW1. (680 particles measured).

VII.4. DISCUSSION AND CONCLUSIONS.

VII.4.a.- *Associations between vector trichodorid species and TRV-serotypes:* The results obtained from the bait tests indicate a substantial degree of specificity between virus serotypes and trichodorid species. However, it is also clear that these associations are more complex than a simple "one species - one serotype" relation. A discussion of these associations is presented below.

VII.4.a.*i.*- *P. pachydermus*: The results of the $F(ab')_2$-ELISA tests on the transmitted isolates show that in general isolates transmitted by *P. pachydermus* belonged to the PRN-serotype. Exceptions were isolates PPK92 and PPK96 which were classified as belonging to the SYM-serotype. Also, these two isolates, like the SYM-strain of TRV, caused systemic symptoms in *C. amaranticolor* and in *C. quinoa*, which distinguished them from all other isolates. The possibility of *P. pachydermus* transmitting SYM-serotype isolates cannot be completely ignored, but the fact that such isolates were only recovered once from numerous transmissions by *P. pachydermus*, even though soil from the same site was repeatedly bait tested, makes this result doubtful. Furthermore, trichodorid nematodes from the same soil from which these isolates were recovered failed to transmit SYM-serotype isolates after having had access to *P. hybrida* source-plants manually inoculated with isolate PPK92 or TRV-strain SYM. Also virus-free *P. pachydermus* did not transmit such isolate types after having had access to *P. hybrida* source-plants manually inoculated with isolate PPK92 or TRV-strain SYM (See Chapter VIII). The most likely explanation for these anomalous results therefore is that the SYM-serotype isolates

transmitted by *P. pachydermus* resulted from a contamination of the bait-plants or virus indicator plants with TRV-strain SYM.

The transmission of PRN-serotype isolates by *P. pachydermus* confirms the conclusion from Chapter VI that this vector species is associated with the PRN-serotype. Other workers (See Table 8, Chapter IV) also reported the transmission of TRV by *P. pachydermus*, but the serological affinities between the isolates recovered in this study and those obtained by others are unknown. Van Hoof (1962) reported the transmission of PEBV from the Netherlands by this vector, but this result was not confirmed here. From the results obtained in this study it can be concluded that whenever *P. pachydermus* is transmitting TRV, the virus will most probably belong to the PRN-serotype. Conversely, whenever PRN-serotype TRV is found, the vector is most likely to be *P. pachydermus*.

VII.4.a.*ii*.- *P. teres*: The association between vector species and serological characteristics of transmitted isolates can however be more complex. *P. teres* from two populations in the Netherlands transmitted serologically distinct isolates of TRV, the population from Wieringermeer ORE-serotype isolates and the population from Julianadorp N5-serotype isolates. However, the isolates transmitted within the populations were similar. Individual *P. teres* from the Wieringermeer transmitted isolates which reacted with the TY-antiserum, but which, unlike the isolates transmitted by *P. pachydermus*, failed to react with the PRN-antiserum. The ORE-strain of TRV, reacting with the TY- but not with the PRN-antiserum (See V.4.c.*ii*), was therefore serologically similar to the *P. teres* transmitted isolates. Although these isolates were not tested in $F(ab')_2$-ELISA against the ORE-antiserum, because no

such antiserum was available in reasonable amounts, the results obtained by trapping particles of isolate PTW1 onto EM-grids with antiserum ORE (See Table 36) indicated the affinity of this antiserum for this isolate. It can therefore be concluded that isolate PTW1 is serologically very similar to the ORE-strain of TRV and that antiserum TY should be regarded as a mixed antiserum, reacting with at least two serologically distinct types of TRV (PRN- and ORE-serotype isolates).

P. teres from the population from Julianadorp transmitted isolates reacting with antiserum prepared against the N5-strain of TRV. The N5-strain however is considered an anomalous TRV-strain, originated from recombination between PEBV and TRV, having RNA-2 sequence homologies with the Dutch strain of PEBV, and an RNA-1 sequence homologous to that of TRV. Indeed, the N5- antiserum recognizes isolates of the Dutch serotype of PEBV (Robinson *et al.*, 1987). Inoculation of the *P. teres* transmitted isolates PTJ21, PTJ28, PTJ58 and PTJ59 onto *P. vulgaris* produced symptoms typical of TRV rather than PEBV. Furthermore, in a spot-hybridization test, RNA of isolate PTJ21 reacted specifically with a cDNA probe derived from TRV RNA-1, implying that this isolate should be classified as a TRV- and not as PEBV-isolate (D.J.Robinson, pers. comm.). Therefore, like the N5-strain of TRV, isolate PTJ21, should be considered an anomalous TRV-isolate having RNA-2 sequences homologous to the Dutch strain of PEBV and an RNA-1 sequence typical of TRV. There is no reason to assume that the other isolates transmitted by *P. teres* from the same population are different.

Transmission of ORE- and PEBV-D-serotype isolates by *P. teres* corresponds with results obtained by Jensen *et al.* (1974), who reported transmission of ORE-serotype TRV by *P. teres* in the USA, and those by van Hoof (1962) who reported

transmission of PEBV by *P. teres* in the Netherlands. *P. teres* therefore is able to transmit tobravirus-isolates belonging to at least two distinct serotypes. It is unknown if there is any specificity within *P. teres* regarding the transmission of isolates belonging to the two serotypes. However, in this study the *P. teres* population which transmitted ORE-serotype isolates contained about equal numbers of males and females, whereas no males were found in the populations associated with the N5-serotype (See also Table 11). Whether this observation is coincidental or reflects a true association between population type and TRV-serotypes should be tested using individual *P. teres* obtained from both population types given access to virus source-plant roots infected with tobravirus-isolates belonging to either serotype.

VII.4.a.*iii*.- *P. anemones*: The association of an uncharacterized tobravirus-isolate with *P. anemones* as concluded in VI.4 was confirmed by bait testing single *P. anemones* nematodes. As the isolates transmitted by this species failed to react in $F(ab')_2$-ELISA, they could initially not be identified as belonging to the tobravirus group. However, the fact that they were transmitted by a trichodorid vector, the bipartite rod shaped nature of the particles and the modal length of the longer particles as observed in the electron microscope made it improbable for it to be anything other than a tobravirus. Symptoms resulting from inoculation of isolates PAY27 and PAY68 onto *P. vulgaris* indicated that both were TRV- rather than PEBV-isolates. Isolate JVY2, transmitted by a juvenile, was shown to be TRV in a cDNA-hybridization test (D.J. Robinson, pers. comm.). Transmission of the English strain of PEBV by *P. anemones* was reported by Harrison (1967) and of TRV from the Netherlands by van Hoof (1968). A reaction of isolates PAY27 and PAY68 with

antiserum SP5 (= English PEBV-serotype) in a plate trapped antigen (PTA)-ELISA (results not shown), was not confirmed in $F(ab')_2$-ELISA. The report by Harrison (1967) on the transmission of the English strain of PEBV by *P. anemones* is therefore not necessarily in disagreement with our results, as $F(ab')_2$-ELISA is a relatively recent, highly specific serological technique which separates the *P. anemones* transmitted isolates from the English PEBV-serotype, whereas in less discriminating techniques (e.g. PTA-ELISA) these isolates clearly reacted with the PEBV-E antiserum. The same isolates gave an inconsistent cross reaction with antiserum RQ in $F(ab')_2$-ELISA. Thus, although isolates transmitted by *P. anemones* appear serologically distantly related to the English strain of PEBV and possibly to the RQ-strain of TRV, they could not be clearly classified as belonging to a particular serotype.

The symptoms in *C. quinoa* after inoculation with isolates transmitted by *P. anemones* were similar to those caused by TRV-strain SYM, consisting in systemic top necrosis. However, *P. anemones* transmitted isolates could be symptomatologically distinguished from TRV-strain SYM as they failed to cause systemic symptoms in *C. amaranticolor*. The fact that systemic top necrosis in *C. quinoa* did not occur when infected with NM-type isolate PAY68, suggests that the RNA-2 part of the virus genome is at least involved in the production of this symptom. A similar result was obtained by Kurppa *et al.* (1981) who found that the systemic invasion of *C. amaranticolor* by strain SYM was controlled by the RNA-2.

VII.4.a.*iv*.- *P. nanus*: *P. nanus* failed to transmit virus. Van Hoof (1973) however reported *P. nanus* to be an efficient vector of TRV in Dutch bulb fields and Cooper

and Thomas (1970) reported the transmission of PRN-serotype TRV by this species in Scotland. Particularly the latter observation does not correspond with our results as *P. nanus*, extracted from soil which also contained *P. pachydermus* transmitting PRN-type isolates (See Table 28), failed to transmit such isolates. The conclusion from Chapter VI that *P. nanus* may be associated with N5-serotype (= Dutch PEBV-serotype) isolates, was not confirmed. A likely explanation for the discrepancy between results obtained from pot bait tests in Chapter VI and those obtained using single nematodes is that, in addition to *P. nanus* also *P. teres* was present in numbers too low to be readily detected, but sufficient to account for the virus transmission. The likelihood of this explanation is increased by the observation that *P. teres* was the most efficient vector of the species tested and that one of two juveniles, identified as a *Paratrichodorus* spp. but not *P. nanus*, transmitted an N5-serotype isolate, whereas 105 adult *P. nanus* individuals failed to transmit any virus (See Table 30).

VII.4.a.*v*.- <u>*Trichodorus* spp. general</u>: There is less evidence for the occurrence of specific associations between *Trichodorus* vector species and TRV serotypes. Isolates reacting with the RQ-antiserum were transmitted by three *Trichodorus* species: *T. cylindricus*, *T. primitivus* and *T. viruliferus*. Whether each of the three species are able to transmit isolates originally transmitted by one of the other species, or whether the species recognize differences within the RQ-serotype, is unknown. If the RQ-antiserum was a single antiserum rather than an antiserum raised against a mixture of serologically distinct isolates, then it can be concluded that there is no specific association between the vector species *T. cylindricus*, *T. primitivus* and *T. viruliferus*

and serotypes of transmitted TRV-isolates. However, the RQ-antiserum was raised against TRV obtained from roots of a cucumber bait-plant grown in trichodorid nematode containing field soil (Cooper, 1972), and the possibility of the bait-plant having been infected with different TRV-serotype isolates cannot be completely excluded. In order to unequivocally establish the lack of specificity or otherwise, it will be necessary to demonstrate that an RQ-serotype isolate transmitted by any one species cannot be transmitted by the others.

VII.4.a.*vi.*- *T. cylindricus*: Apart from RQ-serotype isolates, *T. cylindricus* also transmitted an isolate (TCS44) which reacted with the antiserum to the SP5-strain of English PEBV, three isolates reacting with the TCB2-8-antiserum and, thirdly, an isolate (TCB4) which remained uncharacterized. When the latter isolate was tested against the TCB2-8 antiserum it failed to react, but it did show a previously not observed reaction with the PRN-antiserum. This indicated that the TCB4-isolate was no longer present in its original form, either due to contamination with PRN from an outside source or, less likely, because a PRN-serotype isolate arose in the original TCB4-culture and multiplied to the exclusion of the original form. Isolate TCS44, classified as belonging to the English PEBV-serotype, gave symptoms typical of TRV when inoculated onto *P. vulgaris*, and was subsequently confirmed to be TRV in a cDNA-hybridization test (D.J. Robinson, pers. comm.). Isolate TCS44 therefore is like the isolates transmitted by *P. teres* from 'tZand, with RNA-1 like TRV but with RNA-2 sequences homologous to PEBV. Thus, *T. cylindricus* naturally transmitted several serologically distinct isolates of TRV even within one population

(isolates TCS26 and TCS44). The only other record of *T. cylindricus* transmitting TRV is by van Hoof (1968).

VII.4.a.*vii*.- *T. primitivus*: The association between *T. primitivus* and serotype RQ as observed in Chapter VI was confirmed by the transmission by individual *T. primitivus*. The same association was reported by Cooper (1972) who isolated the RQ-strain from a cucumber bait-plant grown in field soil containing a *T. primitivus* population. The same species has also been reported to transmit the English strain of PEBV (Gibbs and Harrison, 1964a) and recently this was also found by Brown and Robinson (pers. comm.) using single *T. primitivus* from soil obtained from a field with a PEBV affected pea crop in England. Thus, like *T. cylindricus* , *T. primitivus* naturally transmits serologically distinct tobravirus-isolates, of the RQ- and PEBV-E-serotypes.

VII.4.a.*viii*.- *T. viruliferus*: Results from pot bait tests did not reveal a clear association between *T. viruliferus* and TRV-serotypes (See Chapter VI), but individual *T. viruliferus* from two populations transmitted RQ-serotype isolates. *T. viruliferus* has been reported to transmit TRV in the Netherlands (van Hoof, 1968) and in Italy (van Hoof *et al.*, 1966) and PEBV in England (Gibbs and Harrison, 1964a) but serological affinities between TRV isolates transmitted by *T. viruliferus* are unknown and transmission of PEBV by this species was not found in this study.

VII.4.a.*ix*.- *T. similis*: *T. similis* transmitted isolates which initially failed to react with the available antisera. This rendered these isolates indistinguishable from those

transmitted by *P. anemones*. Furthermore, symptoms in *C. amaranticolor* and in *C. quinoa* resulting from inoculation with isolates transmitted by these species were identical. However, antiserum TS, prepared against isolate TSZ34 by C.J. Asjes, recognized all isolates transmitted by *T. similis* but failed to recognize those transmitted by *P. anemones*. Cremer and Schenk (1967) reported the transmission of TRV causing notched leaf symptoms in Gladiolus by *T. similis*, and prepared an antiserum against this virus. This antiserum strongly decorated isolate TSZ34 in an EM decoration test (C.J. Asjes, pers. comm.) which indicates the serological similarity of the isolate transmitted by *T. similis* causing notched leaf and isolate TSZ34 transmitted by *T. similis* found in this study.

VII.4.a.*x*.- T. *sparsus*: *T. sparsus* did not transmit virus in tests during this study. However, transmission of TRV by this species has been reported by van Hoof (1968; 1970a).

VII.4.a.*xi*.- Overall species - serotype associations: A schematic representation of the associations between tobravirus-serotype and vector species as found in this study is shown in Fig. 19.

It can be concluded that serotypes are either associated with *Paratrichodorus* or *Trichodorus* species but not with both. Within *Paratrichodorus* different species were associated with different serotypes, although different serotypes were associated with the same species. In contrast, within *Trichodorus* the same species was found to transmit several serologically distinct TRV-isolates and, moreover, serologically similar isolates were transmitted by several *Trichodorus* species.

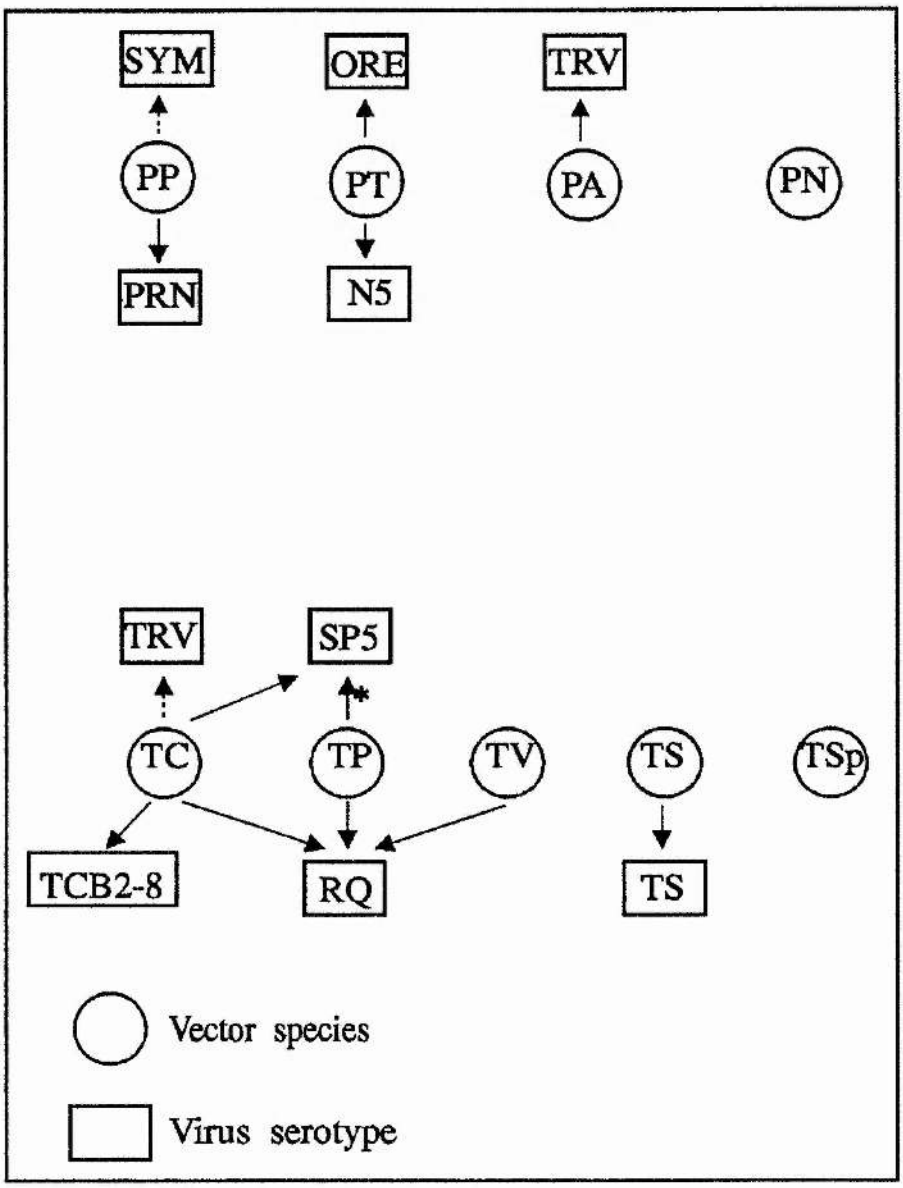

Figure 19. Associations between trichodorid species and TRV-serotypes. Species: PP: *P. pachydermus*, PT: *P. teres*, PA: *P. anemones*, PN: *P. nanus*, TC: *T. cylindricus*, TP: *T. primitivus*, TV: *T. viruliferus*, TS: *T. similis*, TSp: *T. sparsus*.
* from Brown and Robinson (pers. comm).

This situation is also reflected in the results obtained from bait testing individual trichodorids from mixed species populations as *P. pachydermus* and *T. cylindricus*

from the same population transmitted TRV-isolates which could be serologically categorized according to the vector species. The same was true for *P. pachydermus* and *T. viruliferus* from the same population. However, isolates transmitted by *T. primitivus* or *T. cylindricus* from the same population could not be serologically separated according to vector species. The inability of *Paratrichodorus* spp. to transmit TRV-isolates naturally transmitted by *Trichodorus* species and vice versa is also illustrated by the significant differences in transmission rates between *Paratrichodorus* and *Trichodorus* spp. present in the same population e.g. *P. pachydermus* and *T. similis* (Table 16), *P. pachydermus* and *T. cylindricus* (Table 17), *P. teres* and T. *primitivus* (Table 25) and *P. teres* and *T. similis* (Table 27). Although such a difference was also observed between *T. cylindricus* and *T. primitivus* in the same population, the serological similarity of isolates transmitted by these two species indicates that one species was merely a slightly more efficient vector of the same type of isolate (See Table 21).

VII.4.b.- *Transmission efficiency*: There were no significant differences between transmission rates of females and males in any of the bait tests. However, the overall transmission rate of juveniles was significantly higher than that of males and that of females. This was mainly caused by the results from the bait test on soil from Wieringermeer (See Table 25) in which 13 out of the 22 juveniles, transmitted TRV. Within this bait test however the juveniles did not have a significantly higher transmission rate than males or females. My results are, therefore, similar to those obtained by Ayala and Allen (1966) who found males, females and juveniles of *T. allius* almost equally efficient vectors. Transmission frequencies for transmitting

adults only, per species per population ranged from 1% for the least effective population of *P. pachydermus* to 78% for the most effective population of *P. teres*, with an average transmission rate of transmitting adults per species of 21%. When including non-transmission, significant differences in transmission frequencies between species were observed, with *P. teres* having a significantly higher transmission frequency than the other species (See Table 34). The overall transmission frequency of *T. cylindricus* (7%) and *T. similis* (5%) each was significantly lower than that of the other TRV transmitting species, higher than the non-transmission of *P. nanus* but not significantly higher than the non-transmission of *T. sparsus*. Summarizing, it can be concluded that, in my tests, *P. teres* was the most efficient vector, the species group with *P. anemones*, *P. pachydermus*, *T. viruliferus* and *T. primitivus* transmitted TRV relatively efficiently and the group with *T. cylindricus*, *T. similis*, *T. sparsus* and *P. nanus* was characterized by low or non-transmission. The relevance of this classification may however be questioned as the populations were obtained at different times of the year from a variety of habitats with a range of plant species. Particularly the differences in plant species may have played an important role in the ability of the nematodes to acquire and retain virus. Furthermore, it must be kept in mind that the classification of *T. sparsus* and *P. nanus* as inefficient vectors is based on results from one population of each species only and that, when testing other populations, these species may be found to be efficient vectors of TRV. Also, the fact that these species failed to transmit may be the result of the bait-system itself. The possibility that e.g. these species do not feed on *P. hybrida*, which would explain the lack of virus transmission, cannot be

excluded. On the other hand it is clear that in particular *P. teres*, even when present in low numbers in the soil, may cause high TRV incidence in a susceptible crop.

VII.4.c.- *Particle length distribution*: The length distribution of isolates PPK20, PTW1, TCS44 and PAY68 revealed the bipartite nature of these isolates. The long-particle length varied between 185 and 200 nm which corresponds with that given for TRV (*c.* 190 nm; Robinson and Harrison, 1989). The short-particle length was *c.* 107 nm for PAY68, *c.* 97 nm for PTW1, *c.* 92 nm for TCS44 and *c.* 102 nm for PPK20. ORE-strains of TRV, serologically related to isolate PTW1, had short-particle lengths of 81, 90 or 100 nm (Lister and Bracker, 1969). Short particles of strain PRN, serologically similar to isolate PPK20, were 78 nm long (Harrison and Nixon, 1959) and those of anomalous TRV-isolate I6, similar to isolate TCS44, were 105 nm (Robinson and Harrison, 1989). Although too few data on particle lengths have been collected, it is unlikely that it is involved in determining vector specificity or transmissibility, since particle length is merely a reflection of the sizes of the RNA genome parts of the virus and does not directly relate to the function and number of genes present on the different genome parts.

VII.4.d.- *NM-type isolates:* The recovery of NM-type infections from indicator plants inoculated with the *P. hybrida* bait-plant roots does not necessarily imply that the vector nematodes transmitted NM-type infections as such. An NM-type infection can result from a chance separation of long and short particles. The most likely explanation for the recovery of NM-type infections from indicator plants is that a low virus concentration in the bait-plant roots resulted in the inoculation of long particles

only onto indicator plants, giving rise to NM-type infections. Even when NM-type infections are found in the bait-plant roots, this could have resulted from the nematodes by chance only transmitting injecting long TRV particles, rather than transmitting "naked" RNA-1. Furthermore, successful transmission of NM-type TRV is unlikely as the naked RNA is very sensitive to RNase activity and therefore very unstable. As a result of this transmission of NM-type infections is usually only successful when precautions are taken to protect the RNA from RNase enzymatic activity.

VII.4.e.- *Symptom variation:* Finally, it was shown that variations in symptom development in plants inoculated with a range of TRV-isolates are not necessarily related to variations in serological characteristics of the isolates or to differences in the virus vector as serologically indistinguishable isolates transmitted by *P. teres* from one population caused a range of symptoms in *N. glutinosa* plants. This implies that damage in a crop resulting from TRV infection will not only depend on characteristics of the crop cultivar, the presence and composition of trichodorid vector populations, environmental and physical factors, but also on the variation in pathogenicity present in the pool of transmissible TRV-isolates.

CHAPTER VIII

ACQUISITION AND SUBSEQUENT TRANSMISSION OF TOBACCO RATTLE VIRUS ISOLATES BY TRICHODORID NEMATODES.

VIII.1. INTRODUCTION.

The data presented in Chapter VII suggest that there is a considerable degree of specificity between trichodorid vector species and TRV-serotypes. The results were, however, obtained from bait tests using naturally viruliferous trichodorid populations, and are therefore merely observations that particular species naturally transmit TRV-isolates belonging to a particular serotype. Whether true specificity occurs, i.e. whether a particular vector species will only transmit those isolates belonging to the serotype with which this species is naturally associated, remained unknown. Thus, van Hoof (1968), found that initially virus-free *P. pachydermus* only successfully acquired and subsequently transmitted one TRV-isolate out of several serologically similar isolates tested, and that this isolate, in contrast with the others, originated from the same locality as the nematodes. He concluded that "TRV will not readily be spread by infected plant material, since there is only a small chance that the TRV strain will suit the *Trichodorus* population in the new habitat". Harrison (1966) also found that virus-free *T. primitivus* acquired and transmitted a British isolate of PEBV obtained from the same area as the nematodes, but failed to do so when given access to a serologically distinct Dutch PEBV-isolate. Furthermore, Harrison (1967) reported that two serologically related isolates of British PEBV were transmitted by *P. anemones*, but that a Dutch PEBV-isolate was not transmitted by this species.

In the experiments described in this chapter initially virus-free trichodorids were allowed access to a series of TRV-isolates and subsequently tested for their ability to transmit these isolates. Also, the effect on the rate of transmission of allowing naturally viruliferous trichodorids access to a TRV-source prior to using the nematodes in transmission tests was studied.

VIII.2.- MATERIALS AND METHODS.

VIII.2.a.- *Nematode populations:* The nematodes used in acquisition and transmission tests were extracted from Kinshaldy (*P. pachydermus*, *T. similis*), Barry (*P. pachydermus*, *T. cylindricus*) or Woodhill (*P. pachydermus*, *T. primitivus*, *T. cylindricus*) (See also Chapter VI, Table 13). In addition, nematodes from soil from Wageningen, the Netherlands, sampled in April 1990 from grass underneath apple trees, were used. In this sample *P. pachydermus* (*c.* 85/100 g soil) was the only trichodorid species found.

VIII.2.b.- *TRV-isolates and -strains:* TRV-strain SYM and isolate PPK92, belonging to the SYM-serotype, strain ORE and isolate PTW1, belonging to the ORE-serotype, strains PRN, PLB and isolates PPK20, PPB10, PPM10, PPW10, and PPS49, belonging to the PRN-serotype, isolates TVC49, TPO37 and TPE11, belonging to the RQ-serotype, isolate TCS44, belonging to the SP5-serotype, isolate TCB2-8 belonging to the TCB2-8 serotype and isolate PAY68 which failed to react with any of the available antisera (See also V.4.c.*ii* and Chapter VII, Table 35) were used as virus source for the nematodes. Also, a Danish TRV-isolate from an infected

potato tuber (cv. Bintje), was provided by D.J. Robinson and included in a acquisition and transmission test. This isolate reacted in $F(ab')_2$-ELISA with the PRN-antiserum (D.J. Robinson, pers. comm.).

VIII.2.c.- *Acquisition and transmission by bulk nematodes:* The general methods used for acquisition and transmission experiments have been described in V.3. The design of the different experiments is given below:

Experiment 1: A bulk nematode suspension from Woodhill was given access to *c.* 6 week-old *P. hybrida* plants (*c.* 45 trichodorids/plant) inoculated with TRV-isolates PPB10, PPK20, PPM10, PPS49, PPK92, PAY68, PTW1, TCB2-8, or TRV-strain PRN. Each isolate was inoculated onto six *P. hybrida* plants. As a control, nematodes were also added to 12 virus-free *P. hybrida* plants. One week after inoculation, prior to adding the nematodes, the presence of virus in the roots of the source-plants was assessed by inoculation of a part of each root system onto a *C. amaranticolor* indicator plant. After a 3 week acquisition period the nematodes were extracted from the source-plants and the numbers of trichodorids recovered counted. The complete root system of each source-plant was again tested for presence of virus as described above. The nematode suspension extracted from each source-plant was subsequently transferred to a virus-free *P. hybrida* bait-plant. Three weeks later the root system of each bait-plant was tested for the presence of TRV.

Experiment 2: As *P. hybrida* appeared not to be an equally suitable source-plant with all TRV-isolates, the effect of using *N. tabacum* cv. White Burley or *P. hybrida*

plants as source- or bait-plants on acquisition and transmission was studied. Nematodes extracted from Woodhill were given access to virus source-plants (*c.* 50 trichodorids/plant) inoculated with TRV-isolates PPK20, PPB10, PPM10, PPS49, PPW10, the Danish TRV-isolate or TRV-strains PRN or PLB. All strains and isolates were serologically similar, reacting with the PRN-antiserum. With each isolate or strain twelve 6 week-old *P. hybrida* and twelve 6 week-old *N. tabacum* cv. White Burley plants were inoculated to be used as virus source-plants. As controls twelve *P. hybrida* and twelve *N. tabacum* cv. White Burley plants remained uninoculated. Presence of virus in the roots before and after the acquisition period was assessed as described with Experiment 1. Nematodes were transferred from source-plants to virus-free *P. hybrida* or *N. tabacum* cv. White Burley bait-plants so that each combination of source- and bait-plant species occurred six-fold.

VIII.2.d.- *Acquisition by a virus-free nematode population and transmission by single trichodorids:* Nematodes from Woodhill (*P. pachydermus*, *T. primitivus*, *T. cylindricus*) and Wageningen (*P. pachydermus*)were individually bait tested to obtain additional information on the transmission frequency of individual trichodorid vector species following access to virus source-plants. Nematodes from both populations had been extensively tested for virus transmission in pot-bait tests and were found not naturally to transmit virus (results not shown). *P. hybrida* was used as source- and bait-plant. TRV-strains ORE and PLB and isolates PPK20, PPK92, PPB10, PPW10, TVC47 and TPE11 were used as virus sources. Before adding the nematodes to the source-plants, the presence of virus in the source-plant roots was confirmed by inoculation of a part of the root systems onto *C. amaranticolor* indicator plants. After

a 3 week acquisition period, the nematodes were extracted from the source-plants and trichodorid nematodes were individually bait tested for 10 days (See V.2.b). The isolates obtained from bait-plant roots were propagated in *N. clevelandii* and tested in $F(ab')_2$-ELISA as described in V.4.c.*iii*.

VIII.2.e.- *Acquisition by a viruliferous nematode population and transmission by single trichodorids:* To assess whether the transmission frequency of a naturally viruliferous trichodorid population could be increased by concentrating their feeding on a single plant, nematodes from Barry (*P. pachydermus*, *T. cylindricus*) and Kinshaldy (*P. pachydermus*, *T. similis*) were allowed access for 3 weeks to virus-free or virus-inoculated *P. hybrida* plants prior to testing single trichodorids for virus transmission. TRV-isolate PPB10 and PPK92 were used as virus sources for nematodes extracted from soil from Barry and Kinshaldy respectively.

VIII.3. RESULTS

VIII.3.a.- Acquisition and transmission of TRV by a virus-free nematode population: The results showed that differences occurred between isolates regarding the movement of the virus into the *P. hybrida* source-plant roots, with some isolates moving less rapidly than others. Furthermore, the transmission experiment with a virus-free nematode population from Woodhill (Experiment 1, VIII.2.c.) showed that PRN-serotype isolates which were originally obtained from transmissions by single *P. pachydermus* (isolates PPB10, PPK20, PPM10 and PPS49) were all successfully acquired and transmitted by the virus-free population from Woodhill. However, non-

PRN-serotype isolates (PPK92, PAY68, PTW1, TCB2-8 and TPO37) which had been obtained from transmissions by individual trichodorid nematodes and the PRN-strain which had been maintained in the laboratory at SCRI for many years, were not transmitted.

Table 37. Transmission of TRV to *P. hybrida* bait-plants by a nematode population from Woodhill after having had access to manually inoculated *P. hybrida* virus source-plants.

	TRV source									
	PPB10	PPK20	PPM10	PPS49	PPK92	PAY68	PTW1	TCB2-8	PRN	TPO37
P. hybrida sources with TRV in roots.										
before acquisition	2/6*	6/6	1/6	6/6	5/6	0/6	2/6	6/6	1/6	6/6
after acquisition	2/6	6/6	3/6	6/6	6/6	6/6	6/6	6/6	5/6	6/6
P. hybrida baits infected.	2/6	6/6	2/6	3/6	0/6	0/6	0/6	0/6	0/6	0/6

*) Numerator is number of *P. hybrida* plants from which virus was detected from the roots, denominator is number of *P. hybrida* plants set up.

No virus was detected in the roots of twelve virus-free *P. hybrida* "source"-plants after the acquisition period, nor did the nematodes, transferred from those plants to virus-free *P. hybrida* bait-plants, transmit virus to the roots of these bait-plants. This indicates that the population was not naturally viruliferous. The numbers of trichodorids present in the suspensions extracted from the source-plants and transferred to the bait-plants ranged between 12 and 34, but were not related to the virus source isolate.

Table 38. Transmission of TRV to *P. hybrida* or *N. tabacum* cv. White Burley bait-plants by a nematode population from Woodhill after having had access to manually inoculated *P. hybrida* or *N. tabacum* cv. White Burley virus source-plants.

Bait/source combination	TRV source PPB10	PPK20	PPM10	PPS49	PPW10	PRN	PLB	TRV-Den
P. hybrida source *P. hybrida* bait								
Virus in source roots before acquisition	6/6*	6/6	3/6	6/6	6/6	2/6	3/6	3/6
Virus in source roots after acquisition	6/6	6/6	5/6	6/6	6/6	4/6	4/6	5/6
***P. hybrida* baits infected.**	3/6	6/6	1/6	4/6	4/6	0/6	0/6	0/6
P. hybrida source White Burley bait								
Virus in source roots before acquisition	6/6	6/6	2/6	6/6	6/6	2/6	3/6	2/6
Virus in source roots after acquisition	6/6	6/6	3/6	6/6	6/6	3/6	3/6	3/6
White Burley baits infected.	3/6	6/6	1/6	4/6	5/6	0/6	0/6	0/6
White Burley source *P. hybrida* bait								
Virus in source roots before acquisition	6/6	6/6	5/6	6/6	6/6	6/6	6/6	5/6
Virus in source roots after acquisition	6/6	6/6	5/6	6/6	5/5**	6/6	6/6	5/5**
***P. hybrida* baits infected.**	2/6	6/6	4/6	5/6	5/5	0/6	0/6	0/5
White Burley source White Burley bait								
Virus in source roots before acquisition	6/6	6/6	6/6	6/6	6/6	5/6	5/6	6/6
Virus in source roots after acquisition	6/6	6/6	6/6	6/6	6/6	5/5**	5/6	5/5**
White Burley baits infected.	2/6	6/6	0/6	5/6	6/6	0/6	0/6	0/5
Overall bait-plant infection	10/24	24/24	6/24	18/24	21/23	0/23	0/24	0/22

*) Numerator is number of plants from which virus was detected from the roots, denominator is number of plants set up.

**) One virus source-plant died during acquisition period.

No virus was detected from roots of twelve virus-free *P. hybrida* or twelve virus-free White Burley plants on which nematodes had been allowed to feed for 3 weeks. Numbers of trichodorids extracted from source-plants and added to bait-plants ranged from 17 to 48 but were not related to virus source isolate or bait-plant/source-plant combination.

When amalgamating the transmission data for the eight TRV source-isolates, the number of bait-plants which became infected was not significantly different for the four source-plant/bait-plant combinations (χ^2_3=1.15 ns). Also, using *P. hybrida* or White Burley tobacco as bait- or source-plants did not significantly affect the acquisition and transmission of isolates PPB10, PPK20, PPS49, PPW10 strains PRN, PLB or the Danish TRV-isolate. However, using White Burley tobacco as virus source- and *P. hybrida* as bait-plants for isolate PPM10 resulted in significantly more bait-plants becoming infected by the nematodes than when using White Burley as both a source- and bait-plant for the virus (χ^2_2=6 P≤0.05). Overall, isolate PPK20 was most efficiently acquired and transmitted although not significantly better than isolate PPW10. Transmission and acquisition of isolate PPS49 was less efficient than that of isolate PPK20, but not than that of isolate PPW10. Transmission and acquisition of isolates PPB10 and PPM10 was less efficient than that of isolates PPK20, PPW10 and PPS49, but greater than that of the Danish TRV-isolate and the PLB and PRN strains.

Table 39. Results of pairwise comparisons of transmission rates of TRV-isolates after acquisition by virus-free nematodes. (χ^2-test; *** significant at P≤0.001, ** significant at P≤0.01, * significant at P≤0.05.)

TRV-isolates							
	PPK20	PPW10	PPS49	PPB10	PPM10	TRV-Den	PRN
PPW10	ns						
PPS49	**	ns					
PPB10	***	***	*				
PPM10	***	***	***	ns			
TRV-Den	***	***	***	***	*		
PRN	***	***	***	***	*	ns	
PLB	***	***	***	***	***	ns	ns

VIII.3.b.- *Acquisition by a virus-free nematode population and transmission by single trichodorids:* Bait tests on individual trichodorids from Woodhill or Wageningen showed that *P. pachydermus* from both populations, after having had access to TRV source-plants, transmitted PRN-serotype isolates PPK20 and PPW10, but failed to transmit PRN-serotype strain PLB. *P. pachydermus* from Woodhill also transmitted PRN-serotype isolate PPB10, but failed to transmit TRV-isolates belonging to other serotypes (PPK92, TVC47, TPE11, ORE-strain). *T. primitivus* did not transmit any virus. All isolates obtained from bait-plant roots reacted with the PRN-antiserum in $F(ab')_2$-ELISA.

Table 40. Transmission of TRV to *P. hybrida* by individual trichodorids from Woodhill and Wageningen after having had access to manually inoculated *P. hybrida* virus source-plants.

TRV-source	Trichodorid population Woodhill	Wageningen
PPK20	24/46* Pp** (52%) 0/6 Tp (<17%)	18/30 Pp (60%)
PPK92	0/75 Pp (<2%) 0/6 Tp (<17%)	n.d
PPB10	14/78 Pp (18%) 0/6 Tp (<17%)	n.d
PPW10	18/47 Pp (38%) 0/3 Tp (<33%)	7/32 Pp (22%)
PLB	0/54 Pp (<2%) 0/4 Tp (<25%)	0/48 Pp (<2%)
TVC47	0/48 Pp (<2%) 0/6 Tp (<17%)	n.d
TPE11	0/36 Pp (<3%) 0/20 Tp (<5%)	n.d
ORE	0/41 Pp (<2%) 0/36 Tp (<3%)	n.d

*) Numerator is number of nematodes transmitting, denominator is number of nematodes tested.
**) Pp: *P. pachydermus*, Tp: *T. primitivus*.

The earlier findings that the populations from Woodhill and Wageningen were not naturally viruliferous are confirmed here, as no transmission occurred after allowing the nematodes access to strain PLB. When comparing frequencies with which the different source-isolates were transmitted by *P. pachydermus* in χ^2-tests, the transmission frequency of isolate PPK20 was not significantly greater than that of isolate PPW10 with *P. pachydermus* from Woodhill (χ^2=1.81 ns) but was with *P. pachydermus* from Wageningen (χ^2=9.35 P≤0.01). The transmission frequency of

PPW10 was significantly greater than that of strain PLB with both populations.(χ^2=25.17 P≤0.001 and χ^2=11.51 P≤0.001). The transmission frequencies of these isolates were not significantly different between the two populations (PPK20 Woodhill-Wageningen χ^2=0.45 ns, PPW10 Woodhill-Wageningen χ^2=2.37 ns).

VIII.3.c.- *Acquisition by a viruliferous nematode population and transmission by single trichodorids:* Bait tests on single trichodorid nematodes from Kinshaldy after having allowed the nematodes access to *P. hybrida* source-plants inoculated with SYM-serotype isolate PPK92, resulted in 14 of 68 *P. pachydermus* and none of 29 *T. similis* transmitting. The transmitted isolates reacted in F(ab')$_2$-ELISA with PRN- but not with SYM-antiserum, indicating that the nematodes had not acquired and transmitted the SYM-serotype PPK92 source isolate, but TRV which they naturally carried. When the SYM-strain was used as a TRV-source 37 out of 69 *P. pachydermus* and none of 49 *T. similis* transmitted. Of the 37 isolates transmitted after access to TRV source SYM, 18 were picked and tested in ELISA. They also reacted with PRN- but not with SYM-antiserum.

Bait tests on single trichodorids from Barry, having had access for 3 weeks to virus-free *P. hybrida* plants, resulted in 65 out of 96 (69%) *P. pachydermus* transmitting (no *Trichodorus* spp. found). When allowing the nematodes access to *P. hybrida* source-plants inoculated with TRV-isolate PPB10, 38 out of 69 (55%) of *P. pachydermus* transmitted (no *Trichodorus* spp. found).

VIII.4. DISCUSSION AND CONCLUSIONS.

The idea that transmissibility of TRV by trichodorid nematodes is largely dependent on the combination of vector species and TRV-serotype is, at least for *P. pachydermus*, supported by the results obtained from acquisition and transmission tests presented here. Thus, virus-free *P. pachydermus* only acquired and transmitted TRV-isolates belonging to the serotype (PRN-serotype) with which this species was found to be naturally associated. Also, successful acquisition and transmission was not related to geographical factors, as virus-free *P. pachydermus* from Woodhill, Scotland transmitted TRV-isolates PPS49 and PPW10 which were originally obtained from transmissions by naturally viruliferous *P. pachydermus* from Sweden and the Netherlands respectively, and as *P. pachydermus* from Wageningen, the Netherlands transmitted isolate PPK20 from Scotland. Therefore, the conclusion by van Hoof (1968) that a trichodorid nematode population is unlikely to transmit a newly introduced TRV-isolate, can not be accepted as a general rule, as a population may efficiently acquire and transmit a newly introduced TRV-isolate when the vector species "matches" the TRV-serotype.

Significant differences were found in the efficiencies with which PRN-serotype TRV-isolates were acquired and transmitted by *P. pachydermus* individuals or by populations containing *P. pachydermus*. TRV-isolates PPK20, PPS49 and PPW10 were transmitted efficiently, PPB10 and PPM10 less efficiently and strains PLB, PRN and the Danish TRV-isolate were not transmitted. Similar results were obtained by Brown *et al.* (1989) in acquisition and transmission tests using *Longidorus attenuatus* vectors of tomato black ring nepovirus (TBRV) isolates. They also found

that significant differences occurred in the frequency with which serologically indistinguishable TBRV-isolates were transmitted by this longidorid vector species. Brown *et al.* (1989) therefore concluded that differences, which they failed to detect in serological tests, did exist between isolates, and which in some way affected the transmissibility. Such differences may also explain the results obtained in the acquisition and transmission tests with *P. pachydermus* and the PRN-serotype TRV-isolates. Furthermore, such differences appear to affect the transmissibility of isolates irrespective of the *P. pachydermus* vector population. Isolate PPK20 was about equally well transmitted, isolate PPW10 had an intermediate transmissibility and strain PLB was not transmitted by *P. pachydermus* from both the Woodhill and the Wageningen population. Whether the observed differences in transmissibility of these serologically similar isolates is a reflection of differences in the ability of the vector nematodes to acquire, retain or release these isolates is unknown. Trudgill *et al.* (1981) attributed low transmission of raspberry ringspot nepovirus by *L. elongatus* and by *L. macrosoma* to different mechanisms. In electron microscopy many particles retained within the odontostyle lumen of *L. macrosoma* could be observed, but in *L. elongatus* this was only rarely found. Trudgill *et al.* (1981) therefore concluded that *L. macrosoma* was an inefficient vector because of poor virus release, whereas with *L. elongatus* poor retention of particles caused the low transmission rates. A similar approach may reveal whether the differences in transmissibility of TRV-isolates PPK20, PPW10 and the PLB strain are caused by differential acquisition, retention or release of particles by *P. pachydermus*.

Whereas transmissibility of TRV-isolates PPK20, PPW10 and strain PLB was similar with the two *P. pachydermus* populations tested, in studies on transmission of

nepoviruses by longidorid vector it was often found that different populations of a vector species were not equally able to transmit a particular nepovirus (Brown, 1985; Brown and Trudgill, 1983; van Hoof, 1966). As only two *P. pachydermus* populations were tested here, it can as yet not be concluded that this situation will also occur with trichodorids.

Transmissibility of a PRN-serotype isolate or strain by *P. pachydermus* was correlated with the origin of the isolate or strain: Strains PLB and PRN and the Danish TRV-isolate were originally isolated from an infected potato tuber, and were not transmitted. The isolates which were acquired and transmitted all were originally obtained from transmission by a single *P. pachydermus* to a bait-plant. Whether this correlation has any biological significance, e.g. whether TRV-isolates lose vector transmissibility upon infection of potato tubers is unknown. If it can be established that originally transmissible isolates indeed become non-transmissible after infection and subsequent isolation from potato tubers, this would be of great practical interest as it would suggest that infected potato seed does not provide a source of TRV for the vector nematodes.

The failure of *P. pachydermus* to acquire and transmit non PRN-serotype TRV-isolates corresponds with the conclusion of Chapter VII that *P. pachydermus* is associated with PRN-serotype isolates only. However, *T. primitivus*, also present in the Woodhill population, failed to transmit any of the TRV-isolates, including isolate TPE11 originally transmitted by *T. primitivus*, and isolate TVC47 which was originally transmitted by *T. viruliferus* but serologically indistinguishable from isolates transmitted by *T. primitivus*. This could indicate that *T. primitivus* recognizes differences between isolates, which were not detected in $F(ab')_2$-ELISA. The fact

however that isolate TPE11 was not transmitted by virus-free *T. primitivus*, although it was originally obtained from a transmission by this species, suggests that acquisition and transmission of TRV by *T. primitivus* is more specific than that by *P. pachydermus*, as the latter species was able to transmit all PRN-serotype isolates originally obtained from transmissions by *P. pachydermus*. The failure of virus-free *T. primitivus* to acquire and transmit any of the TRV-isolates, might however have resulted from the fact that the acquisition and transmission system itself is unsuitable for this species.

Allowing viruliferous nematodes from Barry to feed for 3 weeks on uninoculated *P. hybrida* or on *P. hybrida* inoculated with TRV-isolate PPB10, resulted in 69% and 55% of *P. pachydermus* respectively transmitting in a subsequent bait test. When individual trichodorids from this population were bait tested immediately after extraction of the nematodes from soil, without allowing them to feed on *P. hybrida*, less than 16% of *P. pachydermus* transmitted TRV (See Table 17, Chapter VII).

P. pachydermus from a viruliferous population from Kinshaldy did not acquire and transmit SYM-serotype isolate PPK92 or the SYM-strain of TRV. This supports the hypothesis from Chapter VII that isolate PPK92 resulted from a contamination with TRV-strain SYM and was not transmitted by *P. pachydermus*.

CHAPTER IX

VECTOR-TRANSMISSIBILITY OF PSEUDO-RECOMBINANT TRV-ISOLATES PRODUCED FROM A TRANSMISSIBLE AND A NON-TRANSMISSIBLE STRAIN.

IX.1. INTRODUCTION.

The first indications that the virus coat-protein might be involved in vector-transmission were obtained by Harrison (1964) who demonstrated that serologically distinctive isolates of raspberry ringspot nepovirus (RRV) and tomato black ring nepovirus (TBRV) were transmitted by different longidorid vector species. Later, the use of laboratory made pseudo-recombinant isolates of bi- or multipartite plant viruses, in which the different genomic species were derived from different "parent" isolates, provided further insight in the molecular basis of vector specificity. Thus, Harrison *et al.* (1974a), by using pseudo-recombinant isolates of RRV, showed that the serological characteristics and vector specificity of this virus was determined by the smaller RNA part of the virus genome which carries the coat-protein gene. Similar results were obtained by Mossop and Francki (1977). Their studies on aphid transmissibility of pseudo-recombinant isolates of the tripartite cucumber mosaic virus showed that transmissibility was determined by the smallest RNA genome part. This part contains two genes, one of which is the coat-protein gene. The important role of the virus coat-protein in transmission by vectors is now well established for several vector-transmitted plant viruses (Harrison and Murant, 1984; Harrison and Robinson, 1988).

The results in previous chapters indicated that serologically distinct TRV-isolates are generally transmitted by different trichodorid vector species. This therefore is consistent with the virus coat-protein playing an important role in the specific transmission of TRV-isolates by trichodorid vector species. To examine whether this is so, transmission by *P. pachydermus* of pseudo-recombinant isolates produced from the non-transmissible PLB-strain and the efficiently transmitted PPK20 isolate was compared.

IX.2. MATERIAL AND METHODS.

IX.2.a.- *TRV-isolates:* TRV-strain PLB was originally obtained from a potato tuber (See V.4.c.*ii*) and isolate PPK20 from a *P. hybrida* bait-plant infected by a single *P. pachydermus* nematode (See Table 16, Chapter VII). Both isolates reacted in $F(ab')_2$-ELISA with antiserum against the PRN-strain of TRV (See Table 9, Chapter V and Table 35, Chapter VII), but only strain PLB gave a positive reaction when particles were trapped with the $F(ab')_2$-fraction of PRN-antiserum and monoclonal antibody (Mab) SCR81 was subsequently used as detecting IgG (1:30 in PBS-Tween). This Mab was raised against strain PLB and kindly provided by J. Legurburu. $F(ab')_2$-ELISA was done as described in V.4.c.*iii*.

IX.2.b.- *Purification of virus particles:* Virus particles were purified according to Robinson and Harrison (1985a) omitting the titration of sap extract to pH 8.0 with NaOH. Systemically infected leaves of *N. clevelandii* inoculated with TRV-strain PLB or isolate PPK20 were harvested 14 days after the plants were inoculated. Sap

was extracted in 67 mM-phosphate buffer, pH 7.3 (1 ml buffer per 2 g leaf) and frozen for 14 days at -20 °C. After thawing, the extract was centrifuged at low speed (10 min. 10.000 rpm), and virus particles were precipitated by adding 100 g polyethylene glycol 6000 and 20 g NaCl per litre of extract. The suspension was stirred for 1 hr. at 4 °C followed by a low-speed centrifugation (10.000 rpm, 15 min.). The pellet, containing the virus particles, was allowed to resuspend overnight in 17 mM-phosphate buffer, pH 7.3, at 4 °C. Virus particles were collected by high-speed centrifugation (50.000 rpm, 90 min. in R65 rotor), followed by another low-speed and high-speed centrifugation. The pellet was then allowed to resuspend overnight in 17 mM-phosphate buffer, pH 8, at 4 °C.

IX.2.c.- *Fractionation of particles:* Short (S-) particle preparations of strain PLB and isolate PPK20 were obtained by fractionation of the purified virus preparations as described by Kurppa *et al.* (1981). The preparations were sedimented for 135 min. at 20.000 rpm through sucrose density gradients. Gradients were prepared by freezing 25% sucrose in 17 mM-phosphate buffer, pH 8, in Beckmann SW 27 tubes (35 ml/tube). The solution in the tubes was allowed to thaw overnight at 4 °C, and used the following morning. After centrifugation the gradients were fractionated by upward displacement, using an ISCO Model 640 density gradient fractionator. Fractions containing S-particles were collected, diluted *c.* 3 times in 17 mM-phosphate buffer, pH 8, and particles sedimented by a high-speed centrifugation (50.000 rpm, 90 min. in R65 rotor). The resulting pellet was allowed to resuspend overnight in 17 mM-phosphate buffer, pH 8, at 4 °C, and particles were further fractionated and purified by an additional sedimentation through sucrose density

gradients. After final sedimentation of particles by high-speed centrifugation (50.000 rpm, 90 min. in R65 rotor), the pellet was allowed to resuspend overnight in 17 mM-phosphate buffer, pH 7.3, at 4 °C. The resulting solution was used as the S-particle preparation for production of pseudo-recombinant isolates and reconstitution of "parent" isolates.

IX.2.d.- *Production and testing of NM-type isolates:* NM-type isolates were obtained as described by Robinson and Harrison (1985a). Sap of systemically infected leaves of *N. clevelandii* plants inoculated with strain PLB or isolate PPK20 was diluted in tap water (1:40.000). The diluted sap was inoculated onto *C. amaranticolor* plants (3 plants for PLB and 3 for PPK20). Five days after inoculation well-spaced single lesions were individually cut, comminuted in a small mortar with a pestle, adding a drop of water and individually inoculated onto *N. clevelandii* plants. Ten days after inoculation sap extracted from those plants was inoculated onto a *C. amaranticolor* plant prior to, and after subjecting the sap extract to a threefold cycle of freezing and thawing. Also, the infectivity of phenol extracts of leaves was compared to that of water extracts of leaves. The inoculation of phenol extracts of leaves infected with NM-type TRV usually results in a greater number of lesions on indicator plants than when using water extracts, as phenol protects the uncoated RNA-1 of NM-infections against activity of RNases and other inactivators (Harrison and Robinson, 1978). Phenol extracts of leaves were prepared by grinding *c.* 1 g infected leaf tissue in a cold mortar an pestle with phenol-cresol (3 ml) and 17 mM-phosphate buffer, pH 7.8. A little coarse sand was added to facilitate grinding. The emulsion was spun at 3000 rpm for 5 min. and the aqueous phase collected. By

shaking the aqueous phase three times with an equal volume of diethylether the phenol was removed. Remaining ether was then removed by bubbling N_2-gas through the suspension and the solution was used for manual inoculation (wearing plastic gloves, suspension on ice) of *C. amaranticolor* plants.

IX.2.e.- *Extraction of RNA from NM-infected N. clevelandii plants:* RNA was extracted from NM-infected *N. clevelandii* plants as described by Harrison and Robinson (1982). Infected leaf tissue (*c.* 1 g) was ground in a glass homogenizer with 10 ml phenol-cresol, 10 ml 0.01 M Tris buffer, pH 7.6, containing 0.05 M sodium chloride, 0.1 g tri-iso-propylnaphthalene sulphonate and 0.6 g sodium-4-amino-salicylate. The emulsion was broken by centrifugation (3000 rpm, 5 min), the aqueous phase separated, 0.3 g sodium chloride added and extracted again in 10 ml phenol-cresol. The aqueous phase was collected after another low-speed centrifugation and RNA precipitated with 25 ml ethanol at -20 °C. This RNA preparation was used for the production of the pseudo-recombinant isolates.

IX.2.f.- *Infectivity of RNA and S-particle preparations:* For inoculation of *C. amaranticolor* plants with RNA, the RNA preparation was spun (6000 rpm, 15 min) and RNA dissolved in 2 ml bentonite-buffer (25 mM-sodium chloride + 5 mM-Tris-HCl pH 8.0 containing 0.6 mg bentonite per ml). Part of this RNA-solution was used for manual inoculation of *C. amaranticolor* plants, whereas to the other part S-particles of the heterologous strain were added. S-particle preparations of the PLB-strain and isolate PPK20 were manually inoculated onto *C. amaranticolor* plants. Five *C. amaranticolor* plants were inoculated with undiluted S-particle preparations

or with 1:10, $1:10^2$ or $1:10^3$ dilutions of the S-particle preparations. Preparations were diluted in bentonite-buffer.

IX.2.g.- *Production of pseudo-recombinants and reconstitution of "parent" isolates:* "Parent" isolates were reconstituted by mixing PPK20-RNA from NM-type infected plants with the PPK20 S-particle preparation (792 µl RNA + 8 µl S-particle), giving rise to PP_1PP_2 isolates, and by mixing PLB NM-type RNA with the PLB S-particle preparation (720 µl PLB-RNA + 80 µl S-particle), giving rise to PL_1PL_2 isolates. Pseudo-recombinant inocula were prepared by mixing PPK20 RNA-1 extracted from NM-type infected plants with the S-particle preparation from strain PLB (720 µl PPK20-RNA in bentonite buffer + 80 µl PLB S-particle preparation), giving rise to isolates "PP_1PL_2", and vice versa, by mixing PLB RNA-1 extracted from NM-type infected plants with the S-particle preparation from isolate PPK20 (792 µl PLB-RNA in bentonite buffer + 8 µl PPK20 S-particle preparation), giving rise to isolates "PL_1PP_2". Plastic gloves were worn for manual inoculation of carborundum-dusted *C. amaranticolor* leaves. Control plants were inoculated with NM-type PLB RNA-1 or PPK20 RNA-1 solutions and S-particle preparations of PLB or PPK20 separately. Well-separated single lesions in *C. amaranticolor* were excised, three times frozen and thawed, ground up with a drop of water and again inoculated onto *C. amaranticolor*. For both reconstituted "parents", PP_1PP_2 and PL_1PL_2, one isolate was subcultured in *C. quinoa* and subsequently propagated in *N. clevelandii*. For each of the two pseudo-recombinants (PP_1PL_2 and PL_1PP_2), four isolates (I-IV) were subcultured and subsequently propagated. The serological reaction of the

isolates was tested in F(ab')$_2$-ELISA using the F(ab')$_2$-fraction of PRN-antiserum for trapping and IgG of PRN-antiserum or of Mab SCR81 for detection of virus.

IX.2.h.- *Inoculation and testing of virus source-plants:* Each of the eight pseudo-recombinant isolates (PP_1PL_2 I-IV and PL_1PP_2 I-IV) was inoculated onto four 8 week-old *P. hybrida* plants. A further eight *P. hybrida* plants were inoculated with the reconstituted PLB "parent" PL_1PL_2 and eight with the reconstituted PPK20 "parent" PP_1PP_2. Ten days after inoculation a part of the root system of each *P. hybrida* plant was ground in a mortar with a pestle and inoculated onto a *C. amaranticolor* plant. For each pseudo-recombinant virus source two, and for the reconstituted "parents" PL_1PL_2 and PP_1PP_2, four *P. hybrida* plants of which the roots were shown to be infected were used as virus source-plants in acquisition and transmission tests.

IX.2.i.- *Acquisition and transmission tests:* The general method used for acquisition and transmission tests was described in V.3. Five ml of nematode suspension extracted from Woodhill soil (See Table 13, Chapter VI) containing *c.* 120 trichodorid nematodes (mainly *P. pachydermus,* some *T. primitivus*, very few *T. cylindricus*) were added to the *P. hybrida* virus source-plants (four reconstituted PPK20, four reconstituted PLB, eight pseudo-recombinant PP_1PL_2 and eight pseudo-recombinant PL_1PP_2 source-plants). As a control 5 ml nematode suspension was added to each of sixteen virus-free *P. hybrida* plants. After a 3 week acquisition period the nematodes were extracted, and the source-plant roots were tested for presence of virus by inoculation onto *C. amaranticolor*. *C. quinoa* plants were

subsequently inoculated with sap expressed from virus infected *C. amaranticolor* plants and tested in $F(ab')_2$-ELISA against PRN-antiserum and Mab SCR81.

The nematodes extracted from the virus source-plant were subsequently tested for virus transmission. Twenty trichodorids were handpicked from the nematode suspensions extracted from individual pseudo-recombinant virus source-plants. They were added in groups of ten trichodorids to two 0.5 ml plastic capsules, which were processed as described in V.2.b. The nematodes remaining in the suspensions were added in bulk to a 4 week-old virus-free *P. hybrida* bait-plant as described in V.3.c.

The nematodes extracted from the reconstituted "parent" PL_1PL_2 and PP_1PP_2 source-plants were added in bulk only to *P. hybrida* bait-plants. After 14 days, the number of trichodorids present in each capsule was counted, the nematodes were identified to species to assess numbers of *P. pachydermus* and *T. primitivus*, and the *P. hybrida* tested for presence of virus as described in V.3.c. Also after 14 days the nematodes added in bulk to a virus-free *P. hybrida* bait-plant were extracted, counted and the *P. hybrida* bait-plant tested for the presence of virus as described in V.4.a.*i.*

IX.3. RESULTS.

IX.3.a.- *Detection of NM-type isolates:* Out of ten *N. clevelandii* plants inoculated with single lesions cut from PLB-inoculated *C. amaranticolor* leaves, four plants were shown to contain NM-type infections, five to contain M-type infections and one was found uninfected. Three out of ten *N. clevelandii* plants inoculated with single lesions from PPK20-inoculated *C. amaranticolor* leaves were shown to be infected with NM-type virus, five with M-type virus and two were not infected. The "NM-

plants" were characterized by loss of infectivity of sap after a threefold freezing and thawing cycle and by increased infectivity when extracting sap in phenol. A PLB and a PPK20 "NM-plant" was used for RNA extraction.

Table 41. Infectivity of sap expressed from NM-type infected *N. clevelandii* plants used for extraction of RNA.

Inoculum	Plant	Water extract, before freezing and thawing	Water extract, After freezing and thawing	Phenol extract
PPK20	1	13*	0	41
	2	9	0	29
PLB	1	17	0	63
	2	20	0	85

* Number of lesions on four *C. amaranticolor* leaves.

IX.3.b.- *Infectivity of NM-plant RNA, S-particle preparations and pseudo-recombinant isolates:* As preparations of S-particles which are completely free of L particles are difficult to obtain (Harrison and Robinson, 1986), it is necessary to dilute the S-particle preparations in order to reduce the chance of L-particles being present in an aliquot taken from the S-particle preparation. When inoculating such an aliquot on *C. amaranticolor*, the presence of L-particles is revealed by the development of lesions in the inoculated leaves, as S-particles alone are not infective. Inoculation of an aliquot of the PPK20 and PLB S-particle preparation in various dilutions onto *C. amaranticolor*, showed that the PPK20 S-particle preparation diluted 1:100 or more and the PLB S-particle preparation diluted 1:10 or more were virtually free of L-particles.

Table 42. Infectivity of undiluted and diluted S-particle preparations of isolate PPK20 and strain PLB.

S-particle preparation	undil.	1:10	1:100	1:1000
PPK20	39*	6	1	0
PLB	4	0	0	0

* Number of lesions on fifteen *C. amaranticolor* leaves (five plants, three leaves/plant inoculated).

The infectivity of the reconstituted "parent" inocula produced by mixing NM PPK20-RNA with the PPK20 S-particle preparation and by mixing NM PLB-RNA with the PLB S-particle preparation, of the pseudo-recombinant inocula produced by mixing NM PLB-RNA with the PPK20 S-particle preparation (final dilution 1:100) and by mixing NM PPK20-RNA with the PLB S-particle preparation (final dilution 1:10), and of the S-particle and NM-RNA preparations alone was tested by inoculation onto *C. amaranticolor*. Two days after the appearance of lesions in the inoculated leaves, ten single lesions were cut from the leaves inoculated with NM PLB-RNA, NM PPK20-RNA, NM PPK20+PPK20 S-particle, NM PLB+PLB S-particle, NM PLB-RNA+PPK20 S-particle and NM PPK20-RNA+PLB S-particle. The infectivity of these lesions after a threefold freezing and thawing cycle was tested by inoculation onto *C. amaranticolor*.

Table 43. Infectivity of pseudo-recombinant inocula, NM RNA and S-particle preparations.

Inoculum	Number of lesions on *C. amaranticolor* leaves	Number of single lesion isolates which retained infectivity after freezing/ thawing (10 lesions tested)
RNA		
NM PLB-RNA	483°	0
NM PPK20-RNA	243	0
Short particles		
PPK20 S-particles (1:100)	0	n.a.°°
PLB S-particles (1:10)	0	n.a.
Reconstituted parent isolates		
NM PLB-RNA + PLB S-particles (1:10)	415	9
NM PPK20-RNA + PPK20 S-particles (1:100)	126	4
Pseudo-recombinant isolates		
NM PLB-RNA + PPK20 S-particles	397	8
NM PPK20-RNA + PLB S-particles (1:10)	195	4

° Number of lesions on fifteen *C. amaranticolor* leaves (five plants, three leaves/plant inoculated).
°° n.a. : not applicable.

The data from Table 43 show that the infectivity of NM RNA inocula did not survive a threefold freezing and thawing cycle which indicates that these inocula did not contain RNA-2 or S-nucleoprotein particles. The diluted S-particle preparations inoculated alone were not infective, indicating that few, if any, L-particles were present in these preparations. Thus, almost all lesions resulting from the inoculations with the mixed inocula which resisted freezing and thawing, must have contained RNA-1 from the NM-type source and S-particles from the purified S-particle preparation.

One of each reconstituted "parent" isolate (PL_1PL_2 and PP_1PP_2), the four pseudo-recombinant isolates obtained from NM PPK20-RNA + PLB S-particle preparation

(PP_1PL_2 isolates I to IV) and four of the eight pseudo-recombinant isolates obtained by mixing NM PLB-RNA with the PPK20 S-particle preparation (PL_1PP_2 isolates I to IV) were subsequently propagated in *N. clevelandii.*

IX.3.c.- *Serological properties of the reconstituted "parent" and pseudo-recombinant isolates:* The eight pseudo-recombinant isolates, both the reconstituted "parent" isolates, isolate PPK20 and strain PLB gave positive reactions in $F(ab')_2$-ELISA when virus-particles were trapped with the $F(ab')_2$-fraction and detected with IgG of PRN-antiserum. However, when Mab SCR81 was used instead as detecting antibody, the four PP_1PL_2 pseudo-recombinant isolates, the reconstituted "parent" PL_1PL_2 and the PLB-strain gave a positive reaction whereas the four PL_1PP_2 pseudo-recombinant isolates, the reconstituted "parent" PP_1PP_2 and PPK20 failed to react. This shows that the pseudo-recombinant and reconstituted "parent" isolates were serologically indistinguishable from the respective parent isolate or strain used for the purification of S-particles.

IX.3.d.- *Presence of virus in source-plant roots and serology of virus obtained from those roots:* Inoculation of a part of root system of *P. hybrida* plants which had been inoculated ten days earlier with the reconstituted "parent" isolates PL_1PL_2 or PP_1PP_2, or with the four PP_1PL_2 or PL_1PP_2 pseudo-recombinant isolates, onto *C. amaranticolor* plants showed that in most of the *P. hybrida* root systems infective virus was present.

Table 44. The presence of virus in roots of *P. hybrida* plants to be used as virus-source-plants for trichodorid nematodes.

Inoculum	Isolate	Nr. of *P.hybrida* plants with virus in roots/nr. of *P. hybrida* plants tested.
Reconstituted "parent" isolates		
PL_1PL_2	n.a.*	8/8
PL_1PL_2	n.a.	6/8
Pseudo-recombinant isolates		
PP_1PL_2	I	2/4
	II	3/4
	III	4/4
	IV	3/4
PL_1PP_2	I	4/4
	II	4/4
	III	4/4
	IV	4/4

* n.a. : not applicable.

For the reconstituted "parent" isolates PL_1PL_2 and PP_1PP_2 four, for each pseudo-recombinant isolate two *P. hybrida* plants of which the roots were shown to be infected, were picked and used as virus source-plants for the nematodes. During the acquisition period one *P. hybrida* source plant inoculated with PP_1PL_2 pseudo-recombinant isolate IV and one inoculated with PL_1PP_2 pseudo-recombinant IV died.

Inoculation of the complete root system of each virus source-plant onto a *C. amaranticolor* after the acquisition period confirmed that all source-roots had contained infective virus. $F(ab')_2$-ELISA on the isolates obtained from the source-plant roots furthermore showed that they were serologically indistinguishable from the isolates used to inoculate the respective source-plants. Inoculation of root systems of the sixteen non-inoculated *P. hybrida* plants onto *C. amaranticolor* after the

acquisition period showed that no virus was present in these root systems, confirming that the nematodes were not naturally viruliferous.

IX.3.e.- *Transmission of pseudo-recombinant and "parent" isolates to P. hybrida bait-plants:* Nematodes allowed access to the reconstituted "parent" PP_1PP_2 or to PL_1PP_2 pseudo-recombinant isolates subsequently transmitted virus to *P. hybrida* bait-plants. However, nematodes which had been allowed access to the reconstituted "parent" PL_1PL_2 or to PP_1PL_2 pseudo-recombinant isolates failed to subsequently transmit virus to *P. hybrida* bait-plants.

Table 45. Transmission of pseudo-recombinant and "parent" TRV-isolates to *P. hybrida* bait-plants by trichodorid nematodes.

TRV source	Isolate number	Source-plant	Replicate	Experiment*	Trichodorids recovered**	Infection of bait-plant***
PP_1PP_2	n.a.	1	n.a.	bulk	85	+
		2	n.a.	bulk	68	+
		3	n.a.	bulk	101	+
		4	n.a.	bulk	78	+
PL_1PL_2	n.a.	1	n.a.	bulk	98	-
		2	n.a.	bulk	109	-
		3	n.a.	bulk	71	-
		4	n.a.	bulk	61	-
PL_1PP_2	I	1	1	10 tricho's	8 Pp, 1 jv	+
			2	10 tricho's	9 Pp	+
			n.a.	bulk	75	+
		2	1	10 tricho's	9 Pp	+
			2	10 tricho's	8 Pp	+
			n.a.	bulk	85	+
	II	1	1	10 tricho's	6 Pp, 1 Tp, 2 jv	+
			2	10 tricho's	7 Pp, 3 jv	+
			n.a.	bulk	59	+
		2	1	10 tricho's	8 Pp	+
			2	10 tricho's	5 Pp, 2 jv	+
			n.a.	bulk	82	+
	III	1	1	10 tricho's	6 Pp, 1 jv	+
			2	10 tricho's	6 Pp, 1 jv	+
			n.a.	bulk	65	+
		2	1	10 tricho's	6 Pp, 2 jv	+
			2	10 tricho's	5 Pp, 2 jv	+
			n.a.	bulk	72	+
	IV	1	1	10 tricho's	7 Pp, 2 jv	+
			2	10 tricho's	6 Pp, 1 Tp, 2 jv	+
			n.a.	bulk	48	+
		2****				

Continued next page.

Table 45 continued

TRV source	Isolate number	Source-plant	Replicate	Experiment*	Trichodorids recovered**	Infection of bait-plant***
PP_1PL_2	I	1	1	10 tricho's	9 Pp	-
			2	10 tricho's	6 Pp, 2 jv	-
			n.a	bulk	98	-
		2	1	10 tricho's	5 Pp, 1 jv	-
			2	10 tricho's	6 Pp, 1 jv	-
			n.a.	bulk	67	-
	II	1	1	10 tricho's	8 Pp, 1 jv	-
			2	10 tricho's	7 Pp	-
			n.a	bulk	101	-
		2	1	10 tricho's	5 Pp, 2 jv	-
			2	10 tricho's	10 Pp	-
			n.a.	bulk	98	-
	III	1	1	10 tricho's	6 Pp, 1 jv	-
			2	10 tricho's	8 Pp, 2 jv	-
			n.a	bulk	83	-
		2	1	10 tricho's	8 Pp	-
			2	10 tricho's	7 Pp	-
			n.a.	bulk	70	-
	IV	1****				
		2	1	10 tricho's	5 Pp, 2 jv	-
			2	10 tricho's	5 Pp, 1 jv	-
			n.a.	bulk	59	-

* bulk: nematode suspensions extracted from virus source-plants added in bulk to *P. hybrida* bait-plants. 10 tricho's: ten trichodorids handpicked and added to *P. hybrida* bait-plant.
** number of trichodorids recovered from bait-plants. Pp: *P. pachydermus*, Tp: *T. primitivus*, jv: juvenile trichodorid.
*** +: comminuted bait-plant roots causing lesions in inoculated *C. amaranticolor* leaves. -: no lesions developing in *C. amaranticolor* leaves after inoculation with comminuted bait-plant roots.
**** virus source-plant died during acquisition period.

There was no significant difference in the number of trichodorid nematodes recovered from the capsules (t-test, t_{26}=1.31 ns) between PL_1PP_2 and PP_1PL_2 pseudo-recombinant sources or in the number of trichodorids in the bulk suspensions recovered from bait-plants corresponding with PP_1PP_2, PL_1PL_2, PL_1PP_2 and PP_1PL_2 sources (ANOVA, F^3_{18}=1.12 ns). Thus, the failure of bait-plants to become infected

can not be attributed to differences in the numbers of trichodorid nematodes having had access those plants.

All isolates recovered from bait-plant roots gave a positive reaction in $F(ab')_2$-ELISA when detecting IgG was from PRN-antiserum but failed to react when Mab SCR81 was used as detecting antibody. Thus, the transmitted isolates reacted similar to isolate PPK20 and were serologically indistinguishable from the corresponding virus source-isolates.

IX.4. DISCUSSION AND CONCLUSIONS.

Pseudo-recombinant TRV-isolates produced by mixing RNA-1 from a transmissible isolate (PPK20) with S-particles from a non-transmissible isolate (PLB), were not transmitted by *P. pachydermus* vector nematodes. Conversely, pseudo-recombinants in which the RNA-1 originated from a non-transmissible (PLB), and the S-particles from a transmissible (PPK20) isolate were efficiently transmitted. Thus, transmissibility of pseudo-recombinant isolates corresponded with that of the S-particle donor "parent"-isolate. It can therefore be concluded that transmissibility is a function of the RNA-2 genome part of the virus, which is contained in the S-particles. The serological properties of the pseudo-recombinant isolates were also determined by the S-particles, which corresponds with the coat-protein gene being carried in these particles (Robinson and Harrison, 1989).

Whether transmissibility by vectors and serological properties are both manifestations of the same gene (coat-protein gene) is yet unproven. The RNA-2 of strain PLB, which was used as a "parent"-isolate from which the pseudo-

recombinants were produced, contains two genes, one of which is the coat-protein gene (Angenent *et al.*, 1989). The other gene codes for a 16K protein and is of diploid nature as it is also found on the RNA-1 genome part of the PLB-strain. Although it was initially hypothesized that the 16K protein might play a role in vector transmission (Boccara *et al.*, 1986), this idea was later rejected by Guilford *et al.* (1991). It is unknown if the RNA-2 of the other "parent"-isolate, PPK20, contains genes additional to the coat-protein gene. Thus, the lack of genes on RNA-2 of TRV apart from the coat-protein gene which may be involved in determining vector transmission, makes it likely that the coat-protein itself is the major factor involved in vector specificity and transmission.

The first evidence that the coat-protein of a plant virus determined transmissibility by vectors was provided by Rochow (1970) working with aphid transmitted barley yellow dwarf virus. At present the involvement of the coat-protein in vector transmission has been demonstrated for a number of plant viruses (Harrison and Murant, 1984). Harrison and Robinson (1988), by comparing results obtained from studies on aphid transmission of potyviruses, pointed out that only those isolates which had an Asp-Ala-Gly amino acid triplet near the N-terminal region in the coat-protein were aphid transmissible. Recently, the hypothesis that this amino acid triplet is involved in aphid transmission, was confirmed by Atreya *et al.* (1991) who found that substituting one amino acid of the Asp-Ala-Gly triplet for another strongly reduced the transmission rate by aphids.

A comparison of the coat-protein amino acid sequences of the non-transmissible PLB-strain and the efficiently transmitted isolate PPK20 which were used to produce the pseudo-recombinants, revealed a similarity of 94% (Mathis and Bol, unpublished

results). However, which of the observed differences in the amino acid composition of these two coat-proteins are relevant for transmission by *P. pachydermus* and how they possibly affect the recognition between the vector and the virus coat-protein remains to be studied.

The evidence presented here that the RNA-2 of TRV determines transmissibility by a trichodorid vector is a first step in locating the factor(s) which interact with the trichodorid vector nematodes, resulting in virus transmission. Further work, e.g. acquisition and transmission studies using TRV-isolates constructed to have a recombinant coat-protein gene, derived from the coat-protein genes of a transmissible (PPK20) and a non-transmissible (PLB) TRV-isolate, is needed to specifically locate the region on the coat-protein involved in the transmission process.

CHAPTER X

SUSCEPTIBILITY OF TRANSGENIC TOBACCO PLANTS EXPRESSING TOBACCO RATTLE VIRUS COAT PROTEIN TO INFECTION WITH TRV BY MANUAL INOCULATION AND BY TRICHODORID NEMATODES.

X.1. INTRODUCTION.

Protection of a plant by one strain of a virus against infection with a second is known as cross-protection. (Matthews, 1991). Genetically engineered cross-protection is similar phenomenon, achieved by expression of a part of a virus genome, usually the coat-protein (cp) gene, integrated into the plant genome. An increased resistance of such transgenic plants, which accumulate viral cp, to infection by the homologous virus has been reported for a number of different viruses (Beachy, 1990).

For the tobravirus group cross-protection was reported to occur within TRV (Cadman and Harrison, 1959) and PEBV (Gibbs and Harrison, 1964a). Later, van Dun and Bol (1988) and Angenent *et al.* (1990) demonstrated that transgenic tobacco plants expressing TRV-cp were resistant to infection by manual inoculation of homologous or serologically similar TRV-isolates, but not to manual inoculation with viral RNA or a serologically distinct isolate.

Beachy (1990), reviewing results obtained with different tobamoviruses, concluded that cp-mediated genetically engineered cross-protection is partly based on structural similarities between the protecting cp and the challenge virus, and that resistance decreased when the cp-gene donor virus and the challenge virus were less related. It is thought that the uncoating of virus particles, preceding virus replication, is affected

by the endogenous cp, resulting in the decreased susceptibility of the transgenic plants (Angenent *et al.*, 1990; Register and Beachy, 1988).

Most research on cp-mediated resistance of transgenic plants has been done by manual inoculation of the challenge virus. Because TRV is naturally transmitted by *Trichodorus* and *Paratrichodorus* nematode species (trichodorids), it is important to know if TRV-cp expressing transgenic plants show a decreased susceptibility to nematode transmitted TRV. Here the susceptibility of transgenic tobacco plants to infection with TRV-isolates transmitted by trichodorid vectors and to infection by manual inoculation with TRV-isolates that had been obtained in transmission tests from individual trichodorid nematodes is compared.

X.2. MATERIALS AND METHODS.

X.2.a.- *Virus-isolates:* TRV-strain PLB was originally isolated from potato (Angenent *et al.*, 1989) and TCM from tulip (Cornelissen *et al.*, 1986) in the Netherlands and were kindly provided by J.F.Bol. TRV-isolate TCS44 was obtained from transmission by a single *T. cylindricus* nematode from Scryne, Scotland (See Chapter VII, Table 20). Isolates PPK20, PPW10 and PPS49 were obtained from transmissions by single *P. pachydermus* from Scotland, the Netherlands and Sweden, respectively (See Chapter VII, Table 16, 24 and 31). Virus was grown in *N. clevelandii* plants. Isolates PPK20, PPW10 and PPS49 are serologically similar to the PLB-strain of TRV (See Chapter V, Table 9 and Chapter VII, Table 35). Isolate TCS44 is serologically similar to the N5-strain of PEBV (See Chapter VII, Table 35), but reacts with probes derived from TRV RNA-1 in nucleic acid hybridization

tests (Robinson, pers. comm.); it is probably a recombinant isolate similar to strain TRV-strain I6 (Robinson *et al.*, 1987).

X.2.b.- *Plant material:* Transgenic *N. tabacum* Samsun NN plants, expressing the cp of TRV-strain PLB, TCM or TRV-isolate TCS44 (PLBcp-, TCMcp- and TCS44cp-plants) were kindly provided by J.F. Bol. Plants had been grown from seeds of the original transformants. Kanamycin resistant (i.e. transformed) seedlings were selected on medium containing 200 µg/ml kanamycin (J.F. Bol, pers. comm.). The construction of transgenic plants was described by van Dun *et al.*(1987) and Angenent *et al.* (1990). Non-transgenic Samsun NN and White Burley tobacco plants (normal-plants), *C. amaranticolor* and *P. hybrida* plants were grown from seed at S.C.R.I in a heated glasshouse at an average temperature of 20 °C and provided with supplementary sodium vapour lighting for 16 hr/day.

X.2.c.- *Susceptibility of transgenic Samsun NN tobacco plants to infection by manually inoculated TRV:* Leaves of PLBcp- and normal-Samsun NN plants were inoculated with sap expressed from *N. clevelandii* leaves systemically infected with the PLB-strain or the PPK20, PPS49 or PPW10 isolates of TRV. Manual inoculation of this sap onto *C. amaranticolor* indicator-plants resulted in 75-100 lesions/leaf for each TRV-isolate. Each isolate was manually inoculated onto four carborundum dusted leaves (200 µl sap per leaf) of two normal- and four PLBcp-plants. Infection was assessed visually (symptom development), by back-inoculation of sap expressed from inoculated and non-inoculated leaves (8 and 20 days after inoculation respectively) onto four carborundum dusted leaves of a *C. amaranticolor* plant and

by F(ab')$_2$-ELISA (See V.4.c.) on sap expressed from leaf-disks (1 cm diameter) taken from inoculated and non-inoculated leaves (1 disk per leaf, 4 inoculated and 4 non-inoculated leaves tested per plant) using antiserum prepared against the PRN-strain of TRV.

X.2.d.- *Transmission of TRV by trichodorid nematodes to roots:* Nematodes were extracted from soil from Woodhill, Scotland, containing *c.* 150 trichodorid nematodes (*P. pachydermus*, *T. primitivus*, *T. cylindricus*) per 100 g soil. The nematodes had been previously found not to naturally transmit TRV in extensive bait tests (results not shown). Five ml of the nematode suspension, containing *c.* 15 trichodorids per ml, was added to each of five 25 ml plastic pots. A 5 week-old *P. hybrida* plant, which had been manually inoculated with TRV-isolate PPK20 10 days earlier, was added to each pot as a virus source-plant for the nematodes and processed as described in V.3.b.

After a 3 week acquisition period the nematodes were extracted from the source-plants (See V.3.b.). Single trichodorid nematodes were handpicked from the extracted suspension, added to 200 plastic capsules and bait tested as described in V.2.b. Fifty capsules were each planted with a normal White Burley tobacco-seedling, a PLBcp-, a TCMcp-, or a TCS44cp-Samsun NN tobacco seedling to act as a bait-plant. Angenent *et al.* (1990) reported that TCMcp-plants and normal Samsun NN plants were equally susceptible to infection with the PLB-strain of TRV. As the cp-amino acid sequence of isolate PPK20, which was fed to the nematodes, has a 94% similarity with the PLB cp-sequence (Mathis *et al.*, 1992), the TCMcp-plants were unlikely to be less susceptible than normal Samsun NN plants to infection with

isolate PPK20. Therefore TCMcp-Samsun NN served as cp-control plants. The normal White Burley tobacco plants, known to become readily infected by trichodorid transmitted TRV, were used as an additional control, since the suitability of Samsun NN tobacco as bait-plants was unknown. In addition, another 600 trichodorids were handpicked from the suspension. To each of 30 plastic capsules 20 trichodorids were added. To 10 capsules a PLBcp-, to 10 a TCMcp- and to 10 a TCS44cp- Samsun NN seedling was added. The nematodes were bait tested for virus transmission as described in V.2.b.

The bait-plants from the capsules to which groups of 20 trichodorid had been added were washed from the capsules, transferred into compost, grown for a further 3 weeks and tested for virus presence as described in V.4.a.*i*.

X.2.e.- *Transmission of TRV by nematodes to leaves:* A modification of the leaf-bait-method for testing transmission of TRV by nematodes (van Hoof, 1974) was used. Nematodes were extracted (See V.1.b.) from soil from Kinshaldy, Scotland containing *P. pachydermus* and *T. similis* (See Table 13). The nematodes were added to leaves of Samsun NN tobacco plants either directly after extraction or after having had 4 week access to *P. hybrida* roots, as it was shown that allowing the nematodes access to *P. hybrida* increases the transmission efficiency (See Chapter VIII). For the latter experiment twenty plastic pots (10 cm diameter, 400 ml volume) were filled with soil from Kinshaldy containing *c.* 130 trichodorid nematodes (*P. pachydermus* and *T. similis*) per 100 g soil. In each pot four 4 week-old *P. hybrida* were planted. The pots were placed on moist sand in plastic containers at 17 °C with an 18 hr. light

regime (200 lux) and watered regularly. After 4 weeks the nematodes were extracted from the pots (See V.1.b) and used in the leaf bait test.

From 20 normal Samsun NN tobacco plants and from 20 PLBcp-plants, 8 weeks old and all at a similar stage of growth, one leaf was harvested. Each leaf was placed in a 5 cm diameter plastic petri dish (Sterilin, UK) between two disks of Kleenex tissue paper (Professional wipes - Kimberley-Clark, London) which had been previously cut to fit into the petri dish. Four ml of the nematode suspension, concentrated to contain *c.* 70 trichodorid nematodes, was delivered from a pipette on the top tissue-paper disk. The petri dishes were covered with the lid and placed at 17 °C in the dark. After three days the dishes were transferred to light, and after another 3 days the leaves were removed from the dishes, thoroughly washed under running water and comminuted in a mortar with a pestle. The resulting liquid was manually inoculated onto four carborundum dusted leaves of a *C. amaranticolor* indicator plant and onto a *N. clevelandii* plant. The inoculated leaves of the *C. amaranticolor* plants were checked for the presence of lesions 5 days after inoculation and sap of systemically infected *N. clevelandii* leaves was tested in $F(ab')_2$-ELISA (See V.4.c.) using PRN-antiserum.

Plants from which a leaf had been harvested were manually inoculated with TRV-isolate PPK20 and visually assessed for symptoms 10 days after inoculation.

X.3. RESULTS.

X.3.a.- *Susceptibility of transgenic Samsun NN tobacco plants to infection by manually inoculated TRV:* The results show that plants expressing the PLB cp had a high degree of resistance to manual inoculation with all the TRV-isolates. Occasionally a few lesions developed in the inoculated leaves of these plants and the presence of infective virus in the inoculated leaf could be demonstrated by back-inoculation onto *C. amaranticolor* indicator plants. However, the number of lesions developing on leaves of the indicator plants was low and systemic infection of the transgenic cp expressing plants did not occur.

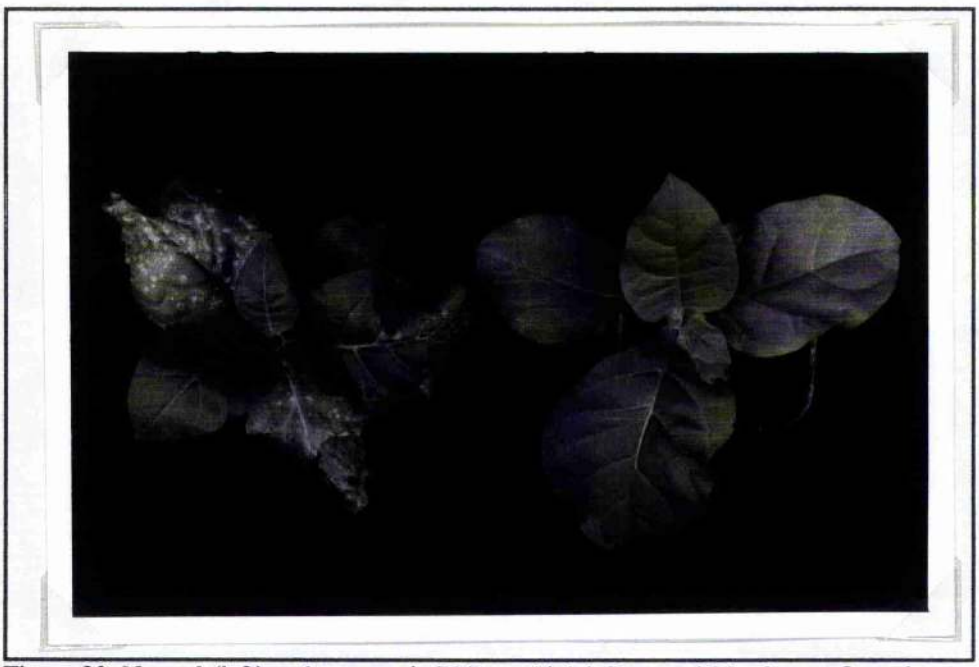

Figure 20. Normal (left) and transgenic PLBcp- (right) Samsun NN tobacco, 2 weeks after manual inoculation with TRV-isolate PPK20.

Table 46. Susceptibility of four PLBcp-transgenic and two normal *N. tabacum* Samsun NN plants to infection by manual inoculation with four TRV-isolates belonging to the PRN-serotype.

TRV-isolate	Plant type[a]	Rep.	Symptoms on Samsun NN[b]	Back test onto *C. amaranticolor* inoc.leaves[c]	Back test onto *C. amaranticolor* syst.leaves[c]	$F(ab')_2$-ELISA value A_{405}sample/A_{405}healthy inoc.leaves	$F(ab')_2$-ELISA value A_{405}sample/A_{405}healthy syst.leaves
PLB							
	normal	1	+++	+++	++	7.3	14.8
	normal	2	+++	+++	+++	9.3	10.9
	PLBcp	1	+	-	-	1.2	0.9
	PLBcp	2	+	-	-	1.0	1.1
	PLBcp	3	+	+	-	1.0	1.1
	PLBcp	4	+	-	-	1.3	1.2
PPK20							
	normal	1	+++	+++	+++	3.5	11.9
	normal	2	+++	+++	+++	4.3	11.3
	PLBcp	1	-	+	-	1.8	1.2
	PLBcp	2	-	-	-	1.5	1.2
	PLBcp	3	-	+	-	1.5	1.6
	PLBcp	4	-	-	-	1.9	1.5
PPW10							
	normal	1	+++	+++	+++	4.5	7.3
	normal	2	+++	+++	+++	5.9	5.7
	PLBcp	1	-	-	-	1.0	1.3
	PLBcp	2	-	-	-	1.4	1.3
	PLBcp	3	-	-	-	1.4	1.3
	PLBcp	4	-	-	-	1.2	1.3
PPS49							
	normal	1	+++	+++	+++	3.0	13.6
	normal	2	+++	+++	++	2.9	10.5
	PLBcp	1	+	+	-	1.1	1.3
	PLBcp	2	-	-	-	1.2	1.3
	PLBcp	3	-	-	-	1.1	1.3
	PLBcp	4	-	-	-	1.2	1.2

[a] normal: non-transgenic Samsun NN plant; PLBcp: transgenic Samsun NN plants expressing PLB coat-protein.

[b] +++ >50 necrotic lesions per leaf followed by severe stem and vein necrosis; ++ 11-50 necrotic lesions per leaf followed by less severe stem and/or vein necrosis; + 1-10 necrotic lesions per leaf, no stem or vein necrosis developing; - no lesions.

[c] +++ >100 lesions per leaf; ++ 11-100 lesions per leaf; + 1-10 lesions per leaf; - no lesions.

X.3.b.- *Transmission of TRV by trichodorid nematodes to roots:* Whereas the transgenic plants expressing the PLB cp were highly resistant to mechanical leaf inoculation of TRV-isolate PPK20, the roots of such plants became readily infected with TRV when groups of 20 trichodorid nematodes carrying the PPK20-isolate were given access to these roots (See Table 47). In similar tests with single trichodorid nematodes, TRV was transmitted by adult *P. pachydermus* (*c.* 85% of the adult population) and by juvenile trichodorids but not by adult *T. primitivus*. Overall, the lowest rate of transmission (11%) was to PLBcp-plants, but this transmission frequency was not significantly lower than that for the control normal plants or for those expressing TCM or TCS44 cp (χ^2_3=4.08 ns).

Table 47. Transmission of TRV-isolate PPK20 to transgenic PLBcp- and to normal tobacco bait-plants, measured by the infectivity of their root extracts, by *P. pachydermus* and *T. primitivus* nematodes, having fed on *P. hybrida* roots infected with the PPK20-isolate.

Bait-plant	single trichodorids[a] (50 set up)	groups of 20 trichodorids[b] (10x)
Normal White Burley	1/13 Pp[c]	n.d
	0/4 Tp[c]	
	8/22 juv.[c]	
Overall	9/39 (23%)	
PLBcp	3/25 Pp	8/8 (100%)
	0/6 Tp	
	2/4 juv	
Overall	5/45 (11%)	
TCMcp	6/28 Pp	8/8 (100%)
	0/4 Tp	
	3/14 juv.	
	9/46 (20%)	
TCS44cp	8/21 Pp	10/10 (100%)
	0/3 Tp	
	3/15 juv.	
	11/39 (28%)	

[a] nominator: nr. of nematodes transmitting, denominator: nr. of nematodes recovered.
[b] nominator: nr. of plants infected, denominator: nr. of plants tested.
[c] Pp: *P. pachydermus*, Tp: *T. primitivus*, juv.: juvenile.

The results of F(ab')$_2$-ELISA using antiserum prepared against the PRN-type strain of TRV on isolates obtained from bait-plant roots (four isolates tested for each type of bait-plant) show that all isolates obtained from the bait tests with groups of 20 trichodorids reacted with the PRN-antiserum. However, of the 16 isolates transmitted

by single trichodorid nematodes that were tested, only 10 reacted; the remaining six were shown to be NM-type isolates.

Table 48. F(ab')$_2$-ELISA results of trichodorid transmitted TRV obtained from bait-plant roots.

Bait-plant	Experiment*	Number of isolates (4 tested) reacting with PRN-antiserum
Normal White Burley	singles	4
PLBcp	singles	3
	20's	4
TCMcp	singles	2
	20's	4
TCS44cp	singles	1
	20's	4

* singles: trichodorid nematodes individually bait tested, 20's: groups of 20 trichodorids bait tested.

X.3.c.- *Transmission of TRV by nematodes to leaves:* All leaves taken from normal Samsun NN plants were infected with TRV, but only two out of 10 and seven out of 10 of the leaves taken from the transgenic PLBcp-plants were infected by the nematodes when used directly from field soil and when used after 4 weeks access to *P. hybrida* respectively.

Table 49. Transmission of TRV to detached leaves of PLBcp- and to normal Samsun NN tobacco plants by a viruliferous nematode population i) directly from field soil and ii) after 4 weeks access to *P. hybrida.*

	Leaf source	Nr. leaves infected (10 tested)
i) Nematodes directly from field soil		
	Normal	10
	PLBcp	2
ii) Nematodes after 4 weeks access to P. hybrida		
	Normal	10
	PLBcp	7

A F(ab')$_2$-ELISA test on sap expressed from *N. clevelandii* plants inoculated with sap expressed from the 29 virus infected leaves from the bait test, showed that all these leaves were infected with PRN-serotype TRV.

Manual inoculation with TRV-isolate PPK20 caused numerous local lesions in the inoculated leaves of all twenty normal Samsun NN plants, followed by systemic symptoms. In the inoculated leaves of the 20 PLBcp plants however, a few lesions developed on only some plants and systemic symptoms were not observed, indicating that all 20 transgenic plants were expressing the PLB cp.

Overall, TRV transmission by trichodorids to PLBcp-leaves was significantly less than transmission to normal leaves (χ^2=15.17 P≤0.001). Also, significantly fewer PLBcp-leaves than normal leaves became infected when the trichodorids were used directly from field soil (χ^2=13.33 P≤0.001). However, transmission by the nematodes

which had been allowed access to *P. hybrida* prior to adding them to the bait-leaves, was not significantly different for PLBcp- and normal leaves (χ^2=3.53 ns).

X.4. DISCUSSION AND CONCLUSIONS.

TRV-isolates PPK20, PPW10 and PPS49 react with PRN-antiserum in $F(ab')_2$-ELISA and in that respect are indistinguishable from TRV-strain PLB (See Chapter VII, Table 35 and Chapter V, Table 9). Therefore, the fact that PLBcp-plants resisted infection with these isolates when manually inoculated, corresponds with results obtained by van Dun and Bol (1988) who also found that transgenic plants expressing coat protein of one TRV-strain were resistant to infection by another serologically similar strain. However, individual *P. pachydermus* nematodes and groups of twenty trichodorids (mainly *P. pachydermus*) transmitted TRV-isolate PPK20 to the roots of transgenic plants expressing the PLB, TCM or TCS44 cp. No other reports are available describing resistance of transgenic plants to virus transmission by nematodes, but studies on aphid transmitted viruses showed that the resistance of transgenic plants expressing viral cp was generally not overcome by the aphid vectors (Lawson *et al.*, 1990; Quemada *et al.*, 1991; van der Wilk *et al.*, 1991). Lawson *et al.* (1990) however, also found that some lines of transgenic plants, expressing cp of potato virus Y, were resistant to this virus when manually inoculated but not when transmitted by aphids. A possible explanation for the lack of resistance to nematode transmission of TRV to the roots of transgenic TRV coat-protein expressing plants could be that expression of the PLB cp is lower in roots than in leaves. When this was explored, PLB cp could not be detected in $F(ab')_2$-

ELISA from roots or from leaves (data not shown). Angenent *et al.* (1990) also failed to detect viral cp serologically in PLBcp-plants and estimated that less than 0.01% of soluble protein in such plants consisted of viral cp. A possible lower expression of viral cp in roots can, however, not fully explain the results obtained in this study since the nematodes were also able to transmit TRV, serologically similar to strain PLB, directly to PLBcp-leaves. Fewer PLBcp-leaves became virus infected by nematodes which had been added to these leaves immediately after extraction than by nematodes which had been allowed to feed for 4 weeks on *P. hybrida*. It was shown that allowing nematodes 4 weeks access to virus-free *P. hybrida* resulted in an increase in the proportion of viruliferous nematodes in the population from 3-15% to 69% (See Chapter VIII). This suggests that the transmission of TRV to PLBcp-plants increases with an increasing inoculum pressure. A number of studies involving cp mediated resistance in transgenic plants also demonstrated that resistance could be overcome by increasing the virus inoculum concentration (Hemenway *et al.*, 1988; Nelson *et al.*, 1988; Powell Abel *et al.*, 1986; Stark and Beachy, 1989; Tumer *et al.*, 1987). This phenomenon was also observed with PLBcp-plants and PLB inoculum (Bol, unpublished results). As PLBcp-plants are not resistant to infection with PLB RNA (Angenent *et al.*, 1990), an alternative explanation is that the nematodes injected viral RNA into the plant cells. The latter explanation, however, seems unlikely as the TRV cp appears to play an important role in the recognition between virus and vector and because TRV RNA is unstable and its infectivity much less than that of intact nucleoprotein (Harrison and Robinson, 1986). Thus it can be concluded that trichodorid vector nematodes can effectively overcome cp-mediated resistance in transgenic tobacco plants, possibly by efficiently concentrating virus inoculum (i.e.

injection of a relatively large number of TRV particles into very few cells). It may be questioned therefore whether genetically engineered cp-mediated resistance will be of use for controlling TRV under field conditions considering the large variation in serological characteristics between TRV-isolates and the fact that the natural trichodorid vectors seem able to effectively overcome such resistance. However, whether endogenous cp in transgenic plants affects the nematodes ability to acquire virus, e.g. by modifying the recognition or binding between virus and vector receptor sites, remains to be studied.

CHAPTER XI.

GENERAL DISCUSSION AND CONCLUSIONS.

XI.1. THE ASSOCIATION BETWEEN TRV SEROTYPES AND TRICHODORID VECTOR SPECIES.

Few studies have been done to examine the transmission of TRV by trichodorids due to misconceptions about the difficulties for handling and working with these nematodes. In this study a bait test technique was developed (See V.2.b.) in which individual trichodorid nematodes were allowed to transmit virus. This technique is essential for studying associations between trichodorid vector species and TRV-serotypes. A major advantage of the technique is that each transmitted TRV-isolate corresponds with one trichodorid nematode only and that each nematode is specifically identified. By using this technique the individual vector species in a mixed population can be tested for virus transmission. Furthermore, serological characteristics of isolates transmitted by individual nematodes or species can be determined. In addition, this technique allows the transmission efficiencies of populations, species or sexes (male, female, juvenile) to be determined.

The results from chapter VI and VII show that a considerable degree of specificity occurs between TRV serotypes and trichodorid vector species. The results indicate that the virus coat-protein is likely to play an important role in the recognition between vector and virus. This hypothesis is confirmed by results from chapter IX where it is shown that by replacing RNA-2 from a transmissible isolate with RNA-2 from a non-transmissible isolate, the originally transmissible isolate is no longer

transmitted. Conversely, replacing the RNA-2 from a non-transmissible strain with RNA-2 from an efficiently transmitted isolate results in the efficient transmission of the originally non-transmissible strain. This is the first unequivocal evidence demonstrating that the RNA-2 of TRV determines vector transmissibility.

Whilst evidence obtained revealed that transmissibility of TRV is determined by the RNA-2 genome part, and therefore most likely by the virus coat-protein, it is also clear that the serological characteristics of TRV-isolates, as determined in $F(ab')_2$-ELISA using polyclonal antisera, are not directly related with transmission by a specific vector. Particularly within the genus *Trichodorus*, no clear correlation exists between serological characteristics of transmitted TRV-isolates and trichodorid vector species. For example, TRV-isolates reacting with the RQ antiserum are transmitted by *T. cylindricus*, *T. primitivus* and *T. viruliferus* (See Fig. 19, Chapter VII). Also, several isolates, from a group of serologically indistinguishable PRN-serotype isolates, are not acquired and/or transmitted by *P. pachydermus*, whereas other isolates belonging to this serotype are efficiently transmitted. Similar results were obtained in studies on the transmission of nepovirus-isolates by longidorid vectors (Brown *et al.*, 1989). As with TRV, transmissibility of nepoviruses is generally regarded as being dependant on the virus coat-protein (Harrison *et al.*, 1974a). Thus, the vector nematodes recognize differences between isolates which are not detected in serological tests. This could mean that the part of the virus coat-protein which is recognized by the nematodes is not very antigenic, and that only a small proportion of the IgG molecules in a polyclonal antiserum specifically react with this part of the coat-protein.

The results from bait-tests using individual nematodes (See Chapter VII) reveal that isolates transmitted by *Trichodorus* spp. are serologically distinct from those transmitted by *Paratrichodorus* spp. TRV-isolates obtained from individual trichodorid nematodes extracted from mixed species populations can be serologically characterized as having been transmitted by *Paratrichodorus* or *Trichodorus* spp. Furthermore, isolates transmitted by *Trichodorus* spp. were not transmitted by *P. pachydermus*. This implies that isolates transmitted by *Paratrichodorus* spp. have coat-proteins substantially different from those of isolates transmitted by *Trichodorus* spp. Within the group of isolates transmitted by *Trichodorus* spp. however, isolates which are not distinguished by the available antisera are associated with different vector species. This could imply that vector specificity is less prominent with TRV-*Trichodorus* than with TRV-*Paratrichodorus* associations and that *Trichodorus* vector species are able to transmit "each others" isolates. The results could however also be explained by supposing that the *Trichodorus* vectors recognize differences in coat-proteins of different isolates which are not being detected in $F(ab')_2$-ELISA using polyclonal antisera.

Further experimental data are required to confirm unequivocally the observation that certain isolates transmitted by one species can be also transmitted by another species. The possibility that vector specificity within *Trichodorus* transmitted TRV occurs, is however not unlikely as it was shown that TRV-isolates with a similar reaction in $F(ab')_2$-ELISA differed considerably in their transmissibility by *P. pachydermus* (See Chapter VIII). Atreya *et al.* (1991) demonstrated that a change in one coat-protein amino acid only, resulted in the loss of aphid transmissibility of

tobacco vein mottling virus and a similar change in coat-proteins of TRV-isolates may have similar consequences for their transmissibility by trichodorids.

In addition to the observations that serologically similar isolates are associated with different *Trichodorus* vector species, it was also shown that serologically distinct TRV-isolates are associated with the same *Trichodorus* species. This again may imply that the most antigenic regions on the coat-protein are not those which are recognized within the vector nematodes. However, the possibility that the coat-protein is only one component in determining the transmissibility and vector specificity of TRV should not be excluded. Harrison and Robinson (1986) concluded that, because of the large antigenic diversity of TRV-isolates, there has been little selection pressure acting to conserve the amino acid sequence of surface exposed regions of the TRV coat-protein. They therefore speculated that the coat-protein was unlikely to have a major function in determining vector transmissibility and specificity (Harrison and Robinson, 1986). Harrison (1987) suggested that, because of the antigenic diversity of TRV-isolates and the existence of a viral gene with unknown function, a helper factor might be involved in the transmission process. Guilford *et al.* (1991) later rejected the hypothesis that this gene, coding for a 16K protein, was involved in vector transmission.

The information presented in this study shows that a considerable degree of vector specificity occurs with the transmission of TRV by trichodorids and that the virus RNA-2, most probably the coat-protein, determines transmissibility. However, serological characteristics of TRV-isolates did not always correspond with the transmission of these isolates by specific vector species. This may be attributed to serological methods using polyclonal antisera, which fail to detect minor coat-protein

differences important for the recognition between virus and vector. Furthermore, factors other than the virus coat-protein, may play an important role in the transmission process.

XI.2. THE EFFICIENCY OF TRANSMISSION.

Male, female and juvenile trichodorid nematodes are all equally able to transmit TRV (See Chapter VII), and in this respect again resemble longidorid vectors of nepoviruses (Taylor and Cadman, 1969). Substantial differences were observed in the transmission efficiency of different trichodorid populations and of species within mixed populations (See Chapter VII). The fact that only one trichodorid species from a mixed species population transmitted virus, whereas the other(s) failed to do so can be attributed to vector specificity. Thus, within a mixed species population the different species each transmit their "own" associated serologically distinct isolate (See Chapter VII), and only one species out of a species mixture, is able to acquire TRV from manually inoculated source-plants and to subsequently transmit the virus to bait-plants. Furthermore the proportion of *P. pachydermus* in a trichodorid population transmitting TRV can be increased by allowing the population to feed on a *P. hybrida* plant prior to transferring individual nematodes to bait-plants (See Chapter VIII). Walkinshaw *et al.* (1961) observed a similar increase in the transmission efficiency of *P. minor* after allowing the nematodes to feed on maize prior to exposing them to tobacco bait-plants, and Harrison *et al.* (1961) maintained *L. elongatus* populations with a high proportion of viruliferous nematodes by allowing the nematodes access to virus infected potato plants.

Taylor and Cadman (1969) attributed differences in transmission efficiency of field populations of vector species to a number of factors such as the virus concentration in the plant roots, growth of infected plants, soil temperature etc. Thus, it is not clear whether the observed differences between vector populations in the proportion of nematodes transmitting virus to bait-plants reflect differences in the proportion of viruliferous vector nematodes or result from differences in the proportion of the viruliferous population able to reach and subsequently infect the bait-plant roots.

It is unlikely that differences in the transmission rates of vector species and populations (See Chapter VII) can be attributed to differences in environmental conditions (e.g. soil moisture in the capsules, bait-plant age or species, temperature etc.), as these were kept constant throughout this study. Therefore, the most likely explanation for the differences in transmission rates is that indeed the results reflect the proportion of viruliferous nematodes within a vector population. This indicates that even within the small volume of a soil sample (*c.* 2 kg) some individual vectors carry virus whereas others do not. The proportion of viruliferous nematodes increases when they are allowed to feed on virus infected source-plants, and therefore the availability of infected roots is an important factor in determining the proportion of viruliferous nematodes within a vector population when other factors e.g. soil temperature, soil moisture etc. are not sub-optimal. Furthermore, it shows that the failure of a part of the vector population to transmit virus is not genetically determined. Brown (1986) showed that differences in transmission efficiency of arabis mosaic nepovirus by two populations of *Xiphinema diversicaudatum* were genetically controlled. He therefore concluded that the occurrence of receptor sites

complementary to virus coat-protein surface structures is an inherited factor. In this study individual virus-free *P. pachydermus* from the Netherlands and from Scotland did not differ in the efficiency with which they acquired and transmitted PRN-serotype TRV-isolates. However, it is not known if genetic differences between trichodorid vector populations exist which may affect their ability to transmit TRV-isolates.

XI.3. PLANT/VIRUS/VECTOR INTERACTIONS.

Like nepoviruses, tobraviruses are mainly viruses of wild plants and weeds. TRV was shown experimentally to have a wide host range and it has been recovered from a variety of naturally infected crops and plant species (Robinson and Harrison, 1989; Schmelzer, 1959). Similarly, trichodorid nematodes also have been found associated with a wide variety of crops. Even so, much less information is available on the host status of plant species or crop cultivars for both the virus and the vector. As TRV was frequently recovered from the roots of Samsun NN and White Burley tobacco and *P. hybrida* plants used in this study, these plants appear to be suitable hosts for the vector and the virus. Whether these plants are indeed good hosts for the vector nematodes can however be questioned as successful transmission of TRV is perhaps more likely when the nematodes only briefly probe the root cells of the bait-plants. When the nematodes feed on the same cell for prolonged periods of time, the cell is destroyed making it impossible for the virus to become established in such a cell (Wyss, 1975). The failure of *P. nanus* to transmit TRV to *P. hybrida* bait-plants (See Chapter VII) may have been due to either the nematodes having been virus-free or to

P. hybrida being an unsuitable bait-plant for detecting TRV transmission by *P. nanus*. Conversely, *P. pachydermus* transmitted TRV to roots of White Burley, Samsun NN and *P. hybrida* plants as well as to detached Samsun NN leaves. The fact that transgenic Samsun NN plants, expressing the TRV coat-protein gene, are resistant to manual inoculation with TRV but not to infection with TRV by *P. pachydermus* vector nematodes (See Chapter X) indicates that vector transmission may be a very efficient process when a suitable bait-plant is used.

The importance of the choice of bait-plants was demonstrated by Trudgill and Brown (1980). They found that *X. index* acquired grapevine fanleaf nepovirus from *C. amaranticolor* source-plants and subsequently transmitted the virus to grapevine seedlings but not to virus-free *C. amaranticolor* bait-plants. Jones *et al.* (1989) obtained similar results showing that *P. hybrida* plants were more suitable as bait-plants than raspberry plantlets when testing the transmission of raspberry ringspot or arabis mosaic nepovirus by their longidorid vector nematodes.

The choice of source- or bait-plant species may also affect the transmission of TRV by trichodorids. Van Hoof (1968) reported that White Burley tobacco plants were unsuitable as bait-plants when testing the transmission of TRV by *P. nanus*, and used *Aster chinensis* instead. Engsbro (1976) found that growing potatoes at a site for seven consecutive years, using potato seed tubers 100% infected with TRV, failed to make the trichodorid population present at that site viruliferous. The nematode vectors present at this site and the TRV-strain introduced with the infected potatoes may not have been compatible or the majority of TRV-infected potato tubers may have contained NM-type isolates (Harrison and Robinson, 1986). In this study none of three PRN-serotype TRV-isolates obtained from potato tubers were acquired and

transmitted by *P. pachydermus*, whereas all other isolates, serologically indistinguishable from the potato originated isolates, were acquired and transmitted by this vector species (See Chapter VIII). Therefore even M-type infected potato plants may not act as virus sources for trichodorid nematodes. Whether TRV-infected flower bulbs may act as a virus source for the nematodes is at present unknown. The infection process in bulbous crops however seems different from that in potato, as M-type infections are common in flower bulb crops (Asjes, pers. comm.; Harrison and Robinson, 1986).

XI.4. DETECTION OF TRICHODORID TRANSMITTED TRV.

In this study vector transmitted TRV was detected from field soil by pot bait-tests (See V.2.*a*). *P. hybrida* or White Burley tobacco plants used as virus bait-plants gave similar results regarding the detection of vector transmitted TRV from soil samples, although generally more virus was obtained from *P. hybrida* than from White Burley roots as determined by inoculation onto *C. amaranticolor* indicator plants. Also, it was demonstrated that results obtained from pot bait-tests may underestimate the TRV transmitting ability of certain species. For example, when comparing results obtained from pot bait-tests with those from individual bait-tests from several soil samples only one serotype was detected from bait-plants grown in pots, whereas using individual trichodorids revealed that two serologically distinct types of isolates were present and being transmitted. An explanation for this apparent discrepancy could be that a plant, once infected with TRV, becomes immune to subsequent infection. Cross protection within TRV has been reported to occur (Cadman and

Harrison, 1959), although the serological similarities of the cross protecting isolates were not fully determined. Plants infected with the Dutch strain of PEBV however, were protected from subsequent infection by the serologically distinct English strain of PEBV (Gibbs and Harrison, 1964a). Another possibility, suggested by Cadman and Harrison (1959) is that when plants are infected with a mixture of TRV-isolates, one isolate-type becomes predominant to the exclusion of the other type(s).

The conclusion by Harrison and Robinson (1986) and by Robinson (1989) that serological tests are of limited value for the detection of TRV was confirmed in this study. Several TRV-isolates were recovered from bait-plants which failed to react in $F(ab')_2$-ELISA with any of the antisera used. A major drawback of the bait-test system as described in V.2.*a* is that it is time and labour consuming. Results by van Hoof (1974) and those presented in Chapter X show that allowing the nematodes to feed directly on leaves and subsequently testing these leaves for the presence of TRV may be used as an alternative to the pot bait-test for the detection of trichodorid transmitted TRV from soil. At present the use of this "bait-leaf" method has several advantages over the pot bait-test. Whereas with the latter method the time from setting up the bait-tests to obtaining the results is *c.* 4 weeks, with the bait-leaf method only 6 days are required. Furthermore the bait-leaf method requires less attention (no watering of pots) and space. Van Hoof (1974) buried the bait-leaves in the soil without extracting the nematodes. In this study nematodes were extracted from the soil and subsequently added to the bait-leaves in order to increase the number of nematodes having access to the leaves. However, the relative efficiencies of using the bait-leaf method or the pot bait-test for detecting trichodorid transmitted TRV are unknown.

XI.5. ASSOCIATIONS BETWEEN TRICHODORID NEMATODES AND TOBRAVIRUSES: PRESENT AND FUTURE.

The main mode of transmission of tobraviruses appears to be by *Paratrichodorus* and *Trichodorus* vector species. Other modes of transmission have however been reported but seem less common. For example, TRV has been shown to be transmitted through seeds of weed hosts (Cooper and Harrison, 1973) and pea plants infected with PEBV yielded infected pea seeds (Bos and van der Want, 1962; Harrison and Robinson, 1981). The transmission of TRV in soil in absence of vector nematodes has also been reported by several workers (Fritzsche *et al.*, 1985; Sol, 1963) but is most likely to occur only exceptionally. TRV may also be spread by the dissemination of infected vegetatively propagated plant material (i.e. potato seed, bulbs). However, TRV is probably self-eliminating when vegetatively propagated material is planted in a field in which the vector nematodes do not occur (Asjes, 1989; van Hoof and Silver, 1976). Thus, although seed-borne transmission and spread of TRV following the use of infected material for planting may be important as a means of introducing the virus into new sites, the presence of trichodorid vector nematodes at these sites is crucial if the virus is to become established. This is strongly supported by the observation that disease caused by TRV is seldom encountered when growing crops on soil types which are not naturally inhabited by trichodorid nematodes.

The distribution of the virus being dependent on the distribution of the vector is reflected in the results of a literature review on the occurrence of trichodorids and tobraviruses in Europe. In all European countries from which tobraviruses have been

recorded, trichodorid nematodes have also been found (See Fig. 4, Chapter IV). However, in a number of countries from which trichodorids have been recorded, the presence of TRV has not been confirmed. Furthermore, only a small proportion of the total number of trichodorid species has been shown to be able to transmit virus (See Fig. 3, Chapter IV). Studies in Belgium, the Netherlands and Great Britain show that almost all trichodorid species naturally occurring in these countries, are associated with the transmission of TRV. It is therefore likely that TRV will also be shown to be present in areas or countries where trichodorid nematodes naturally occur, but from which at present no virus associated with these nematodes has been found, and that the number of species shown to be able to act as virus vectors will increase.

XI.6. THE INTERACTION BETWEEN TRV AND TRICHODORID VECTORS: SIMILARITIES AND DIFFERENCES WITH OTHER VIRUS-VECTOR ASSOCIATIONS.

Substantial differences occur in the ecology, genome strategy and chemical and physical properties of vector transmitted plant viruses. Furthermore, the virus vectors are quite diverse including fungi, insects and nematodes. Most information is available on virus transmission by insects. The interaction between insect, particularly aphid, vectors and transmitted viruses can be classified into three types:

1. propagative
2. circulative, non-propagative
3. non-circulative.

The propagative interaction is characterized by the multiplication of the virus inside the vector and by the long persistence of the virus transmitting ability of the vector (often the lifespan of the vector). This type of interaction is therefore also known as persistent. The circulative, non-propagative interaction is characterized by virus particles circulating within the vector prior to being released in the saliva of the vector. The virus does not, however, replicate in the vector. After having acquired the virus, the vector usually stays infective for a considerable time (many days). This type of interaction is also known as semi-persistent. The non-circulative, or non-persistent, type of interaction is characterized by the direct attachment of virus particles to the mouthparts of the vector. The vector usually looses its infectivity soon after virus acquisition (hours to a day).

Harrison (1987), reviewing the transmission of plant viruses by vectors, only briefly mentions the transmission of tobraviruses by trichodorid nematodes. However, the interaction between nepoviruses and longidorid vector nematodes was considered to be of a non-circulative type (Harrison, 1987). The interaction between tobraviruses and trichodorids has several characteristics in common with that of nepoviruses by longidorids: there is no indication of virus replication inside the vector body and virus particles do not circulate in the body prior to transmission. The infectivity of the vectors is lost when they moult, and there are no indications that virus is passed through the eggs of the vectors to the progeny. Both longidorid and trichodorid vector nematodes have been shown to remain infective for long periods of time after having acquired the virus (Taylor and Robertson, 1975). Thus, one could suggest that, like longidorids and nepoviruses, the interaction between trichodorid vectors and tobraviruses also is non-circulative. With aphid transmitted plant viruses the non-

circulative type of interaction is usually synonymous with non-persistent transmission. This is clearly not so with nematode transmitted plant viruses. Although only short periods are required (*c.* 1 hr) for the nematodes to acquire the virus (Ayala and Allen, 1966; Teliz *et al.*, 1966), they may remain infective for long periods, several weeks or months (Taylor and Raski, 1964; van Hoof, 1970b). Therefore, the transmission of plant viruses by nematode vectors cannot be satisfactorily categorized according to the interactions found with the transmission of plant viruses by insect vectors. Indeed, Taylor and Cadman (1969), reviewing virus transmission by nematodes, concluded that any similarities between insect and nematode virus vectors were "superficial and fortuitous". However, although insect and nematode vectors differ greatly in their ecology, which has major implications for the way in which the viruses transmitted by these vectors are spread, the nature of the interactions between the vectors and their associated viruses shows considerable homology.

Vector specificity has been demonstrated to occur with many virus-vector associations (Harrison and Murant, 1984). Furthermore, vector specificity and transmissibility has been shown to be determined by virus coded proteins, usually the coat-protein or a protein acting as a helper factor, regardless of the nature of the vector (Harrison and Murant, 1984). The results from this study suggest that the TRV coat-protein plays a major role in determining the transmissibility of this virus by trichodorid vectors. The mechanism underlying coat-protein determined specificity and transmissibility is thought to be based on the recognition of particular regions on the surface of the virus particles by receptor sites in the vector. Thus, it was shown that nepoviruses are retained only at specific sites in the odontophore region and along the inner surface of the guiding sheath of their longidorid vectors (Taylor and

Robertson, 1969; 1970b). Temminck *et al.* (1970) and Harrison and Murant (1984) suggested that transmissibility of tobacco necrosis virus by the fungus *Olpidium brassicae* depended on the ability of specific surface structures on the virus particles to bind with receptor sites on the membrane of the fungal zoospores. A similar mechanism has been suggested to operate in the specific attachment of TRV particles to the oesophageal lining of the vector *P. pachydermus*, involving the interaction of carbohydrate moieties along the vector receptor sites with lectin-like molecules on the virus coat-protein (Martelli and Taylor, 1989; Robertson and Henry, 1986a). The results obtained in this study do not present additional information on the molecular basis of transmissibility and vector specificity.

In conclusion therefore, although the mechanisms determining transmissibility and vector specificity appear to be similar with fungus-virus, insect-virus and nematode-virus associations, the interaction between TRV and trichodorid vectors most closely resembles the one which occurs between nepoviruses and longidorid vectors.

XI.7. FUTURE WORK.

Results obtained in this study reveal for the first time that vector specificity occurs with the transmission of TRV by trichodorid nematodes. The evidence that the RNA-2 genome part of the virus determines transmissibility by the vector suggests that, as with many other vector transmitted plant viruses, the coat-protein plays an important role in the transmission process. This is likely to occur at the level of recognition between virus structural components and receptor sites within the vector. Further work is required to specifically locate the regions of the coat-protein amino acid

sequence involved in this process. A strategy to study this would be to produce TRV-isolates with recombinant RNA-2 genome parts. By constructing these recombinant RNA-2 molecules from the RNA-2's of a non-transmissible (e.g. strain PLB) and a transmissible (e.g. isolate PPK20) TRV-isolate, and subsequently using these recombinants in acquisition and transmission experiments it is likely that the coat-protein regions involved in transmissibility of TRV will be revealed. This type of research would provide valuable information on the mechanism underlying vector specificity. Furthermore, a better understanding of the interactions between virus and vector is likely to be essential for the development of novel strategies aimed at controlling TRV in the field.

Transgenic plants expressing the coat-protein of TRV were readily infected by viruliferous trichodorids. It is therefore concluded that genetically engineered cross protection is unlikely to be a successful way to control TRV. Cadman and Harrison (1959) however observed that NM-type infected *Nicotiana sylvestris* plants were resistant to subsequent inoculation with M-type virus. This suggests that the RNA-1 may also induce cross protection. As the RNA-1 is highly conserved within TRV, a cross protection based on RNA-1 is likely to protect against the total spectrum of TRV-isolates. The molecular mechanism of this RNA-1 induced cross protection is still unknown. The RNA-1 of TRV is not known to carry any structural genes and other workers (Angenent *et al.*, 1990; van Dun and Bol, 1988) have failed to induce virus resistance in transgenic plants expressing non-structural genes. Therefore the mechanism by which NM-type infected plants cross protect against M-type TRV infection and whether this phenomenon can be induced in transgenic plants expressing TRV RNA-1 specific genes requires investigation.

For the future development of alternative strategies to control TRV it is crucial to develop methods which allow the detection of small populations of trichodorid nematodes. Present diagnostic techniques for detection of TRV from soil are laborious and time consuming (bait-tests), subject to errors due to antigenic diversity of the virus (ELISA) or often involve the use of radioactive labels (cDNA hybridization). The detection of TRV by the use of PCR (polymerase chain reaction), in which specific sequences of viral RNA are multiplied to be subsequently visualized on a gel after electrophoresis, may prove of value for a rapid and reliable diagnosis of TRV.

Studies on the ecology of the different trichodorid vector species and their associated TRV-isolates are essential as still much remains unknown about the specific hosts of the vector nematodes, the population dynamics of the different vector species in relation to virus transmission , the influence of crop cultivars on acquisition and transmission of TRV by the vectors and the survival of vectors and of specifically retained virus within the vectors under different environmental conditions.

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APPENDIX A.

A dichotomous key for identification of trichodorid species found in this study.

Trichodorus species.

Females.

1. Sclerotized pieces at vagina close to each other (max. 2 µm) 2
   Sclerotized pieces at vagina more clearly separated (min. 3 µm) 3

2. Sclerotization of vagina rounded triangular. Vagina barrel-shaped ..... *T. cylindricus*
   Sclerotization of vagina triangular. Vagina rhomboid-shaped ..... *T. similis*

3. Sclerotized pieces at vagina two parallel rods ..... *T. primitivus*
   Sclerotized pieces at vagina with different shape 4

4 Cuticular layers separated by irregular or wavy line. Sclerotized pieces at vagina as two small triangular drops. Vagina more or less rectangular. Body length 0.79-1.14 mm. Onchiostyle 45-71 µm ..... *T. sparsus*

   Cuticular layers not clearly separated. Sclerotized pieces at vagina small triangular. Vagina more or less rhomboid shaped. Body length 0.62-0.89 mm. Onchiostyle 38-48 µm. ..... *T. viruliferus*

Males.

1. No cervical papillae in onchiostyle region. Body length 0.79-1.00 mm. Spicules 47-51 µm ..... *T. sparsus*

   Cervical papillae in onchiostyle region 2

2. Spicules distally very thin, so gubernaculum appears to cross spicules. Body length 0.73-0.83 mm. Spicules 33-40 µm ..... *T. primitivus*

   Spicules not so. Gubernaculum not crossing spicules 3

3. Spicules striated. Rudimentary bursa present. Tail and spicules almost straight. Body length 0.63-0.80 mm. Spicules 33-49 µm ..... *T. cylindricus*

   Spicules not striated, no bursa present. Tail and spicules curved ventrally 4

4. Spicules irregualrly shaped, with obvious constriction in middle region. Body length 0.62-0.89 mm, Spicules 31-37 µm ..... *T. viruliferus*

   Spicules with regular shape. No constriction in middle region. Body length 0.76-0.87 mm. Spicules 35-40 µm ..... *T. similis*

Continued next page

Appendix A, Identification key continued.

Paratrichodorus species

Females

1. Oesophagus clearly overlapping intestine ventrally.
   Body length 0.77-1.23 mm. Onchiostyle 41-52 µm. Males uncommon ..... *P. teres*

   Oesophagus not, or only slightly overlapping the intestine 2

2. Spermatheca present. Sperm cells large, with sausage shaped nuclei.
   Body length 0.65-0.97 mm. Onchiostyle 42-53 µm ..... *P. anemones*

   Spermatheca absent 3

3. Sperm cells large with sausage shaped nuclei.
   Excretory pore in oesophagus region. Males common.
   Body length 0.69-1.02 mm. Onchiostyle 44-54 µm ..... *P. pachydermus*

   Sperm cells minute. Excretory pore *c.* 1.5 body width posterior
   to oesophagus base. Males rare. Body length 0.49-0.60 mm.
   Onchiostyle 21-23 µm ..... *P. nanus*

Males

1. No cervical papillae present 2

   One cervical papilla present 3

2. Posterior part of oesophagus ventrally overlapping
   the intestine. Body length 0.72-0.86 mm.
   Onchiostyle 46-60 µm. Spicules 45-52 µm ..... *P. teres*

   Oesophagus not overlapping intestine. Body length 0.49-0.55 mm.
   Onchiostyle 21-23 µm. Spicules 42-44 µm ..... *P. nanus**

3. Tail round, less than anal body width. Spicules thin, kinking.
   Distance from second supplement to anus 38-64% of spicule length.
   Body length 0.71-0.90 mm. Onchiostyle 42-53 µm. Spicules 46-53 µm ..... *P. anemones*

   Tail trapezoid, more than anal body width. Spicules straight.
   Distance from second supplement to anus 75-94% of spicule length.
   Body length 0.61-0.99 mm. Onchiostyle 45-60 µm. Spicules 45-60 µm ..... *P. pachydermus*

* *P. nanus* males were not found in this study.