European Journal of Plant Pathology **100**: 109–122, 1994.

Detection of tobacco rattle virus in nematodes by reverse transcription and polymerase chain reaction

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Accepted 22 February 1994

Key words: Trichodoridae, *Petunia hybrida, Chenopodium amaranticolor, Trichodorus primitivus, Nicotiana tabacum* cv. White Burley

Abstract. An assay, based on amplification of cDNA synthesized from genomic viral RNA, has been developed to detect tobacco rattle virus in infected plant material and viruliferous nematodes. A range of different TRV strains could be detected using the procedure developed. The presence of one to three viruliferous nematodes in a nematode suspension was sufficient for the detection of TRV. The minimum amount of purified virus detectable in the assay was 15 fg, indicating an increased sensitivity of the PCR-based assay as compared to serological detection methods, like ELISA. A dot-blot hybridization procedure was developed for the detection of the PCR products, making agarose gel electrophoresis dispensable.

Introduction

Tobacco rattle virus (TRV) has a world-wide distribution and an extensive host range including economically important crops like potato, sugar beet and several ornamental species [Robinson and Harrison, 1989]. Infection with TRV can cause severe damage and major crop losses. The virus is transmitted by nematodes of the Trichodoridae family. These soil-inhabiting nematodes acquire and transmit the virus while feeding on root cells of their host plants. They can survive in soil without host plants for years and still be able to transmit TRV, which makes it very difficult to control the virus [Van Hoof, 1971].

TRV-isolates can occur as two types [Harrison and Robinson, 1978]. Firstly, M-type TRV isolates, which produce rod shaped particles of two sizes, a long (L) particle and a short (S) particle, containing linear single-stranded RNA molecules of approximately 700 nucleotides (RNA-1) and ranging from 1500 to 4000 nucleotides (RNA-2), respectively. The RNA-2 encodes the coat protein. Secondly, NM-type TRV isolates, which consist only of the RNA-1 and do not produce particles.

Several problems are associated with the detection of TRV in plants. Due to the absence of viral particles and production of coat protein, detection of NM-type isolates using serological methods is impossible, while detection of the M-type isolates is severely hampered by the occurrence of

different serotypes. In the vector nematode TRV particles are found adhered to the oesophageal wall [Taylor and Robertson, 1970]. Detection by serological means of the presence of TRV in its vector is extremely difficult, probably due to low virus titers and absence of efficient sample preparation protocols.

However, detection of TRV in nematodes is a prerequisite for studies on TRV transmission and for an effective control of the virus. Until now TRV has been detected in an indirect manner in nematodes by transmission of TRV to bait plants and subsequent inoculation of test plants [Van Hoof, 1968; Brown et al., 1989] Despite the fact that this method has improved through the years, it is still expensive and time-consuming and requires a great amount of knowledge on TRV symptomatology.

Several polymerase chain reaction (PCR)-based methods for the detection of viruses in plants have been published [e.g. Robertson et al., 1991; Wetzel et al., 1992], including a procedure for the detection of TRV in infected plants [Robinson, 1992]. Since it is of great importance to have a reliable and rapid method for direct detection of TRV in nematodes, we have developed an assay to detect TRV directly in nematodes using a procedure based on PCR. To our knowledge it is the first time that a plant virus was detected in nematodes using a PCR-based method.

Materials and methods

Virus isolates

Purified TRV isolates were kindly provided by C. Cuperus (IPO-DLO). In this study the following TRV isolates were used: PSG [Cornelissen et al., 1986], PRN [Cadman and Harrison, 1959], Lisse Hoof [Van Hoof, 1968], PLB [Angenent et al., 1989], PNP [Linthorst and Bol, 1986], Wageningse Eng and Baexem. The latter two isolates were isolated from tobacco and *Petunia hybrida* plants, respectively, grown in soil from arable fields in the Netherlands and were associated with the vectors *Paratrichodorus pachydermus* and *Trichodorus primitivus*, respectively. TRV isolates used for PCR studies and northern blot analysis were propagated in *Nicotiana tabacum* cv 'White Burley' plants. All strains and isolates showed different but typical TRV symptoms 5 to 7 days after mechanical inoculation. Leaves showing distinct symptoms were directly used in experiments.

Nematodes

Soil containing high numbers of *T. primitivus* nematodes was collected in the early spring from a fallow field near Baexem, the Netherlands and stored for at least three months at 5 °C. Due to this long storage period without feeding, any virus present in the intestine of the nematodes [Van

Hoof, 1967] was most likely to be degraded. No other trichodorid species and only low numbers of other plant-feeding nematodes were found. TRV transmission by several sub-populations from the same field was assessed by planting *P. hybrida* bait-plants on 400 g sub-samples of the soil and testing of the roots after 3 weeks of growth. In this study a non-viruliferous and a viruliferous *T. primitivus* sub-population were used. Bait tests using individual *T. primitivus* nematodes from the viruliferous sub-population were performed according to Brown et al. [1989]. Two week old bait seedlings were exposed to single nematodes for two weeks at 16 °C and subsequently, the whole plantlet was tested on *Chenopodium amaranticolor*. Of 50 individuals incubated with *P. hybrida* and recovered alive afterwards, only 1 transmitted TRV. For PCR studies the nematodes were collected from soil samples by elutriation and filter extraction, essentially as described by Oostenbrink [1960]. Aliquots of the obtained suspensions, containing part of the total nematofauna and known numbers of *T. primitivus*, were stored at −80 °C.

Extraction of nucleic acids from plant material and purified virus

Genomic RNA was isolated from purified virus particles by phenol extraction. A purified virus suspension (200 µl) was successively subjected to phenol extraction at 65 °C, phenol/chloroform (1:1) and chloroform extraction at room temperature. 2 µl of the supernatant was used for RNA-PCR amplification.

N. tabacum cv 'White Burley' leaves with distinct TRV symptoms were used for sample preparation. Leaf material (0.5 g) was thoroughly ground with 500 µl of H_2O in a glass potter tube. The homogenized leaf suspension was transferred to a microfuge tube and nucleic acids were extracted with an equal volume of phenol at 65 °C. The water phase was subjected to phenol/chloroform (1:1) extraction followed by chloroform extraction. 2 µl of the supernatant was used for cDNA synthesis and PCR.

Nucleic acid extraction from nematodes

Two methods were used to extract nucleic acids from nematodes. In the first method, 200 µl of a nematode suspension (containing distinct numbers of 1–200 of *T. primitivus*) was transferred into a microfuge tube which contained 12 glass beads with a diameter of 3 mm. The microfuge tube was placed on a shaker for 6 minutes to squash the nematodes and to obtain a completely homogenized solution. Nucleic acids were extracted from the suspension by phenol extraction with 500 µl phenol of 65 °C. Subsequently the aqueous phase was subjected to phenol/chloroform (1:1) and chloroform extraction. 2 µl of the aqueous phase was used for cDNA synthesis and subsequent DNA amplification.

The second method involved the use of liquid nitrogen to grind the

nematodes. 200 µl of a nematode suspension (1–200 *T. primitivus*) was transferred into a microfuge tube and centrifuged for 2 min at 14000 rpm. Excess water was carefully removed and 0.1 g of sand was added to the nematode pellet. The microfuge tube was incubated in liquid nitrogen for 2 min and the nematodes were ground using a steel rod precooled in liquid nitrogen. A mixture of 200 µl of H_2O and 500 µl of phenol preheated at 65 °C was added to the microfuge tube and incubated for 3 min at 65 °C. The sample was vigorously vortexed. After centrifugation the aqueous phase was removed and subjected to phenol/chloroform (1:1) and chloroform extraction. 2 µl of the aqueous phase was used for cDNA synthesis and subsequent DNA amplification.

Reverse transcription and PCR

The nucleotide sequences of the genomic RNAs of the TRV isolates PSG [Cornelissen et al., 1986], TCM [Angenent et al., 1986], SYM [Hamilton et al., 1987] and PLB [Angenent et al., 1989] were compared using the computer programs GAP and Bestfit [Devereux et al., 1984]. Two sets of primers were chosen in conserved regions located at the 3′ half of the RNA-1 in these isolates. One primer set consisted of pTRV1A (5′-CACCCCCCAATAATCTCTTAGCC-3′) for reverse priming and pTRV1B (5′-GAAGACGATTGAGGCGAAGTATG-3′) for forward priming. For nested PCR, a second primer set consisted of pTRV3A (5′-GTCGGCC AAACGCCAATCTC-2′) just upstream from pTRV1A for reverse priming and pTRV3B (5′-GTCCTTACAGACCAGCTAC-3′) just downstream from pTRV1B for forward priming.

cDNA synthesis and DNA amplification were carried out using the GeneAmp RNA-PCR kit provided by Perkin Elmer Cetus, essentially as described in the suppliers manual. cDNA was synthesized using a downstream primer at a concentration of 0.75 µM. The samples were incubated in a thermal cycler (Perkin Elmer Cetus) programmed for one cycle of 15 min at 42 °C, 5 min at 99 °C and 5 min at 5 °C. The synthesized cDNA was amplified using a downstream and an upstream primer each at a concentration of 1.5 µM and with 4 mM $MgCl_2$ in the reaction mixture. The samples were overlaid with mineral oil and incubated for one cycle of 1.5 min at 94 °C, 2 min at 55 °C and 3 min at 72 °C. This incubation was followed by 34 cycles of 1 min at 94 °C, 2 min at 55 °C and 3 min at 72 °C. After a subsequent incubation of one cycle of 1 min at 94 °C, 2 min at 55 °C and 5 min at 72 °C the samples were subjected to a final incubation at 4 °C.

DNA amplification was carried out using the GeneAmp PCR kit with AmpliTaq DNA polymerase provided by Perkin Elmer Cetus, according to the manual. A downstream and upstream primer were used at a concentration of 1.5 µM each. The samples were overlaid with mineral oil and DNA amplification was performed using the conditions mentioned above. To

visualize the PCR products, 10% of the PCR reaction mixture was loaded onto a 1% agarose gel.

Cloning of the PCR product

PCR products were cloned using the TA Cloning system (Invitrogen). RNA was extracted from purified virus particles as described above and subjected to 25 RNA-PCR amplification cycles. One percent of the PCR product, containing approximately 10 ng of DNA, was ligated into the pCR1000 vector and further handled as described in the TA cloning kit manual. In order to isolate the cloned fragment, the vector was digested with the restriction endonuclease NotI and the mixture was separated on a low melting point agarose gel. A DNA fragment of 750 nucleotides, representing the cloned fragment, was excised from the gel and the agarose fragment was melted at 65 °C. The DNA was isolated by subjecting the melted agarose to a 65 °C phenol, phenol/chloroform (1:1) and a chloroform extraction. After ethanol precipitation the DNA was resuspended in H_2O and stored at –20 °C.

Dot blot hybridization

The TRV cDNA probe was randomly labelled with digoxigenin-dUTP using a commercially available kit (Boehringer Mannheim) and according to the manufacturers instructions.

DNA was spotted onto a nylon membrane (Hybond-N, Amersham) and cross-linked under UV-light for 5 min. The membrane was prehybridized in hybridization solution (5 × SSC, 1% (w/v) blocking reagent, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS) and subsequently hybridized with the digoxigenin (DIG) labelled DNA probe according to procedure of the nucleic acid detection kit (Boehringer Mannheim). The membrane was treated with anti-DIG antibody-AP-conjugate and AMPPD solution in order to visualize the DIG labelled DNA probe by luminography [Kreike et al., 1990].

Estimation of the threshold for detection in nematodes

Throughout the studies the same viruliferous population of *T. primitivus* was used. In a transmission bio-assay, as described by Brown et al. [1989], only one out of 50 single nematodes from this population transmitted TRV. The probability of this observation at various fractions (F) of viruliferous nematodes in the population, as calculated according to the binomial distribution, is less than 0.01 at fractions larger than 0.12.

The sensitivity of the PCR method for detection of TRV in nematodes, expressed as the number of viruliferous individuals required for detection, was evaluated by testing various amounts of nematodes from a viruliferous

population. The probability (P) of detection by PCR in samples of the size used was calculated binomially, assuming fractions (F) of viruliferous nematodes in the population between 0.005 and 0.120, a range of PCR detection levels (T) and equal amounts of virus in each viruliferous nematode. The likelihood of the observed set of detections and non-detections, including the bio-assay results, was calculated by multiplying the probabilities of all events within the set for each combination of F and T (P for detection, 1-P for non-detections). T is the parameter of interest, whereas F is considered an unknown nuisance factor. The bioassay results are neutral with respect to the role of T in the likelihood distribution. Likelihood theory was used to estimate the probable values of T. Although this theory has been derived for continuous parameters, and approximate one-sided 95% confidence interval for T was constructed on the basis of likelihood ratio's [Cox and Hinkley, 1974].

Results

Design of primers and optimizing the conditions for detection of TRV by PCR

Both the complete sequence of the RNA-1 of TRV strain SYM [Hamilton et al., 1987] as well as large parts of the 3′ terminal sequences of the RNA-1 of three other TRV strains, PSG [Cornelissen et al., 1986], TCM [Angenent et al., 1986] and PLB [Angenent et al., 1989] have been determined. The sequences show an extreme high homology in their immediate 3′ terminal ends. Two TRV-specific primers were designed by comparing the genomic nucleic acid sequences of the RNA-1 from the different TRV strains. The upstream primer pTRV1B is complementary to nucleotides 1258 to 1280 of (the published 3′ terminal part of) the RNA-1 of the TRV strain PSG and the downstream primer pTRV1A for reverse priming is identical to the bases 1976 to 1998 of the PSG RNA-1. Using purified TRV-PSG genomic RNA as template, the optimal reaction conditions for cDNA synthesis and PCR were determined. The optimal annealing temperature, primer and magnesium concentrations for PCR proved to be 55 °C, 1.5 μM and 4 mM $MgCl_2$, respectively. Employing these conditions, as described in Materials and methods, a product of the expected size of 741 bp was produced.

Detection of different TRV isolates in leaf material

To determine if the method developed was suitable for the detection of different TRV strains and isolates in infected lead material, the method was tested using seven different TRV isolates. Reverse transcription and amplification (RNA-PCR) of viral RNA segments present in leaves infected with

TRV isolates PRN, PLB, PSG, PNP, Lisse Hoof, Baexem or Wageningse Eng, using the primer set pTRV1A/1B, resulted for all isolates in PCR products of approximately 741 bp which could be clearly visualized on an ethidium bromide stained agarose gel (Fig. 1). No amplification products could be detected in the control reactions, H_2O- or 'mock'-inoculated leaf material (Fig. 1).

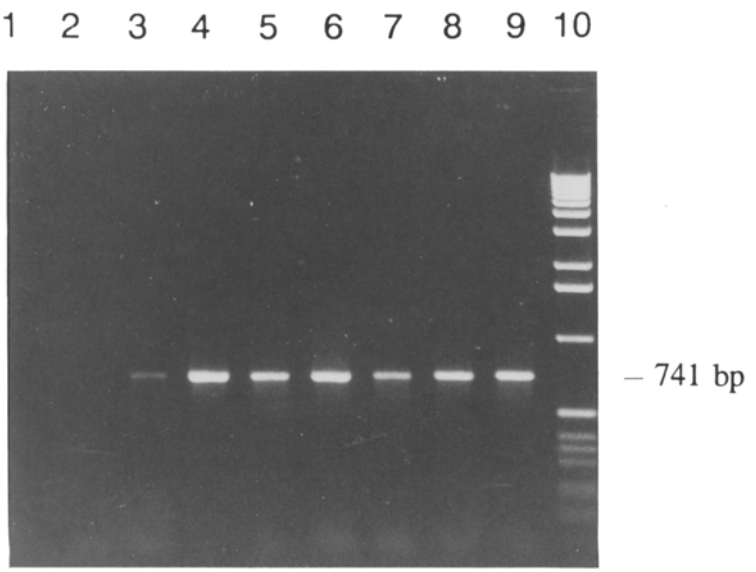

Fig. 1. Reverse transcription and amplification of viral RNA in infected plant material. Lane 1: water control; lane 2: mock inoculated plants material; lane 3 to 9: plants infected with various TRV isolates, PSG, Baexem, Wageningse Eng, Lisse Hoof, PNP, PRN, PLB, respectively; lane 10: DNA marker.

Detection of TRV in viruliferous nematodes

Different methods to isolate nucleic acids from nematodes were tested. The tested procedures involved among others the use of guanidine, phenol, liquid nitrogen and purification columns. Two of the tested methods proved to be useful for the detection of TRV in nematodes using PCR. In the first method the nematodes were ground in liquid nitrogen followed by a phenol extraction to purify the nucleic acids. In the second method, the nematodes were squashed with the help of glass beads and a shaker, followed by phenol extraction of the suspension. Although both methods were effective, the method using glass beads proved to be easier in handling and was used in further experiments.

Reverse transcription and amplification of TRV RNA sequences in nucleic acid extracts derived from both viruliferous and non-viruliferous nematodes resulted in several PCR products of lengths other than the expected product size of 741 bp. Hence, it was concluded that these PCR products were not specific for TRV RNA and that a more specific amplification was required. A second primer set pTRV3A/pTRV3B, located internal to primer set pTRV1A/1B, was introduced into the PCR procedure. The primer pTRV3A was located, upstream from the primer pTRV1A, at position 1865–1884 on the TRV PSG RNA-1, while primer pTRV3B was identical to the nucleotides 1284 to 1302 of the PSG RNA-1, directly downstream to primer pTRV1B.

Reverse transcription of RNA extracts, derived from viruliferous and non-viruliferous nematode populations, were followed by amplification, using the primer set pTRV1A/1B. Subsequently, five percent of the total PCR reaction mixtures was used as templates in a second PCR procedure, using the second primer set pTRV3A/3B. This resulted in the production of only one PCR product of the expected size of 601 bp. No PCR products were observed in the control reactions applied to the nucleic acid extracts derived from the non-viruliferous nematode populations (Fig. 2). Hence, it

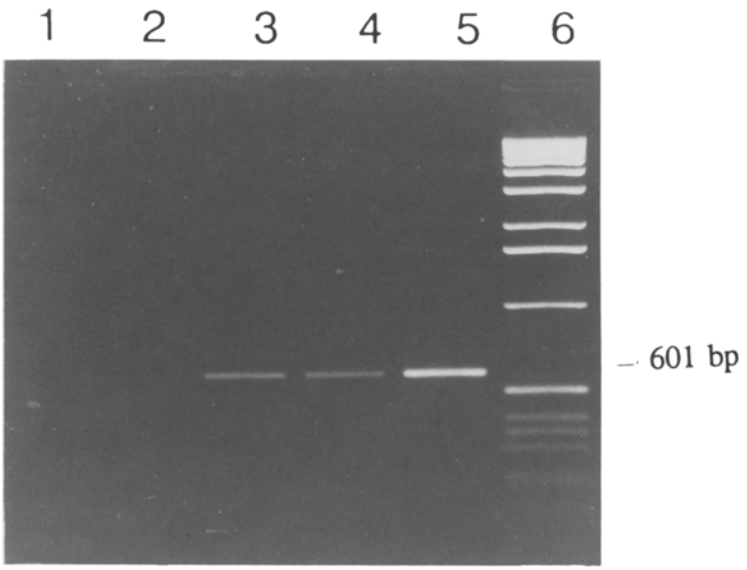

Fig. 2. PCR products after amplification of cDNA synthesized to viral RNA present in nucleic acid extracts derived from nematode populations. Lane 1: water control; lane 2: 100 non-viruliferous nematodes; lane 3 to 5: viruliferous *T. primitivus* population, 50, 75, 100 nematodes, respectively; lane 6: DNA marker.

was concluded that the use of two primer sets instead of one, resulted in a more specific amplification of viral RNA sequences.

Sensitivity of the method

A range of decreasing concentrations of purified virus (TRV strain PSG) was subjected to RNA-PCR amplification using the procedure described above and using both primer sets pTRV1A/1B and pTRV3A/3B. After analysis of 10% of the final PCR reaction mixture on agarose gel, a single PCR product of the expected size could still be detected when 15 fg virus was subjected to cDNA synthesis and amplification (Fig. 3). The viral particles of TRV have molecular weights ranging between 49–50 $\times$ 10^6 (L) and 11–29 $\times$ 10^6 (S) [Robinson and Harrison, 1989]. Consequently, 15 fg of virus commensurates with 300 to 400 viral particles. To determine the sensitivity of the assay for the detection of TRV in nematode extracts, different numbers of *T. primitivus* nematodes obtained from a TRV transmitting population were tested. RNA-PCR tests on 25 samples of 1 to 600 individuals of *T. primitivus* from the viruliferous population gave variable results (Table 1), as was expected from the low number of viruliferous nematodes (1 out of 50) observed in individual transmission bio-assays.

Statistical analysis of the obtained results was carried out to determine the minimum number of viruliferous nematodes needed for the detection of

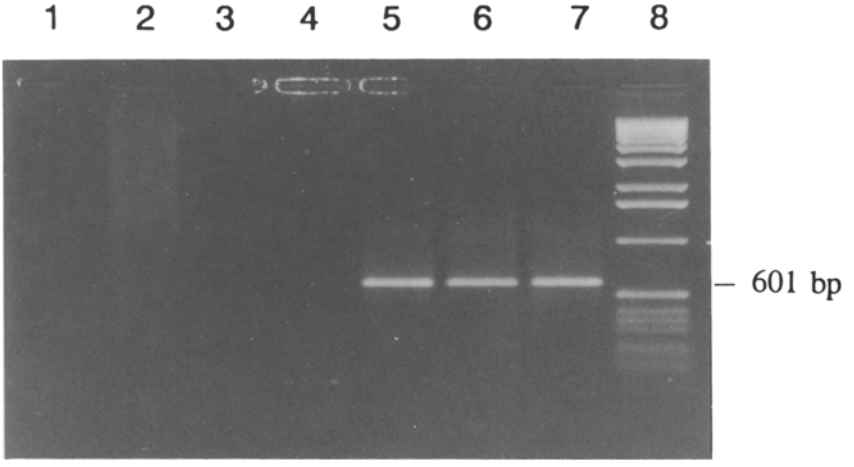

Fig. 3. PCR products derived from RNA-PCR tests performed on different amounts of purified virus. Lane 1 & 2: water controls; lane 3: 0.15 fg, lane 4: 1,5 fg; lane 5: 15 fg; lane 6: 0.15 pg; lane 7: 0.75 pg; lane 8: DNA marker.

Table 1. PCR detections of TRV from a population of *T. primitivus* in batches of different size, and expected values for the same batches at a fraction of 0.02 viruliferous individuals in the population and a detection threshold of 1 viruliferous individual

Number of *T. primitivus* per batch	Number of batches tested	– Number of detections –	
		Observed in PCR	Expected if F = .02 and T = 1
600	2	2	2.000
300	2	2	1.995
120	1	1	0.912
100	2	2	1.735
75	6	4	4.681
60	1	1	0.702
50	4	3	2.543
25	2	1	0.793
20	1	0	0.332
10	1	0	0.183
5	1	0	0.096
1	2	0	0.040

TRV in the PCR-based assay. The likelihood of the combined results of PCR and bio-assay for various combinations of F (fraction viruliferous) and T (PCR detection threshold) is given in Fig. 5. The maximum likelihood was found at T = 1 (F = 0.02), and the 95% confidence interval included T = 1, 2 and 3 viruliferous nematodes.

Dot blot hybridization

For routine analysis of large numbers of RNA-PCR samples agarose gel electrophoresis is laborious and difficult to perform. Therefore, we tested a non-radioactive dot blot hybridization procedure, which allows handling of large numbers of PCR samples. A DNA fragment resulting from reverse transcription of the RNA-1 of TRV strain PSG, followed by amplification of the cDNA and employing the primer set pTRV1A/1B, was cloned and labelled with digoxigenin-dUTP. After the RNA-PCR procedure was carried out, 3 µl of the different total PCR samples were spotted onto a nylon membrane and hybridized with the digoxigenin labelled TRV cDNA fragment. As shown in Fig. 4, all tested amplification products of the different TRV strains or isolates reacted positively in the dot blot procedure.

Discussion

An assay for the detection of TRV by use of reverse transcription of the RNA-1, followed by amplification of the synthesized cDNA has been

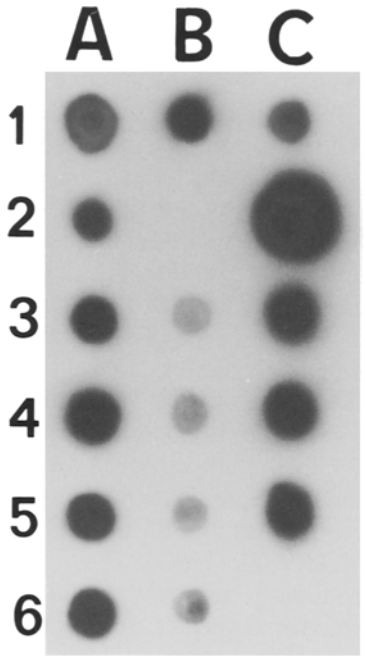

Fig. 4. Dot-blot hybridization test. PCR products derived from experiments using different viruliferous nematode populations or virus concentrations, were spotted onto a membrane and probed with a DIG-labelled cDNA fragment. A1 to A6: PSG, Baexem, Wageningse Eng, Lisse Hoof, PNP, PRN, respectively; B1 to B6: PLB, 100 non-viruliferous nematodes, 35, 37, 61, 73 nematodes (Baexem), respectively; C1 to C6: 5 pg, 500 pg, 100 pg, 50 pg, 25 pg purified virus, H_2O control respectively.

developed. The sensitivity of the assay for the detection of purified TRV has been determined to be 15 fg of purified virus, well below the detection limits of ELISA procedures. Detection of TRV by serological methods is hampered by the occurrence of different serotypes and NM-type isolates. A PCR-based assay allows both the detection of different TRV serotypes and the detection of non coat protein producing TRV strains [Robinson, 1992].

The procedure described in this paper permits the direct detection of TRV in viruliferous nematodes. Until now direct detection of TRV in low numbers of nematodes has shown to be impossible. Therefore, TRV in the vector nematodes is usually detected in an indirect manner by transmission of the virus to bait plants. This indirect detection procedure is time and expertise demanding, expensive and unsuitable for handling large numbers of samples.

With the PCR-based assay large numbers of samples can be processed in a short time span, thus enabling the survey of fields for the presence of viruliferous nematodes. The detection threshold of the PCR method, as determined from probability analysis, is most likely one or two viruliferous nematodes (Fig. 5) and possibly three ($p < 0.05$). Hereby it is assumed that all viruliferous nematodes carry the same amount of TRV, and that all are able to transmit virus in the individual bio-assay. Unequal distribution of virus over the viruliferous nematodes is expected to make higher thresholds only slightly more likely, because of the large number of samples and nematodes tested. The occurrence of considerable numbers of viruliferous nematodes that do not transmit virus in the bio-assay is not very plausible, as Ploeg et al. [1992] demonstrated the high efficiency of this test. The number of TRV particles required for detection by PCR is less than 1 % of the theoretical maximum present in the pharynx and oesophagus of one individual vector nematode (Calculation based on packing and dimensions as published by Taylor and Robertson [1970]. However, field samples frequently contain more than one trichodorid species, only one of which may be able to transmit TRV, but all of which are equally likely to have fed on infected plants and therefore contain virus. Thus, an individual nematode that gives a positive result with this PCR test is not necessarily a potential transmitter of TRV.

TRV can cause severe damage to many agricultural crops especially potatoes and bulbous ornamental species. At this moment, in the bulb-

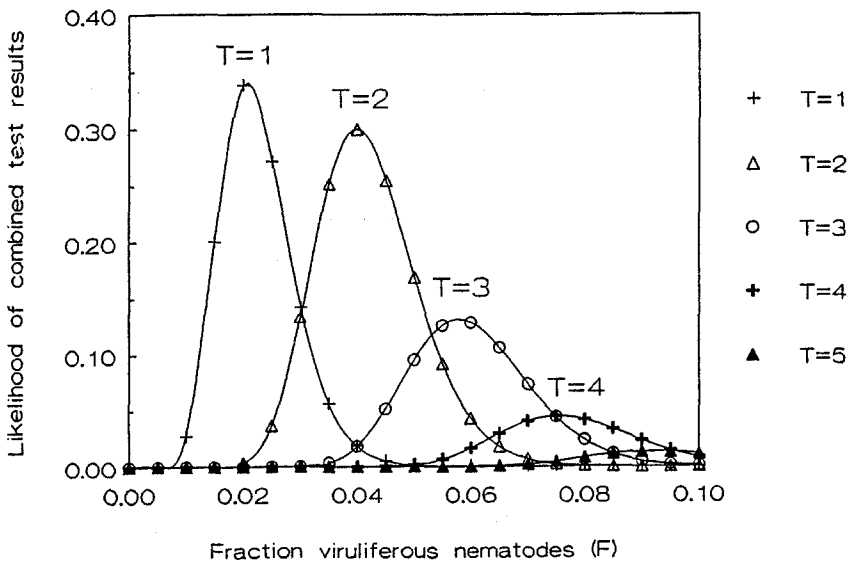

Fig. 5. Likelihood of the set of results of PCR and bio-Assay calculated to combinations of the fraction viruliferous nematodes (F) and the PCR detection threshold (T); (likelihood values multiplied by 10000).

growing and seed potato industry in the Netherlands the presence alone of *Trichodoridae* can be a reason for chemical disinfestation of the soil. Survey of fields for the presence of TRV could possibly lead to a substantial reduction of the use of chemicals.

It is known that non-vector nematodes may take up TRV from plant roots while feeding [Van Hoof, 1976] and thus the detection of TRV in nematode suspensions from soil by itself does not imply the presence of trichodorid vector nematodes. Furthermore, the presence of TRV in plants may be due to seed-transmission [Lister and Murant, 1967; Cooper and Harrison, 1973] or planting of infected material. It therefore seems most practical to conduct a PCR-based assay on nematode suspensions in which *Trichodoridae* were found by microscopic observation. TRV serotypes are transmitted rather specifically by different vector species [Ploeg et al., 1992]. Moreover, there are differences between TRV strains in virulence, symptomatology and other host relations. Therefore, extension of our method towards discrimination of different strains or genotypes of TRV by restriction analysis of the PCR products [Robertson et al., 1991], combined with specific identification of trichodorid nematodes, might improve the evaluation of the potential risk of TRV transmission to certain crops.

Acknowledgements

The authors wish to thank J. de Bree for help with statistical analysis and A.T. Ploeg for helpful discussions and critical reading of the manuscript. This work was supported by the Ministry of Agriculture, Nature Conservation & Fisheries in the framework of the Multi-Year Crop Protection Plan 1990–2000 (MJP-G) and by the Urgency Programme for Flower bulb Breeding and Disease Research.

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