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Short communication

The use of collagenase to improve the detection of plant viruses in vector nematodes by RT-PCR

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A B S T R A C T

Tomato ringspot virus (ToRSV), *Tobacco ringspot virus* (TRSV) and *Tobacco rattle virus* (TRV) are transmitted to healthy plants by viruliferous nematodes in the soil. A method was developed for extraction of genomic viral RNA from virus particles carried within nematodes and a sensitive nested RT-PCR detection assay. The procedure has been adapted to microscale for handling multiple samples. This assay is effective for detection of ToRSV or TRSV in *Xiphinema americanum* or TRV in *Paratrichodorus allius*. With this method, viruses can be detected in nematodes fed on infected plants or from field-collected nematodes where the percentage of viruliferous nematodes is unknown. Soil samples from four red raspberry fields infected with ToRSV were collected in 2003 and 2004. Nematodes isolated from these samples were assayed for ToRSV by RT-PCR and compared to cucumber baiting bioassay for virus transmission from the same soil samples. ToRSV was detected in nematodes throughout the season with similar frequencies by the RT-PCR assay and the transmission bioassay.

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Longidorid ectoparasitic nematodes of the species *Xiphinema americanum*, also known as "Dagger nematodes" feed on the roots of a broad range of plants. Although feeding by these nematodes can cause stunting of some plants, e.g. maple (Di Sanzo and Rohde, 1969), they do not cause significant direct damage to raspberry plants (McElroy, 1992). These nematodes can cause plant damage at very low population levels, however, by transmitting viruses. During feeding, *X. americanum* can acquire and subsequently transmit nepoviruses, such as *Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus* (TRSV) and *Cherry rasp leaf virus* (Brown et al., 1994a). In this process, virus particles ingested with plant cytoplasm adhere to cuticular lining of the esophagus and lumen of the stylet (Brown et al., 1994b; Wang et al., 2002). The specificity of transmission among different nepoviruses and nematode vectors (Brown et al., 1995; Wang et al., 2002) is hypothesized to be related to affinity of virus coat protein for specific carbohydrate moieties in the glycoproteins lining the wall of the esophagus (Brown et al., 1995). Upon initiation of feeding, the nematode injects a digestive secretion from the dorsal esophageal gland, which results in some of the virus particles bound to the esophagus being injected into the plant cell. These injected particles can replicate and cause an infection of the plant (Brown et al., 1995; Wang et al., 2002). *X. americanum* has been reported to acquire particles of ToRSV within 1 h of feeding on roots of infected plants and can transfer particles of ToRSV within 1 h of feeding on a healthy plant (Téliz et al., 1966). Viruliferous nematodes that were stored in soil without host plants could transmit ToRSV for up to 2 years (Bitterlin and Gonsalves, 1987). The esophagus and virus are shed with each molt, thus each nematode life stage must feed on a virus-infected host to reacquire the virus (Brown et al., 1995).

Tobacco rattle virus (TRV) is the type member of the genus *Tobravirus*, which includes bipartite, rod-shaped particles with a positive-sense RNA genome. Tobraviruses can be seedborne, but are primarily soilborne and transmitted by several species of trichodorid nematodes in the genera *Paratrichodorus* and *Trichodorus* (Robinsson, 2003). TRV is transmitted by *Paratrichodorus allius*, the stubby root nematode. The virus is attached to esophageal lining and thought to be transmitted as the nematodes egest saliva during feeding in a manner very similar to the nepovirus transmission by *Xiphinema* spp. There is no evidence that the virus multiplies in the nematode vector or that it is retained during molts or transmitted through eggs, though it can be retained by quiescent, non-feeding nematodes for many months. The viruses can have a reasonably

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broad host range and be maintained in weeds during crop rotations if care is not taken to control specific weed hosts. There does appear to be considerable specificity between viruses or virus strains and transmission by a given nematode species. Crop rotation with a non-host for the virus can be an effective means of controlling TRV provided that weed hosts of the virus are controlled (Mojtahedi et al., 2003).

A procedure to extract viral RNA from nematodes must take into account that soil-inhabiting nematodes have evolved to survive extremes in soil structure, moisture, temperature, and salinity. This is accomplished by a complex, tough cuticle that serves as a barrier to the environment, and as a hydrostatic skeleton, against which muscles contract. This cuticle also covers the stylet and stylet extension of the nematodes, and lines the digestive tract. The outermost layer of the cuticle is made primarily of collagen, a cross-linked protein secreted by cells of the inner layers of the cuticle (Johnstone, 1994). Thus, to detect plant viruses carried internally by these nematodes, the nematode cuticle must be disrupted by rather severe means. Previously, researchers have disrupted nematode cuticles mechanically by micro-dissection (Boutsika et al., 2004) or by vortexing for 5 min in the presence of phenol and glass beads (Finetti-Sialer and Ciancio, 2005; Aurelio Ciancio, ciancio@area.ba.cnr.it, personal communication) and these methods were evaluated for the detection of ToRSV in *X. americanum*. None of these methods were suitable for detection of ToRSV in *X. americanum*. Collagenase, a commercially available enzyme (Huang et al., 2004), was evaluated as means to more effectively and efficiently disrupt the cuticle of nematodes and to improve the extraction of viral RNA from nematodes. This communication describes the development of an RT-PCR technique to detect viruses in several nematode vector–virus systems, compare its sensitivity with plant bioassays, and estimate how robust these tests are as diagnostic tools to determine the presence of viruliferous nematodes in field soil.

ToRSV, TRSV and TRV are bipartite viruses, which are important when optimizing detection assays. Four strategies were considered when designing primers to maximize the sensitivity of the RT-PCR detection assays: (1) design a specific primer for use in the RT reaction (versus using random primers) from the 3′ end of the genomic RNA; (2) design this RT primer to bind to identical sequences present in the 3′ non-coding regions of both RNA 1 and RNA 2 molecules of the virus; (3) design PCR primers that bind to identical sequences present in the 3′ non-coding regions of both RNA 1 and RNA 2 and (4) design nested PCR primers within the first set of primers to increase the sensitivity. For ToRSV, CLUSTALW (Thompson et al., 1994) was used to generate a multiple sequence alignment of various isolates of ToRSV to identify regions of RNA 1 and RNA 2 that were identical. An RT primer and several pairs of primers were selected in the regions of identity and tested using RNA extracted from ToRSV-infected raspberries. A similar process was followed to identify identical regions of TRSV RNA 1 and RNA 2. The RT primer for TRV was the "universal Tobravirus cDNA synthesis primer" (Boutsika et al., 2004). TRV PCR primers were designed from the sequence of RNA 1 (accession # X06172) because of the variability observed among RNA 2 molecules of different isolates (Sudarshana and Berger, 1998).

To have nematodes available year-round, a pot culture was developed in the greenhouse of *X. americanum* feeding on Sudangrass (*Sorghum vulgare* var. *sudanense* Hitchc.). To establish this culture, nematodes were collected from Benton County, Oregon, an area free of ToRSV and known to have populations of *X. americanum*. Identity was confirmed by morphometric analysis. Nematodes were hand-picked then transferred to 23 in. pots containing Sudangrass growing in a 2:1 mixture of Willamette sandy loam and sand. The pots were watered daily and populations monitored. For acquisition of ToRSV, soil from the Sudangrass culture was suspended in water. This slurry was decanted through a coarse screen (1 mm). The sieved slurry was decanted through a fine screen (90 μm) and the retentate then applied to a Baermann funnel. After overnight incubation, nematodes were collected from the funnels and added to ToRSV-infected raspberry plants. After a 10-day feeding period, nematodes were reisolated from the soil by the procedure described above. Individuals of *X. americanum* were hand-picked through two water washes. These nematodes were used to test RNA extraction protocols, and subsequently as positive controls in assays of field samples. Nematodes from field samples were prepared in the same manner.

Healthy and TRSV-infected cucumber plants infested with populations of *X. americanum* were obtained from R.C. Gergerich (University of Arkansas, Fayetteville, AR). Tubes containing 50 individuals of *P. allius* fed on healthy alfalfa or 50 individuals of *P. allius* fed on tobacco infected with TRV were provided by E. Riga (Washington State University, Prosser, WA).

Nematodes were hand-picked into 100 μl sterile H_2O in 1.5 ml microfuge tubes. Collagenase (Sigma C-5138, St. Louis, MO) was prepared at 10 mg/ml in 50 mM Tris, pH 7.4, containing 1 mM $CaCl_2$. After the addition of 100 μl of the collagenase solution, the tubes were incubated at 37 °C for 1 h. About 50 mg glass beads (acid-washed, 425–600 μm, Sigma G-8772) were added, then 200 μl 2× extraction buffer (400 mM Tris, pH 8.5, containing 600 mM LiCl, 20 mM EDTA, 3% lithium dodecyl sulfate, 2% deoxycholic acid, 2% tergitol with 2% β-mercaptoethanol added just before use). The closed tubes were vortexed at full speed for 1 min, then 400 μl 6 M potassium acetate pH 6.5 (6 M with respect to acetate and 4 M with respect to potassium) was added. The tubes were vortexed then chilled on ice 30 min. The tubes were then spun in a microfuge at maximum speed for 5 min. The supernatant was transferred to a new tube and 1 μl of glycogen (Invitrogen #10814-010, Carlsbad, CA) was added and mixed. Then an equal volume of isopropanol was added, mixed by inverting the tubes four or five times and then chilled for 30 min at −20 °C. Tubes were centrifuged for 10 min at maximum speed; the supernatant decanted and the RNA pellet was washed with 500 μl 70% EtOH and the final pellet dried under vacuum for 5 min. Pellets were dissolved in 20 μl Molecular Biology Grade (MBG) H_2O (Invitrogen) and put on ice for immediate use, or stored at −20 °C. This procedure was modified from those of Hughes and Galau (1988) and Spiegel and Martin (1993).

To prepare cDNA, 2 μl of the RNA preparation were used in a 20 μl reverse transcription reaction in a 200 μl tube as follows: combine 10.2 μl MBG H_2O, 4 μl 5× first strand buffer, 1 μl 0.1 M DTT, 1 μl 10 mM each dNTP mix, 0.7 μl RnaseOUT (Invitrogen), 0.4 μl of 10 μM primer and 0.7 μl Superscript III (Invitrogen). The tubes were incubated at room temperature for 1 min, then 50 °C for 60 min. One microliter of the reverse transcription reaction was used as a template in a 50 μl polymerase chain reaction (PCR) using the first primer pair for the specific virus and *Taq* polymerase (Invitrogen) as specified by the manufacturer except that primer concentrations used were five times greater than specified. Cycling conditions were as follows: 2 min at 94 °C, followed by 40 cycles of [40 s at 94 °C, 40 s at 56 °C and 1 min at 72 °C] then 4 min at 72 °C. For the nested PCR, 1 μl of first PCR tube was used as template in a 50 μl reaction, with the same parameters and cycling conditions as for the first PCR except the nested primer pair was used. Fifteen microliters of the nested PCR reaction mixture was analyzed by agarose gel electrophoresis (Sambrook and Russell, 2001). Although PCR products from RNA extracted from plant leaves could be detected after the first PCR, amplicons were never detected with RNA templates from nematodes in the first PCR, but always in the nested PCR.

A PCR product was generated from ToRSV cDNA that used a forward primer internal to the RdRP gene of RNA 1 of ToRSV (GCC-

Table 1
Primers used for RT-PCR detection of viruses in nematode vectors.

Virus and primer sequence		Nucleotide start	Primer name	Expected size (bp)
Tomato ringspot virus[a]				
RT primer	GCGAAAACAACGTCCTTGC	8112	ToRSVrt	
1st PCR F	CCGTTAGCAGCTTCCAAAAG	6695	ToRSVf1	512
1st PCR R	GTCCTCATGGAACCTTTCTC	7187	ToRSVr1	
2nd PCR F	GGTTATCCAGCCTTAAGCAAG	6742	ToRSVf2	435
2nd PCR R	CGGTAGGCTATGACAACTAC	7157	ToRSVr2	
Tobacco ringspot virus[b]				
RT primer	GTGTTGGACAAACACGACAC	7520	TRSVr1	
1st PCR F	GGAAGCTGTATAAACTCAGC	7202	TRSVf1	338
1st PCR R	GTGTTGGACAAACACGACAC	7520	TRSVr1	
2nd PCR F	GAGAGCCCAAGAATGTCTGTC	7232	TRSVf2	257
2nd PCR R	GAACAGTGGGCTCAAACAAC	7469	TRSVr2	
Tobacco rattle virus[c]				
RT primer	GGGCGTAATAACGCTTACG	7272	TRV-RT[d]	
1st PCR F	AGGAGGAAGAGAGACCGAAGTA	5421	TRVf1	763
1st PCR R	CTCGTGACCAAGAACAGTGA	6164	TRVr1	
2nd PCR F	CACAGAAGAAGAGAAACTGTC	5445	TRVf2	707
2nd PCR R	AACCCTTGAGTACACACGTC	6132	TRVr2	

[a] Nucleotide numbering is relative to Genbank accession L19655.
[b] Nucleotide numbering is relative to Genbank accession U50869.
[c] Nucleotide numbering is relative to Genbank accession AF166084.
[d] Sequence of the RT primer for TRV from Boutsika et al. (2004).

CATCTTGAAGGTGGTTAC) and the ToRSVrt reverse primer. This PCR product was cloned and used to generate a ToRSV RNA 1-specific sequence. Also, PCR products derived from primers ToRSVf1 and ToRSVr1 were cloned into pCR4Topo (Invitrogen) and sequenced. These sequences were compared to the RNA 1 sequence to determine if the diagnostic PCR products were derived from RNA 1 and RNA 2. Similarly, a PCR product was generated from TRSV cDNA that used a forward primer internal to the coat protein gene of RNA 2 of TRSV (AACCTCCATGTTGTCCATATC) and the TRSVr1 reverse primer for TRSV. This PCR product was cloned and used to generate a TRSV RNA 2-specific sequence. TRSV diagnostic PCR products generated with primers TRSVf1 and TRSVr1 were also cloned and sequenced to determine if amplicons represented RNA 1 and RNA 2 templates. Finally, the TRV diagnostic PCR fragments generated with primers TRVf1 andTRVr1 were cloned and sequenced to confirm their identity.

Bioassay tests were conducted to determine indirectly the ability of nematodes in the soil to vector ToRSV. Soil was collected bi-monthly from each location, subdivided and 125 g was placed in each of six 5 cm pots. A cucumber seedling, newly germinated on moist paper towels, cultivar 'Straight eight', was planted in each pot. Pots representing each location were placed on individual fabric mats on a greenhouse bench and the mats were irrigated to maintain uniform soil moisture in the pots. Plants were grown for 8 weeks at 18–22 °C with 14 h of supplementary light (400 μmol m^2 s^{-1}) per day. Cucumber leaves were collected and the root systems were washed free of soil and both tissues were tested for ToRSV with a double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA, Clark and Adams, 1977). The proportion of cucumber plants grown in soil from each plot that became infected and the proportion of plots in which ToRSV was detected were calculated for each sampling period.

Four red raspberry fields from four separate farms with plants symptomatic of ToRSV were identified in southwest Washington State. Leaves were collected from plants in the areas in which soil samples were to be collected and tested by ELISA to verify the presence of ToRSV. Soil samples were collected every 2 months from three areas sampled in one field, two areas in two fields, and one area in the fourth field. Soil was bioassayed and population densities of *X. americanum* were determined as described above. Individuals of *X. americanum* were hand-picked and transferred twice through sterile water to ensure that the nematodes were free of plant fragments. Replicate lots of 10 nematodes were transferred to 100 μl of sterile water in a 1.5 μl microfuge tube, treated with collagenase, and then RNA was extracted and RT-PCR done as described above.

RNA extracted from virus-infected plants served as an effective substrate for reverse transcription and nested PCR using the primer sets listed in Table 1 (data not shown). Twenty-five clones of ToRSV diagnostic PCR products from four separate reactions were sequenced. Eleven of the cloned diagnostic PCR fragments had sequences identical to that of RNA 1, as determined by the sequence of cloned PCR products that spanned the middle of the RdRp gene through the diagnostic reverse primer in the 3′ untranslated region. Fourteen of the cloned diagnostic fragments were identical to each other but were in a different cluster than the RNA 1 clones when analyzed with CLUSTAL W. These 14 clones likely were derived from the RNA 2 of ToRSV. This shows that both RNA 1 and RNA 2 were amplified in the nested RT/PCR ToRSV assay.

Similarly, the sequence of a PCR product that spanned the region of TRSV RNA 2 from the coat protein gene to the 3′ reverse primer (TRSVr1) was used to provide an unequivocal TRSV RNA 2 sequence. Fourteen nested diagnostic TRSV PCR products from five separate reactions were cloned and sequenced. Four of the cloned PCR fragments had a sequence that was identical to that of RNA 1, as determined by the sequence of a 1.4 kbp PCR product that spanned from the coat protein gene of TRSV RNA 1 to the site of the TRSVr2 primer site, and 10 others that were identical to each other and had a slightly different sequence. This shows that both RNA 1 and RNA 2 were amplified in the nested RT-PCR TRSV assay. Nested PCR products derived from TRV RNA were cloned, sequenced and determined to be very similar to TRV database sequences, confirming the identity of the TRV nested PCR products.

Nematodes from the Sudangrass culture that subsequently were fed on ToRSV-infected raspberry plants were treated with collagenase as described. Microscopic examination of collagenase treated nematodes showed no physical changes. Nevertheless, collagenase treatment was required to allow disruption by glass beads as assayed by the nested RT-PCR assay for ToRSV (Fig. 1). Similarly, collagenase treatment alone did not effectively disrupt the nematodes.

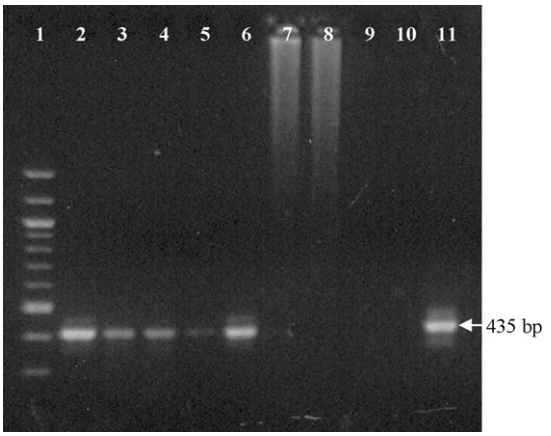

Fig. 1. Detection of ToRSV in *Xiphinema americanum*. Lanes 2–5 and 7–10 RNA from 10, 25, 50 and 60 nematodes collected from root zones of ToRSV-infected plants, respectively. Lanes 6 and 11 RNA from infected plants. Lanes 2–6 samples were pretreated with collagenase prior to RNA extraction whereas samples for lanes 7–11 were not. Lane 1 is 100 bp DNA ladder.

Nested RT-PCR was required to detect ToRSV in nematode preparations regardless of the number of nematodes used (not shown). Comparison of lanes 6 and 11 in Fig. 1 shows that collagenase did not reduce the detection of ToRSV from plant tissues.

Roberts and Brown (1980) estimated that the number of virus particles carried by individuals of various species of *Xiphinema* ranges from 5 to 1300. The smallest number of nematodes that produced a detectable PCR product was 10. Invitrogen suggests that 10,000 copies of the original template are required to detect a PCR product in 30 cycles. Thus, in these 10-nematode RT-PCR reactions, the predicted 50–13,000 viral particles are being detected very efficiently. Thus, the parameters for the RNA extraction and RT-PCR are optimal. The detection of TRSV using the nested RT-PCR assay is shown in Fig. 2A, with lanes 4 and 5 showing the amplicons from 10 and 50 nematodes, respectively, which has a comparable intensity to that obtained from TRSV-infected cucumber leaves (lane 7). Lane 2 in Fig. 2B shows the 707 bp nested RT-PCR amplicons from viruliferous nematodes compared to TRV-infected tobacco (lane 4). The experiments were repeated with a second set of nematodes and a representative experiment shown.

It was possible to detect ToRSV in field-collected viruliferous nematodes throughout the year with the cucumber bioassay and the RT-PCR assay. ToRSV was detected in 66% of the samples by the bioassay and RT-PCR. The two assays were compared for all soil samples. In 17.7% of cases, the RT-PCR assay detected ToRSV in soil samples that were negative in the bioassay. Conversely, in an equal percentage of cases, ToRSV was detected with the bioassay from samples that were negative with the RT-PCR assay. There was no correlation between the frequency of ToRSV detection in nematodes by cucumber bioassay or the RT-PCR test ($R^2 = 0.08$). Therefore, the cucumber bioassay and the RT-PCR assay failed to detect ToRSV in viruliferous nematodes about 18% of the time. Population densities of *X. americanum* were lower throughout the year (<20 per 250 g soil) in these fields than compared to a previous study (Pinkerton et al., 2008), but densities followed the same seasonal trends (data not shown). There was a low correlation between the nematode population density and the frequency of bioassay-positive plants ($R^2 = 0.22$).

The development of techniques to evaluate soil for the presence of viruliferous nematodes before planting a susceptible crop has been one objective of this research. Other researchers have reported from 0% to 100% transmission of ToRSV to cucumber bait plants from field soil (Georgi, 1988; Rosenberger et al., 1983). In this study, ToRSV was detected in 47–97% of soil collected in raspberry field plots where ToRSV was known to occur using this assay depending on the time of year. Converse and Stace-Smith (1971) were less successful, with only 3.5% of cucumbers planted in soil from ToRSV-infected raspberry plantings becoming infected with the virus. Additionally, we developed an RT-PCR test that made it possible to detect ToRSV in lots of 10 individuals of *X. americanum* collected from soil with ToRSV-infected plants. It was determined that RT-PCR tests with hand-picked nematodes extracted from infested soils were as consistent in detecting ToRSV as the cucumber bioassays. Results of the two tests failed to agree in ~36% of cases, with each test giving a negative test in ~18% of the samples that tested positive in the other test. Failure to detect ToRSV in multiple replicates of 10 nematodes suggests that possibly the field populations contained low proportion of viruliferous nematodes.

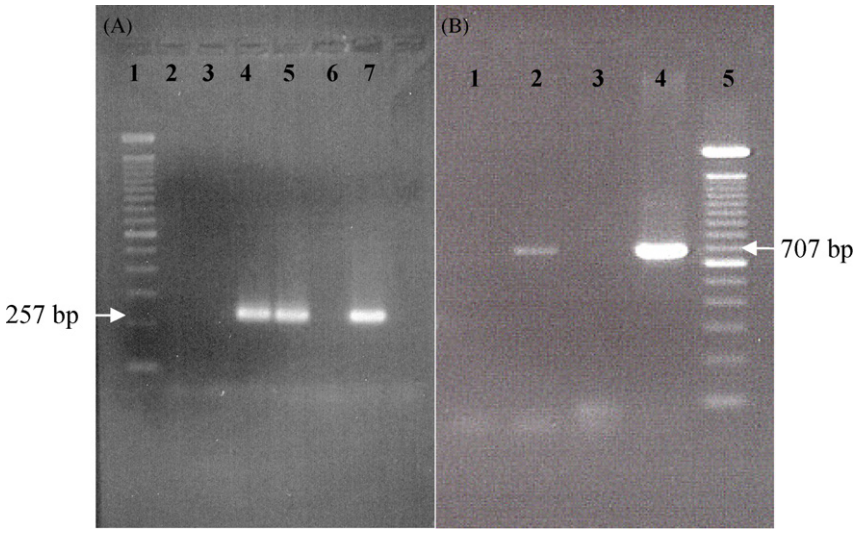

Fig. 2. Detection of TRSV and TRV in *Xiphinema americanum* and *Paratrichodorus allius* extracted using the collagenase. (A) TRSV RT-PCR assay. Lane 1, markers; lanes 2 and 3 are RNA extracted from 10 and 50 nematodes fed on healthy cucumber plants; lanes 4 and 5 are RNA extracted from 10 and 50 nematodes fed on TRSV-infected cucumber plants; lanes 6 and 7 RNA extracted from healthy, TRSV-infected cucumber plants. The experiment was repeated with a second set of nematodes and a representative experiment shown. (B) TRV RT-PCR assay. Lane 1, 50 nematodes fed on healthy alfalfa; lane 2, 50 nematodes fed on TRV-infected tobacco; lane 3, no template; lane 4, RNA extracted from TRV-infected tobacco; lane 5, markers. The experiment was repeated with a second set of nematodes and a representative experiment shown.

Since soil samples were collected in plant rows of raspberry fields where the incidence of ToRSV was 100%, neither test appears to be sensitive enough for diagnostic purposes when viruliferous nematodes are in low population densities. In addition, hand-picking nematodes and the RT-PCR process is time intensive, which limits it utility for diagnosis.

The amount of virus retained by nematodes appears to be very different for various virus nematode combinations. For example, *X. americanum* retains TRSV in the stylet and esophagus (Wang and Gergerich, 1998), whereas, ToRSV is retained only in the posterior part of the esophagus (Wang et al., 2002). Based on the fluorescence observed it appears that much more TRSV is retained by nematodes than ToRSV. They concluded that ToRSV is not as tightly bound as TRSV since ToRSV appears more transient in nematodes. Thus, with a lower titer in the nematodes from the field it is less likely to be detected by either RT-PCR or the bioassay. In contrast, Grapevine fanleaf virus (GFLV) was observed in single particle layers lining the lumen of the odontophore and anterior esophagus (Taylor and Robertson, 1970), appearing to be more similar to TRSV in the amount of virus bound than ToRSV. This is in agreement with the high rate of detection of GFLV in *Xiphinema index* (Demangeat et al., 2004).

The utility of PCR for detection of ToRSV in nematodes or in soil is equivalent to the bioassay using cucumbers. The failure to detect ToRSV with RT-PCR or bioassay in 18% of the samples collected from soil around the crowns of ToRSV infect raspberry plants should not be surprising. It is possible and likely that a percentage of the *X. americanum* nematodes in the soil are not viruliferous and the detection level with either method gives an accurate assessment of the risk of having ToRSV in a field. This failure to detect ToRSV in all nematode samples is not surprising but does emphasize the importance of analyzing multiple samples from a given field. As soil fumigants are being lost as an option to control the nematode vectors of plant viruses other strategies need to be developed. Crop rotation with non-hosts for the virus 'to flush the virus out of the system' can be a promising option when nematode populations are low enough that they are not causing significant crop damage in the absence of virus (Pinkerton and Martin, 2005; Mojtahedi et al., 2003). There are drawbacks to the bioassay or the RT-PCR assay to assess the risk of nematode transmitted viruses prior to planting. The costs of both assays are high due to the number of samples that need to be processed. The cucumber bioassay requires at least a month to complete and access to greenhouse space. The RT-PCR assay described here takes considerably less time, but requires the labor intensive step of hand-picking nematodes for the assay. Given the low titer of ToRSV in *X. americanum*, the routine detection of this virus in field soil will depend on the development of more sensitive technologies before it can be applied widely.

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