

OCCURRENCE OF *TOMATO RINGSPOT VIRUS* AND *TOBACCO RINGSPOT VIRUS* IN HIGHBUSH BLUEBERRY IN NEW YORK STATE

M. Fuchs¹, G.S. Abawi¹, P. Marsella-Herrick¹, R. Cox¹, K.D. Cox¹, J.E. Carroll^{1,2} and R.R. Martin³

¹Department of Plant Pathology and Plant-Microbe Biology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456, USA

²New York State Integrated Pest Management Program, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456, USA

³USDA-ARS, Horticultural Crops Research, Corvallis, OR 97330, USA

SUMMARY

A survey for viruses by DAS-ELISA of highbush blueberry (*Vaccinium corymbosum* L.) showing virus-like symptoms and decline in vigor, showed the occurrence of *Tomato ringspot virus* (ToRSV) and *Tobacco ringspot virus* (TRSV) in New York State (USA). The presence of the two viruses was confirmed in leaf samples by RT-PCR and IC-RT-PCR with appropriate primer pairs that amplify a 320 bp and a 585 bp fragment of the RNA-dependent RNA polymerase gene of TRSV and ToRSV, respectively. Comparative sequence analysis of viral amplicons of New York isolates indicated moderate (80.7-99.7%) and high (90.8-99.7%) nucleotide sequence identities with other ToRSV and TRSV strains, respectively. Analysis of soil samples from the root zone of blueberry bushes for the occurrence of dagger nematodes revealed the presence of *Xiphinema americanum*-group nematodes at low population densities. Cucumber bait plants potted in soil samples containing *X. americanum* became infected with ToRSV or TRSV in a greenhouse. Together, these findings show the occurrence of ToRSV and, to a lesser extent, TRSV in highbush blueberry plantings in New York, as well as of the vector *X. americanum sensu lato*.

Key words: blueberry, ToRSV, TRSV, nepovirus, *Xiphinema americanum*.

INTRODUCTION

Viral diseases are a worldwide problem of blueberry and a major limiting factor for production. Major blueberry viruses are *Blueberry leaf mottle virus* (BILMV), *Blueberry red ringspot virus* (BIRRV), *Blueberry scorch virus* (BIScV), *Blueberry shock virus* (BIShV), *Blueberry shoestring virus* (BISSV), *Peach rosette mosaic virus* (PRV), *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV) (Ramsdell, 1995a, 1995b). In the

spring of 2007, decline and virus-like symptoms were observed on mature highbush blueberry (*Vaccinium corymbosum*) in various plantings in New York State (USA). Symptoms consisted of stunted growth, shoot defoliation, top dieback, leaf distortion and cupping, and dark reddish lesions on apical leaves. Necrosis of leaf and flower buds were also noticed as well as reduced fruit yield. To advance our understanding of the nature of the decline, we carried out a survey of highbush blueberry plantings for the occurrence of viruses and their vectors. We report here on the presence and distribution of ToRSV and TRSV, genus *Nepovirus*, family *Secoviridae* (Sanfaçon, 2008; Sanfaçon *et al.*, 2009), and their nematode vectors *Xiphinema americanum sensu lato* in highbush blueberry plantings in New York.

MATERIALS AND METHODS

Blueberry plantings and sample collection. Twenty plantings of highbush blueberry of cvs Bluecrop, Collins, Herbert and Patriot were selected for this study in 14 farms in New York State. Collins is an early season, Patriot is an early to mid season, Bluecrop is a mid-season, and Herbert is a late-season cultivar. The spatial location of individual blueberry bushes was identified in each planting with coordinates x (row number) and y (location within the row). For example, bush 6-13 was located in position 13 within row 6. Composite samples of eight to twelve apical leaves were collected from branch tips throughout the canopy of individual bushes to determine the occurrence of viruses. Alternatively, fine roots were collected from individual bushes and tested for the presence of viruses. Also, weeds showing virus-like symptoms and growing in proximity to blueberry bushes were collected for testing. We considered a virus isolate as a viral culture recovered from a single infected blueberry bush or weed.

Serological detection of viruses. Viruses were detected by DAS-ELISA in blueberry leaf tissue with specific antibodies (Bioreba, Switzerland). A portion of 8-12 stacked leaves was torn, ground in phosphate buffer saline supplemented with 10 mM sodium sulfite, 1%

Corresponding author: M. Fuchs
Fax: +1.315.7872389
E-mail: mf13@cornell.edu

polyvinylpyrrolidone 40, 1% Tween 20 and powdered egg albumin (2g/l) at a 1:10 ratio (w:v) using a semi-automated ball-bearing HOMEX tissue homogenizer, and tested according to the manufacturer's instructions. Substrate hydrolysis was recorded at 405 nm with an absorbance BioTek ELx808TM microplate reader (BioTek, USA). Samples were considered positive if their optical density (OD_{405nm}) readings were at least twice those of healthy controls. Blueberry root samples and weeds were tested in the same manner.

Spatial distribution analysis of virus-infected highbush blueberry. The spatial distribution of bushes that tested positive for ToRSV or TRSV in DAS-ELISA in blueberry plantings were mapped using the two dimensional contour mapping feature of Sigmaplot version 11.0 (Systat Software, USA). Contour maps indicate the location of infected bushes by coordinates x and y, with the x-axis being the row number and the y-axis being the within-row location (e.g. bush number).

Herbaceous host range of ToRSV and TRSV. The host range of viruses recovered from blueberry and weeds was determined using *Chenopodium quinoa* Willd, *Nicotiana benthamiana* Domin, *Nicotiana glauca* L., *Nicotiana glauca* L. cv. Xanthi, and *Cucumis sativus* L. cv. Marketmore. Twenty plants of each species were inoculated mechanically per virus isolate at the two-leaf stage using leaf homogenates of blueberries and weeds that were positive for ToRSV or TRSV in DAS-ELISA. Inoculated plants were monitored for symptom development over three weeks and tested by DAS-ELISA to confirm infection.

Characterization of partial ToRSV and TRSV RNA-1 segments by RT-PCR in blueberry and nematode samples. Blueberry leaf samples and dagger nematodes extracted from blueberry soil samples were assayed for TRSV by RT-PCR with total RNA and appropriate primers. Total RNA was extracted from leaf tissue (100 mg) or individual nematodes using the RNeasy mini plant kit (Qiagen, USA) as described (Fuchs *et al.*, 2009). A segment of the TRSV RNA-1-encoded RNA-dependent RNA polymerase (RdRp) gene was amplified with primers MF05-21-R (5'-CAATACGGTAAGTG-CACACCCCG-3') and MF05-22-F (5'-CAGGGGCGTGAGTGGGGGCTC-3'). A primer pair specific to the ribulose 1,5-bisphosphate carboxylase chloroplast (*Rbc1*) ribosomal gene [Rbc1-R 5'-CTGCATGCATTGCACGGTG-3' and Rbc1-F 5'-TACTTGAACGCTACTGCAG-3' (Sanchez-Navarro *et al.*, 2005)] was used as internal control to amplify the corresponding mRNA in blueberry leaf tissue in standard and multiplex RT-PCR. For ToRSV, a segment of the RNA-1-encoded RdRp gene was amplified from blueberry leaf tissue and dagger nematodes by IC-RT-

PCR with primers ToRSV-R (5'-CCACCACACTCCACCTACC-3') and ToRSV-F (5'-ACTTCTGAAGGCTACCCGTT-3') as described (Fuchs *et al.*, 2009).

RT-PCR was carried out using the OneStep kit (Qiagen, USA) with 50 pmoles of specific primers in a 50 µl final volume according to the manufacturer's protocol. Single tube RT-PCR used a 30 min heating step at 50°C and a 15 min heating step at 95°C followed by 35 cycles of 30 sec melting at 94°C, 1 min annealing at 50°C, and 1 min elongation at 72°C with a final extension of 10 min at 72°C. The reaction products were resolved by electrophoresis in 1.5% agarose gels in 40 mM Tris-acetate, 10 mM EDTA, pH 8.0, stained with ethidium bromide and visualized under UV light.

Viral sequence determination and analysis. ToRSV and TRSV DNA amplicons obtained by RT-PCR from blueberry leaf tissue and dagger nematodes were extracted from agarose gels with the QIAquick® purification kit (Qiagen, USA) and sequenced bidirectionally using the Big Dye Terminator kit, AmpliTaq-FS DNA polymerase and an Applied Biosystem Automated 3730xl DNA Analyzer at the DNA Sequencing facility at Cornell University. Sequences were analyzed and compared using the DNASTAR Lasergene v7.2 software package using TRSV bud blight strain (GenBank accession No. U50869) (Zaloua *et al.*, 1996) and ToRSV raspberry strain (GenBank accession No. L19655) (Rott *et al.*, 1995) as reference strains. The program CLUSTAL W was used for alignment of nucleotide sequences (Thompson *et al.*, 1994). Phylogenetic relationships were determined with the neighbor-joining method using 1,000 bootstrap replicates (Saitou and Nei, 1987).

Soil samples from blueberry plantings and dagger nematode recovery. Soil samples collected from the root zone of blueberry bushes were analyzed for the occurrence of dagger nematodes. Juveniles and adults were extracted from soil samples by using a combination of the decanting-sieving and pie-pan (modified Baermann funnel) methods (Southey, 1986). Nematodes were identified based on morphological parameters (Lamberti *et al.*, 2002) and counted under a dissecting microscope at 40X magnification. Individual nematodes were picked with a fine needle, transferred to 2.0 ml microfuge tubes, and stored at -80°C for further characterization of their viruliferous status by RT-PCR.

Cucumber baiting assays. Soil samples collected from the root zone of blueberry bushes with known populations of dagger nematodes, were used in cucumber baiting assays in a greenhouse. Three to five subsets of each soil sample (200 g) were mixed with sand at a 3:1 ratio, placed in clay pots, seeded with four cucumbers, and held at 25°C day, 22°C night (16/8 h photoperiod). Cucumber leaf samples were collected and tested

for ToRSV and TRSV by DAS-ELISA after 60 days. Cucumber baiting assays were duplicated.

RESULTS

Disease symptoms. Decline and virus-like symptoms were observed in 8- to 20-year-old mature highbush blueberry cvs Bluecrop, Collins, Herbert, and Patriot in various plantings in New York State in 2007-2009. Symptoms in cvs Collins, Patriot, and Herbert consisted of severely reduced vigor, shoot defoliation (Fig. 1A), distorted and crinkled apical leaves with dark reddish lesions (Fig. 1C). Some bushes also showed a top dieback with poor blossom development (Fig. 1A) or necrotic flower buds in comparison to symptomless bushes (Fig. 1B). Symptoms were more severe in Patriot than in cvs Collins and Herbert bushes. Symptoms in cv. Bluecrop consisted of mosaic or dark reddish lesions on apical leaves and a general decline. Fruit yield was

substantially reduced in all cv. Patriot plantings (Fig. 1D) and to a lesser extent in the cvs Bluecrop, Collins, and Herbert plantings.

Virus identification. Preliminary DAS-ELISA showed the presence of TRSV and ToRSV but not of BLSV, and BLSV, two viruses prevalent in blueberry plantings in the United States, in leaf samples from randomly selected cvs Patriot and Bluecrop bushes in September 2007. Since tests were run late in the season, an extensive survey was conducted in the spring of 2008 and 2009 to confirm preliminary findings, and determine the incidence and distribution of TRSV and ToRSV. Across cultivars, plantings and year, 10% (65 of 650) of the leaf samples tested reacted to ToRSV (5%, 35 of 650) or TRSV (5%, 30 of 650) in DAS-ELISA (Table 1). Absorbance values were higher for infected compared to healthy blueberry leaf samples with $OD_{405\text{ nm}}$ readings of 1.16 ± 0.879 vs 0.085 ± 0.003 (ToRSV) and 1.92 ± 0.93 vs 0.079 ± 0.013 (TRSV), after 1 h of substrate hydrolysis.

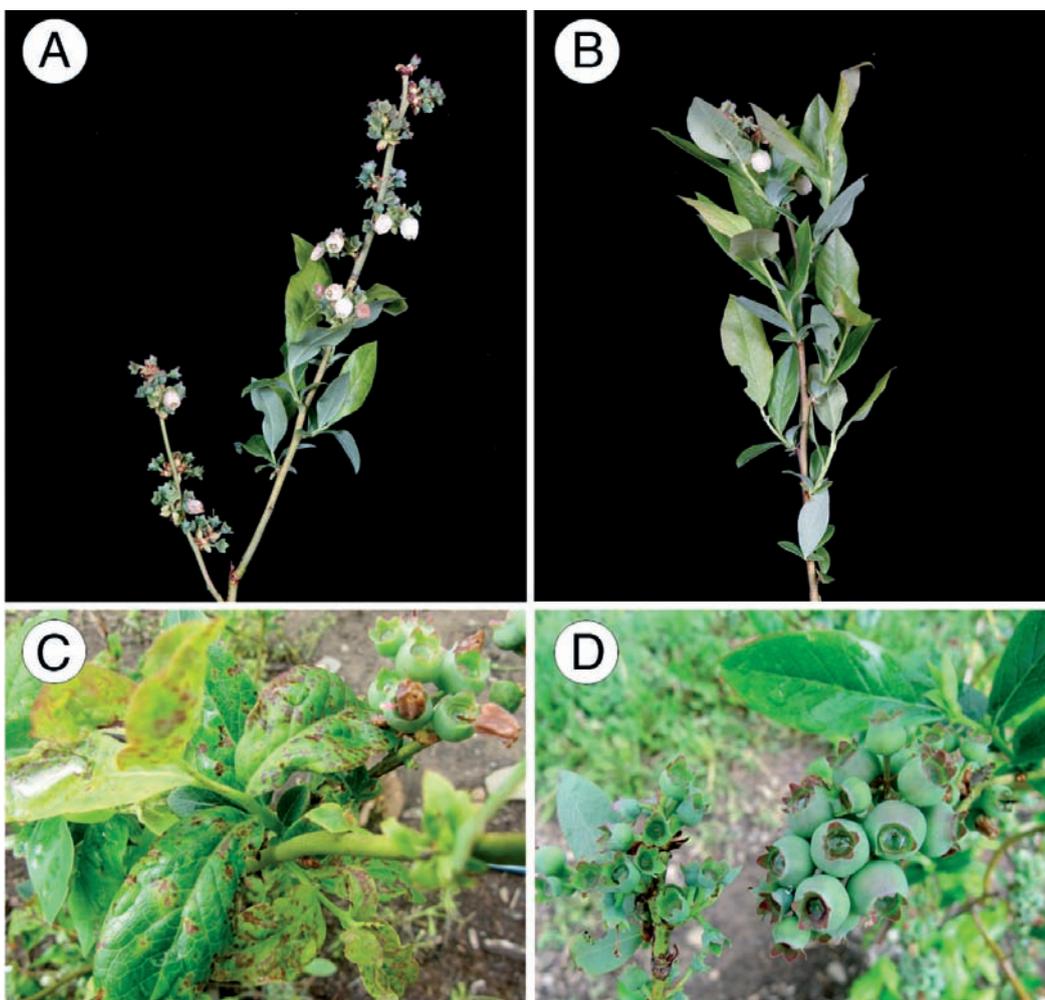


Fig. 1. Virus-like symptoms on highbush blueberry cv. Patriot. (A) Apical dieback and defoliated branch of a symptomatic compared to (B) An asymptomatic bush, (C) Apical leaf chlorosis, crinkling, cupping and dark reddish lesions on a symptomatic bush, and (D) Fruit production on a symptomatic (left) and asymptomatic (right) bush.

Table 1. Incidence of *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV) in highbush blueberry plantings surveyed in this study.

Blueberry cultivar	No. bushes sampled	TRSV infection ^a		ToRSV infection	
		No. infected	%	No. infected	%
Patriot	533	22	4	20	4
Bluecrop	89	8	9	8	9
Collins	18	0	0	4	22
Herbert	10	0	0	3	30
Total	650	30	5	35	5

^aTRSV and ToRSV were detected in blueberry leaves by DAS-ELISA in 2008 and 2009.

None of the samples tested was co-infected with ToRSV and TRSV. Analysis of varied blueberry tissues in a subset of bushes showed a more reliable ToRSV detection in fine roots (40%, 40 of 100) than apical leaves (10%, 10 of 100) while no virus was detected in middle (0%, 0/40) and lower (0%, 0 of 40) leaves. Similar data were obtained for TRSV with 35% (35 of 100) and 15% (15 of 100) of the fine roots and apical leaves testing positive in DAS-ELISA, respectively. ToRSV was detected in half of the plantings (7 of 14) surveyed while TRSV was found only in two plantings.

Spatial distribution of virus-infected bushes. Analysis of the spatial distribution of virus-infected blueberry bushes using the two dimensional contour mapping feature of Sigmaplot was conducted to detect clustering in one cv. Patriot (Fig. 2A) and one cv. Bluecrop (Fig. 2B) planting. TRSV-infected blueberry bushes were distributed in the central and northern areas of cv. Patriot (Fig. 2A) and throughout cv. Bluecrop (Fig. 2B) plantings. ToRSV-infected bushes were identified mainly in the southern half and central areas of cv. Patriot (Fig. 2A), and in the central and northern areas of cv. Bluecrop (Fig. 2B) plantings.

Virus host range. The herbaceous host range of TRSV and ToRSV from cvs Bluecrop, Collins, Herbert and Patriot bushes was typical of corresponding isolates from other crops. TRSV from highbush blueberry caused necrotic lesions on inoculated leaves and apical dieback on *C. quinoa*, necrotic lesions and systemic chlorosis on *C. sativus*, necrotic ringspots on inoculated leaves and mosaic symptoms on the new leaves of *N. tabacum*, necrotic local lesions on *N. clevelandii*, and systemic chlorosis on *N. benthamiana*. ToRSV induced necrotic local lesions on *C. quinoa*, chlorotic local lesions on *C. sativus*, necrotic ringspots and chlorosis on *N. tabacum*, necrotic local lesions and systemic chlorosis on *N. clevelandii*, and mottling and apical necrosis on *N. benthamiana*. The presence of TRSV and ToRSV was confirmed in symptomatic herbaceous plants by DAS-ELISA (data not shown).

Viruses in weeds. Among the weeds collected in close spatial proximity of highbush blueberry, only samples of *Rumex acetosella* L. (red sorrel) with dark red-

dish lesions reacted to ToRSV in DAS-ELISA and induced the same symptoms on herbaceous hosts as ToRSV isolates from blueberry (data not shown). These results were consistent with a ToRSV transmission from

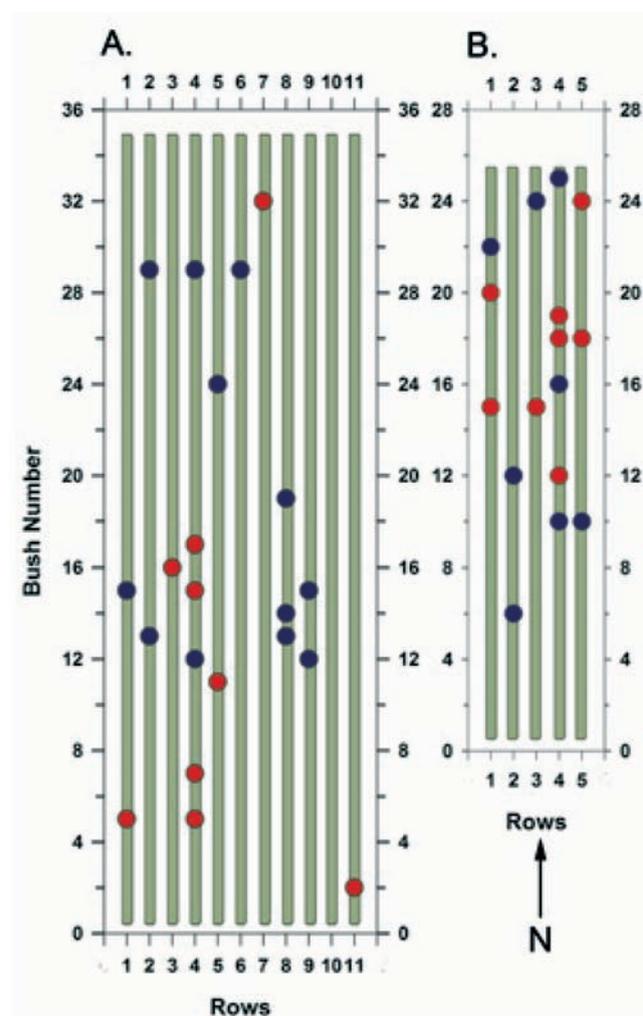


Fig. 2. Spatial distribution of TRSV- and ToRSV-infected highbush blueberry in a (A) Patriot and (B) Bluecrop planting. Bush rows are indicated on the x-axis and bush numbers are indicated on the y-axis. Bushes reacting positively to TRSV and ToRSV in DAS-ELISA are shown in blue and red circles, respectively. Highbush blueberry with no detectable TRSV and ToRSV are in green. Three rows of healthy bushes are not shown on the east side of the Patriot planting. North (N) is indicated by an arrow.

highbush blueberry to these weeds or vice-versa, providing indirect evidence of the presence of nematode vectors carrying ToRSV. TRSV was not found in any of the weed samples collected and tested by DAS-ELISA.

Search for vector nematodes. Sixteen soil samples from two cv. Patriot plantings and three from one cv. Bluecrop planting were tested for the presence of nematodes in 2008 and 2009. Species identified were primarily *Pratylenchus* spp., *Helicotylenchus* spp. and *Hoplolaimus* spp. *Xiphinema americanum*, the vector of TRSV and ToRSV, was found only in five of the 19 (26%) soil samples tested from a cv. Patriot planting (Table 2). *X. americanum* populations were low and varied from 10 to 280 individuals per kg of soil collected (Table 2). Comparatively, higher dagger nematode population densities (1,200-1,800 per kg of soil) are found routinely in a cherry orchard at Cornell University by using the identical recovery method from soil samples.

Cucumber baiting assays with four soil samples containing the highest *X. americanum* populations demonstrated transmission of ToRSV from soil samples A and B, and TRSV from samples C and D (Table 3). No virus transmission was obtained in assays using soil samples without identified dagger nematodes (data not shown). These results were consistent with the occurrence of viruliferous *X. americanum* in the cv. Patriot planting. The viruliferous status of *X. americanum* specimens isolated from soil sample C was confirmed by RT-PCR with am-

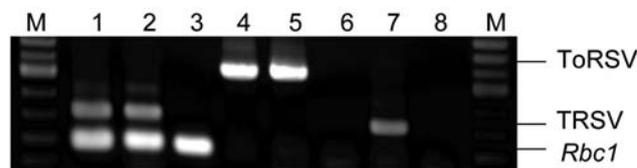


Fig. 3. Agarose gel analysis of TRSV and ToRSV amplicons obtained by RT-PCR assays. Total RNA extracted from blueberry leaves was used along with primer pairs specific to the TRSV RdRp gene and the *Prunus Rbc1* gene in multiplex RT-PCR assays. For ToRSV, IC-RT-PCR using primers for the RdRp gene was used. Lanes 1-2: Patriot 4-12 and Bluecrop 2-6 infected with TRSV (plant locations are from Fig. 2); lanes 4-5: Patriot 5-11 and Bluecrop 1-15 infected with ToRSV. Total RNA extracted from *X. americanum* recovered from Patriot 8-8 used for (lane 7) TRSV and (lane 8) ToRSV detection by RT-PCR; lanes 3 and 6: healthy highbush blueberry; and lane M: 100 bp DNA size standard (Promega). The TRSV and ToRSV RdRp gene amplicons are shown at 320 bp and 580 bp, respectively, and the *Rbc1* amplicon is shown at 183 bp.

plification of a 320-bp long TRSV RdRp gene fragment from six individuals, as illustrated for one of them (Fig. 3). No ToRSV amplicon was obtained from individual nematodes of soil sample C (Fig. 3). The presence of amplifiable viral gene products was not tested by RT-PCR in dagger nematodes from soil samples A, B, and D.

Virus detection by RT-PCR. The presence of TRSV was confirmed by RT-PCR in leaf samples of a subset of

Table 2. Incidence of plant-parasitic nematodes in a declining Patriot blueberry planting.

Blueberry soil sample ^b	Bush location ^c	No. of plant parasitic nematodes/kg soil ^a	
		<i>Xiphinema americanum</i>	Other species ^d
A	4-6	40	600
B	7-30	280	360
C	8-8	40	200
D	8-18	40	100
E	3-46	10	120

^aNematodes were extracted from soil samples using a combination of sieving/decanting and pie-pan protocols.

^bSoil samples were collected in the root zone of blueberry bushes in the Patriot planting shown in Fig. 2A.

^cThe planting row number followed by the plant number within the row are indicated.

^dOther plant parasitic nematodes included lesion (*Pratylenchus* spp.), lance (*Hoplolaimus* spp.) and spiral nematodes (*Helicotylenchus* spp.).

Table 3. Transmission of *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV) by *Xiphinema americanum* from blueberry soil samples to cucumber plants in baiting assays.

Virus	Soil samples ^b	First experiment ^a	Second experiment	Total
		Infected/Tested	Infected/Tested	
ToRSV	A, B	3/11	3/23	6/34
	C, D	0/30	0/22	0/52
TRSV	A, B	0/11	0/23	0/34
	C, D	2/30	1/22	3/52

^aTwo independent baiting experiments were performed with blueberry soil samples containing identified *X. americanum*. Data represent the number of cucumber plants that were positive for TRSV or ToRSV in DAS-ELISA over the total number of plants tested.

^bSoil samples were collected from the root zone of Patriot bushes 4-6 (A), 7-30 (B), 8-8 (C), and 8-18 (D).

blueberry bushes (six cv. Patriot isolates and five cv. Bluecrop isolates) with amplification of a 320-bp DNA fragment (Fig. 3). As expected, no product was amplified from total RNA of healthy blueberry leaves while a 183 bp long fragment corresponding to the plant internal control *Rbcl* was obtained (Fig. 3). Similarly, the presence of ToRSV was confirmed in leaf tissue of highbush blueberry cvs Patriot (11 isolates) and Bluecrop (5 isolates) by IC-RT-PCR and appropriate primers that amplified a 585 bp fragment of the RdRp gene (Fig. 3). IC-RT-PCR was preferred to RT-PCR because preliminary tests had shown that the latter assay was not reliable for ToRSV detection (data not shown).

Sequence and phylogenetic analyses. The amplicons obtained by PCR from four ToRSV and eleven TRSV isolates were sequenced and their sequences were deposited in GenBank with accession Nos GQ141525 to GQ141539. The ToRSV isolates characterized in this study had 93.2-99.7% sequence identity at the nucleotide level and 96.9-99.5% at the amino acid level. A multiple alignment of ToRSV sequences from blueberry and those available in GenBank, including the corresponding RNA-1 fragment of the reference strain from raspberry (Table 4), revealed 80.7-99.7% and 90.2-99.5% sequence identity at the nucleotide (585 nts) and amino acid (194 residues) levels, respectively.

TRSV isolates characterized in this study had 93-99.1% sequence identity at the nucleotide level and 94.3-100% at the amino acid level. A multiple align-

ment of TRSV sequences from blueberry and those available in GenBank, including the corresponding RNA-1 segment of the reference strain bud blight (Table 4), showed 90.8-99.7% and 91.4-100% sequence identity at the nucleotide (316 nts) and amino acid (105 residues) levels, respectively.

Phylogenetic analyses showed a clustering of ToRSV isolates into three groups (Fig. 4A) and TRSV isolates into one group (Fig. 4B). The same grouping of virus isolates was obtained with phylograms obtained from deduced amino acid sequences (data not shown). Phylogenetic analyses further inferred no grouping of genetic variants based on blueberry cultivar because ToRSV and TRSV haplotypes from cvs Patriot and Bluecrop grouped together. ToRSV haplotypes from blueberry did not group with corresponding haplotypes from other crops (Fig. 4A). These results expand on previous data on ToRSV diversity (Wang and Sanfaçon, 2000) and suggest a genetic differentiation according to host. In contrast, all TRSV haplotypes clustered together into a single phylogenetic group, regardless of the host, although isolates from small fruits branched closer together.

DISCUSSION

ToRSV and TRSV are known to occur in fruit crops in New York State including grapevines (Gilmer *et al.*, 1970; Uyemoto *et al.*, 1977a) and *Prunus* (Cummins *et*

Table 4. Virus isolates used in this study to determine phylogenetic relationships.

Virus	Isolate ^a	Crop	GenBank accession no.
ToRSV	P4-5	Blueberry	GQ141525
	P5-11	Blueberry	GQ141526
	P11-2	Blueberry	GQ141527
	B4-19	Blueberry	GQ141528
	GYV	Grapevine	AF135407
	PYBM	Peach	AF135408
	Raspberry	Raspberry	AF135409
	T392	Unknown	AF135410
	TRSV	P1-15	Blueberry
P4-29		Blueberry	GQ141530
P5-24		Blueberry	GQ141531
P6-29		Blueberry	GQ141532
P8-14		Blueberry	GQ141533
P9-12		Blueberry	GQ141534
B2-6		Blueberry	GQ141535
B4-25		Blueberry	GQ141536
B4-6		Blueberry	GQ141537
B4-10		Blueberry	GQ141538
B5-10		Blueberry	GQ141539
Bean		<i>Phaseolus vulgaris</i> L.	AJ698718
Cornell		Blackberry	EF528581
Bud blight	Soybean	U50869	

^aIsolates recovered from blueberry cvs. Patriot (P) and Bluecrop (B) are identified, as well as field location. For example ToRSV isolates P4-5 is from bush in position 5 in row 4.

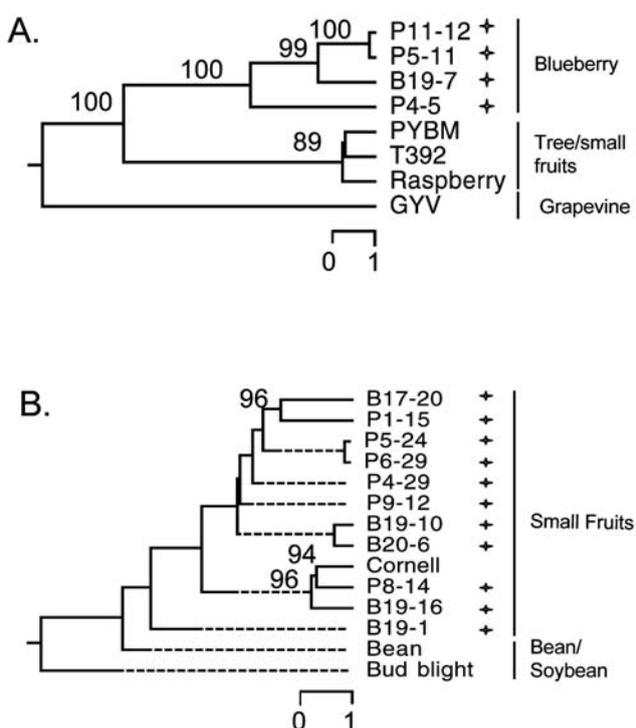


Fig. 4. Phylogenetic trees reconstructed from the partial nucleotide sequence of the (A) ToRSV and (B) TRSV RNA-dependent RNA polymerase gene by the neighbor-joining method with 1,000 bootstrap replicates. Branch lengths represent phylogenetic distances determined by distance matrices of nucleotide sequences. Numbers above critical branches are significant bootstrap values (>80%). The scale bar represents a relative genetic distance of 0.01. TRSV and ToRSV isolates sequenced in this study are indicated by stars.

al., 1982; Uyemoto *et al.*, 1977b). ToRSV was also found in *Malus* (Rosenberger *et al.*, 1989) but, to our knowledge, this is the first report on the occurrence of TRSV and ToRSV in highbush blueberry in New York. Previous reports recorded the presence of TRSV in blueberry from Connecticut, Illinois, Michigan, Arkansas, Oregon, and New Jersey (Ramsdell, 1995a). ToRSV was reported in blueberry in Oregon, Washington, and Pennsylvania (Ramsdell, 1995b). The distribution of the two nepoviruses in the major blueberry producing areas in the United States is likely due to the use of planting material derived from noncertified budwood, stressing the need for a set of national standards for the indexing and certification of small fruit crops, as well as stringent international standards for the safe transboundary movement of blueberry planting material.

The infection rate of TRSV and ToRSV was higher in fine roots compared to apical leaves of cv. Patriot bushes. These results were consistent with findings in red raspberry (Pinkerton *et al.*, 2008). Could gene silencing, a potent plant defense mechanism against viruses (Voinnet, 2008), be involved in the reaction of highbush blueberry to TRSV and ToRSV infection? 'Bluecrop' is

known to recover from TRSV infection (Lister *et al.*, 1963). This recovery phenomenon likely results from RNA silencing, as shown in *Nicotiana* spp. infected with nepoviruses (Ratcliff *et al.*, 1997), particularly with TRSV (Siddiqui *et al.*, 2008) and ToRSV (Jovel *et al.*, 2007). It is conceivable that gene silencing could be active in highbush blueberry infected with TRSV or ToRSV, affecting systemic spread and reducing virus titers in apical leaves.

Mixed infection by ToRSV and TRSV was not detected in any of the highbush blueberry bushes surveyed in this study, nor were nematodes carrying both ToRSV and TRSV detected within any of the soil samples tested. An earlier survey of blueberry in western Oregon described the occurrence of ToRSV and TRSV in plantings of cvs Pemberton, Atlantic and Dixi but mixed infection was not reported in individual bushes (Converse and Ramsdell, 1982). Why was co-infection by the two target nepoviruses not detected in spite of the occurrence of *X. americanum* viruliferous for either TRSV or ToRSV? Knowing that *X. americanum* is a complex nematode species (Molinari *et al.*, 2004) and that TRSV and ToRSV seem to have a distinct mechanism of association with their nematode vector (Wang *et al.*, 2002), could populations of two distinct biotypes, one viruliferous for ToRSV and the other viruliferous for TRSV, simultaneously occur in the highbush blueberry plantings surveyed in this study? Also, could a differential spatial distribution and an exclusion mechanism, whereby a plant cell already infected with one virus strain cannot be superinfected by other strains or other viruses (Dietrich and Maiss, 2003; Takeshita *et al.*, 2004; Takahashi *et al.*, 2007), prevent systemic infection of one or the other virus? More work is needed to address the absence of mixed infection of TRSV and ToRSV in highbush blueberry.

X. americanum, the vector of TRSV and ToRSV, was detected in the root zone of cv. Patriot highbush blueberries. Populations of *X. americanum* were found in vineyards (Uyemoto *et al.*, 1977a) and in apple, peach, and cherry orchards (Molinari *et al.*, 2004) in New York state. To our knowledge, this is the first report of *X. americanum sensu lato* in blueberry in New York. The same nematode species was also reported in blueberry plantings in Pennsylvania (Forer *et al.*, 1982) and Michigan (Griffin *et al.*, 1963). The spring-time population density of *X. americanum* in the blueberry plantings surveyed in this study was low, but similar to findings in other crops (Evans *et al.*, 2007; Pinkerton *et al.*, 2008). In addition, transmission rates were low for either TRSV or ToRSV, as shown by cucumber baiting assays. The limited number of *X. americanum* used per assay (approximately 8-56 per pot) likely accounted for these results, as higher populations (200-300) are usually required for an efficient transmission of nepoviruses by dagger nematodes (Andret-Link *et al.*, 2004; Wang and Gergerich, 1998).

ToRSV was detected in blueberry leaf tissue by IC-RT-PCR but not RT-PCR. Conventional double-stranded RNA extraction protocols routinely applied to several perennial crops known to have high polyphenolic concentrations give poor results with blueberry tissue (Tzanetakis and Martin, 2008). However, it is unlikely that inhibitors unique to blueberry tissue interfered with the effectiveness of RT-PCR since this assay was successful for TRSV. It remains puzzling that RT-PCR was successful for TRSV but not ToRSV. Could there be different levels of RNA silencing between the two viruses, limiting availability of unencapsidated ToRSV RNA for cDNA synthesis? More work is needed to verify this hypothesis. The differential performance of detection assays of TRSV and ToRSV raises concerns about reliable diagnostic tests for nepoviruses in blueberry. Our results suggest a need to test routinely leaves and roots. They also point out the necessity to use several detection techniques for accurate blueberry indexing for these viruses.

'Bluecrop' blueberry is recommended for its resistance to ToRSV infection in Oregon (Pscheidt, 2008). In New York we found this cultivar to be readily infected by ToRSV. Were unique ToRSV haplotypes involved in the infection process? Our comparative sequence and phylogenetic analyses support this hypothesis as ToRSV isolates from cv. Bluecrop had a wider range of nucleotide sequence identity in the RdRp gene with other ToRSV isolates (80.7-99.7%) than TRSV isolates from cv. Bluecrop and other TRSV isolates (91.1-99.7%). It would be interesting to further characterize the genetic variability of ToRSV isolates from blueberry within RNA-2 or other RNA-1 encoded genes to advance our knowledge of diversity in this virus (Wang and Sanfaçon, 2000).

ACKNOWLEDGEMENTS

The collaboration of Tom Gartung, TLC Blueberry Farm in Barton, New York; Mary Pennel, Sugar Shack Blueberry Farm, Penn Yan, New York; Molly Shaw, Tioga County Cornell Cooperative Extension, Oswego, New York; Debbie I. Breth, Cornell Cooperative Extension, Lake Ontario Fruit Team, Albion, New York; and Cathy Heidenreich, Berry Extension Support Specialist, Department of Horticulture, Cornell University, Ithaca, New York is acknowledged. We are grateful to Aracely Ospina, Cheung Mei, and Eric Rockefeller for excellent assistance with sample processing and cucumber baiting assays, and to Joe Ogrodnick for artwork. We are indebted to Dr. L.M. Yepes for critically reading the manuscript and Dr. A. Di Tommaso for identification of weed species. This study was partially funded by state, federal, and institutional funds appropriated to the New York State Agricultural Experiment Station, Cornell University.

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Received October 20, 2009

Accepted January 25, 2010

