Euonymus Chlorotic Ringspot Disease Caused by Tomato Ringspot Virus

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ABSTRACT

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During the spring, young leaves of *Euonymus fortunei* var. *vegetus* and *E. kiautschovicus* exhibited chlorotic rings and oak-leaf patterns. The causal agent was isolated, characterized, and identified as a strain of tomato ringspot virus designated TomRSV-Eu. The virus was transmitted by mechanical inoculation, dodder (*Cuscuta campestris*), grafting, and a nematode (*Xiphinema americanum*). TomRSV-Eu was mechanically transmitted to several euonymus cultivars and infected a wide range of experimental hosts but did not infect tomato plants or systemically infect tobacco. Properties of TomRSV-Eu in crude sap were: thermal inactivation of 59–60 C for 10 min; dilution end point of 1:100–1:1,000 in 0.01 M phosphate buffer, pH 7; and in vitro longevity of 24–28 hr at 25 C. Purified virus preparations were infectious and had an ultraviolet absorption spectrum of a nucleoprotein containing 40% nucleic acid. Sucrose density-gradient centrifugation resulted in two light-scattering zones containing isometric particles 28–30 nm in diameter. Particles from the bottom zone had a sedimentation coefficient of 131S and were highly infectious, whereas particles from the top zone had a sedimentation coefficient of 53S and were not infectious. TomRSV-Eu did not react with antisera to 11 other isometric viruses but did form a single confluent precipitin zone of identity with tomato ringspot virus in reciprocal gel double-diffusion tests.

Several plantings of *Euonymus kiautschovicus* Loes. and *E. fortunei* (Turcz.) Hand.-Mazz. var. *vegetus* (Rehd.) Rehd. on the University of Maryland campus had young leaves exhibiting chlorotic rings, chlorotic concentric ringspots, and chlorotic line-patterns.

Veinal chlorosis, mosaic, and variegation symptoms have been reported on *E. japonicus* L.f. (2), *E. radicans* Sieb. (28), and *E. europaeus* L. (5,23). The agents associated with these symptoms were graft-transmissible but not mechanically transmissible. Barnett and Baxter (1) isolated cucumber mosaic virus from shrubs of *E. japonicus* L. f. var. *microphyllus* Jacq. exhibiting a white mosaic. The euonymus mosaic virus described by Bojnansky and Kosljarova (5) was reported by Mali (15) to be serologically related to tobacco necrosis virus and transmitted by either the nematode *Longidorus euonymus* n. sp. (16) or the chytrid fungus *Olpidium brassicae* (Wor.) Dang. (15).

This paper describes the isolation, characterization, and identification of a nematode-transmitted icosahedral virus, serologically related to tomato ringspot, as the causal agent of the euonymus chlorotic ringspot disease. Preliminary results of these findings have been published (18).

MATERIALS AND METHODS

Source of viruses and inoculation procedures. Young leaves of naturally infected plants of *Euonymus* spp. were the original source of the euonymus strain of tomato ringspot virus (TomRSV-Eu). The virus was maintained and increased in *Petunia hybrida* Vilm. or cucumber *Cucumis sativus* L. 'National Pickling.' *Nicotiana tabacum* L. 'Kentucky 35' (Ky 35) and *Chenopodium quinoa* Willd. were utilized as assay plants. Tomato ringspot virus (TomRSV) isolates used were Price's strain and the apricot strain supplied by E. L. Civerolo. They were maintained in tobacco or cucumber plants. Tobacco ringspot virus (TobRSV) was the American Type Culture Collection (ATCC) isolate 98 and was maintained in cucumber plants.

All mechanical inoculations were made by the Carborundum gauze-pad method, and plant extracts in 0.01 M phosphate buffer, pH 7, with or without additives were used as the inoculum. Unfortunately, during short periods of the summer, temperatures in the greenhouse reached 40 C and it was difficult or impossible to transmit the viruses.

Dodder transmission. *Cuscuta campestris* Yunck. seeds were planted among healthy seedlings of *Vinca rosea* L. or petunia to germinate and become established. Dodder stems 4- to 8-cm long were placed adjacent to petunia or cucumber plants infected with TomRSV-Eu. After the dodder became established on the virus source plants, dodder stems were trained to healthy petunia or cucumber plants. The test plants were observed for symptoms and the leaves of the test plants and dodder stems were indexed for the presence of virus.

Nematode transmission. Studies were conducted with *Xiphinema americanum* Cobb collected from field soil and identified by L. R. Krusberg. Soil containing approximately 100 nematodes per 250 ml of soil was mixed with steam-sterilized soil in clay pots or wooden flats to obtain a final population of about 200 nematodes per acquisition plant (22). Petunia or cucumber plants systemically infected with TomRSV-Eu were transplanted to the nematode-containing soil to serve as virus source plants. Similar plants, not inoculated, were planted in nematode-infested soil to determine that the field collected nematodes were "virus-free." After a 10–14-day acquisition period, the TomRSV-Eu-infected source plants were removed from the containers and two to six young healthy bait plants of petunia or cucumber were planted in each container. Alternatively, three to six bait plants were planted among the acquisition plants. Healthy plants of *E. fortunei* var. *vegetus* were also used in some tests as bait plants. Controls consisted of healthy cucumber and petunia plants growing in steam-sterilized soil without nematodes, healthy bait plants in steam-sterilized soil containing nematodes, and bait plants planted adjacent to TomRSV-Eu-infected acquisition plants in steam-sterilized soil not containing nematodes. Bait and control plants were observed 6 wk for symptoms and indexed periodically for virus by mechanical inoculation of *C. quinoa* or tobacco cultivar Ky 35 with root and leaf tissue extracts.

Physical property assays. The physical properties of thermal inactivation, dilution end point, and longevity in vitro were

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determined by the methods of Ross (21). Crude juice for all tests was obtained from infected leaves of petunia, tobacco, or cucumber 10–14 days after inoculation, treated, and assayed on *C. quinoa* or tobacco cultivar Ky 35.

To determine the relative concentrations of virus, petunia cultivar Red Cascade, cucumber cultivar National Pickling, and fava beans (*Vicia faba* L. 'Long Pod') were inoculated with TomRSV-Eu. The inoculated and uninoculated leaves of test plants were bioassayed for relative virus concentrations at daily intervals on the local lesion host, tobacco cultivar Ky 35.

Purification. Fresh or frozen TomRSV-Eu-infected cucumber, petunia, tobacco, or fava bean leaf tissue, 10–18 days after inoculation was used to obtain purified virus preparations. The following procedure gave highest yields of infectious virus: Infected fresh tissue was macerated in a food chopper and 0.5 M citrate buffer, containing 0.1% thioglycolic acid (TGA) with 0.01 M sodium diethyldithiocarbamate (Na DIECA), pH 6.2, was added to an equal volume of crude juice. The mixture was homogenized in a Waring blender for 2–3 min and the homogenate, after being stored frozen for 1–10 days at −20 C, was thawed and blended 1–2 min in 0.5 volume of extraction buffer. The extract was expressed through cheesecloth, its pH was adjusted to 4.6 with glacial acetic acid and it was allowed to stand at 4 C for 15–30 min. The mixture was centrifuged for 10 min at 10,000 *g*, and the supernatant filtered through Pyrex glass wool. Clarified sap was subjected to two to three cycles of differential centrifugation (high speed of 2 hr at 66,000 *g*; low speed 10 min at 12,000 *g*) and the final pellets were resuspended in 0.01 M tris [tris (hydroxymethyl) amino methane] HCl buffer, pH 7.4. Further purification was obtained by rate-zonal density-gradient centrifugation in 10–40% sucrose in a Beckman SW25.1 rotor for 2 hr at 52,000 *g*. Light-scattering zones were removed and centrifuged for 2 hr at 78,000 *g* or dialyzed 12–16 hr at 4 C against desired buffer. Virus concentration and percent nucleic acid were determined by dry weight and UV spectrophotometry in a Zeiss PMQ II or a Coleman 125 double-beam recording spectrophotometer (11).

Sedimentation coefficients of TomRSV-Eu were estimated by comparing its sedimentation rate with that of purified preparations of southern bean mosaic virus (SBMV) or TobRSV in 10–40% (w/v) sucrose density gradients after centrifugation in a Beckman SW25.1 rotor at 52,000 *g* for 2 hr (6). The sedimentation coefficient value used for SBMV was 115S (14) and values of 53S, 93S, and 128S were used for top, middle, and bottom zones, respectively, of TobRSV (27).

Electron microscopy. Virus preparations were examined in an Hitachi HU electron microscope as chromium-shadowed or negatively stained preparations in 2% phosphotungstic acid. Particle measurements were taken from electron micrographs containing particles of tobacco rattle virus for calibration.

Young leaf tissue from infected and healthy euonymus was fixed in 6% buffered glutaraldehyde, postfixed in 1% buffered osmium tetroxide, dehydrated in a graded series of ethyl alcohol, and embedded in a mixture of Maraglas-Cardolite. Ultrathin sections cut with glass or diamond knives on a Porter-Blum MT-1 microtome were stained with lead citrate and uranyl acetate and examined in the electron microscope.

Serology. Over a period of 7 wk, a rabbit was given 10 intravenous and four intramuscular injections of purified TomRSV-Eu (1.5 mg/ml). The intravenous injections contained 0.5 ml of virus suspension plus 0.5 ml physiological saline, and the intramuscular injections contained 1 ml of the virus suspension emulsified with 1 ml of Freund's complete adjuvant. Twenty days after the last injection, the rabbit was bled by vacuum-ear bleeding and the titer of the serum was determined in a twofold dilution series in microprecipitin tests. Double-diffusion tests in 0.5% Ionagar containing 0.1% sodium azide were conducted to determine the reactions of TomRSV-Eu with antisera to 11 icosahedral viruses.

Virus-host relationship. Twelve tip cuttings 7.5 cm long were made from each of healthy and TomRSV-Eu-infected plants of *E. fortunei* var. *vegetus* and *E. kiautschovicus*. Leaves from each of the four groups of cuttings were indexed for TomRSV-Eu by mechanical inoculation to *C. quinoa* and tobacco cultivar Ky 35. All leaves except the tip leaves were removed from the cuttings. The basal portion of each cutting was dipped in Rootone No. 2 and placed in a sand-vermiculite mixture in a moist chamber for 4 wk. After 4 wk, the cuttings were removed and the relative amount of root development was compared. The cuttings were transplanted to 15-cm-diameter pots containing soil-peat mixture, grown in the greenhouse, and after 16 wk the number of shoots per plant, height of plants, and root development of each plant were compared.

RESULTS

Symptoms in naturally infected euonymus. Young, rapidly expanding leaves of infected plants of *E. fortunei* var. *vegetus* and *E. kiautschovicus* exhibited chlorotic rings, ringspots, and line-patterns during spring growth (Fig. 1). Many of the infected leaves became chlorotic and dropped prematurely, but by early summer the plants had recovered and approximately 90% of the leaves remaining on the plants were symptomless. A few of the persistent or overwintering leaves on both species of euonymus showed reddish-purple ringspots (Fig. 2).

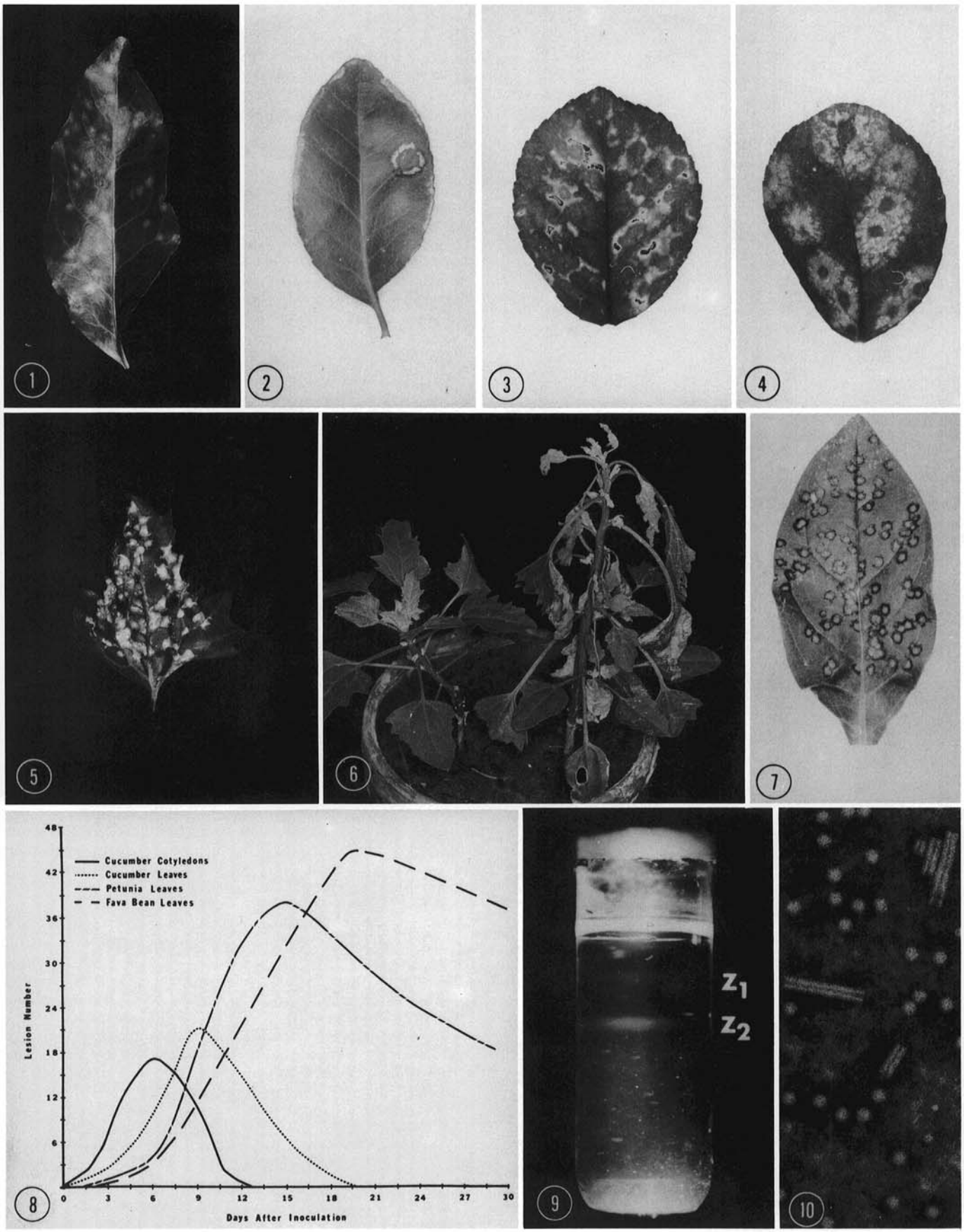
Mechanical transmission of TomRSV-Eu. The initial isolation in 0.01 M sodium phosphate buffer, pH 7, of TomRSV-Eu from young leaves of each symptomatic euonymus species induced only two to three lesions per inoculated tobacco leaf. However, in transmissions with inoculum composed of leaf extracts diluted in a 2% nicotine solution (7) at pH 7.8, the lesion number was increased to 16–18 per inoculated tobacco leaf. The virus was transmitted from euonymus with ease during the spring and early summer but with difficulty during late summer, fall and winter. TomRSV-Eu was maintained in herbaceous hosts and, when mechanically transmitted in the greenhouse to *E. fortunei* var. *vegetus*, chlorotic lesions and rings developed 8–10 days after inoculation. The lesions increased in size and often became slightly necrotic (Fig. 3). Systemic symptoms of chlorotic ringspots and line patterns developed 3–4 wk after inoculation (Fig. 4). Infected plants eventually recovered, but Tom RSV-Eu could be reisolated. This is the first report for mechanical transmission of a virus isolated from euonymus back to euonymus. All euonymus cultivars tested were immune to Price's strain and the apricot strain of TomRSV and to the ATCC isolate 98 of TobRSV.

Experimental host range. One hundred cultivars in 70 species of 52 genera in 18 families, including monocots and dicots, were mechanically inoculated with TomRSV-Eu. Symptoms developed on leaves of 53 cultivars in 32 species of 22 genera in 11 dicotyledonous families. Inoculated and uninoculated leaves of all plants tested were indexed for TomRSV-Eu on *C. quinoa* or tobacco 21–28 days after inoculation. No symptomless infection was detected in any plant cultivar that was tested.

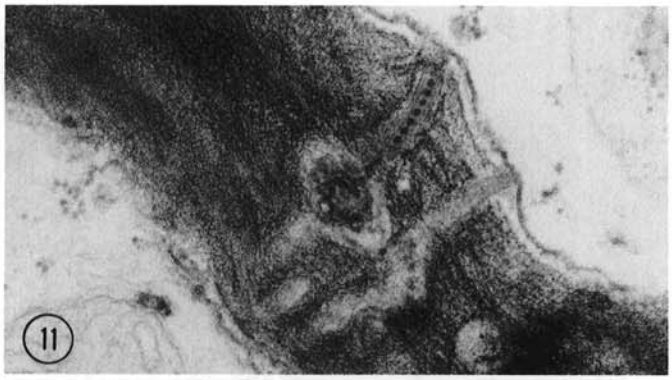
The following plants were susceptible to TomRSV-Eu: *Tetragonia expansa* Murr., *Dianthus chinensis* L., *Gypsophila elegans* Bieb., *Euonymus fortunei* var. *vegetus, E. kiautschovicus, Beta vulgaris* L., *Chenopodium amaranticolor* Coste and Reyn., *C.*

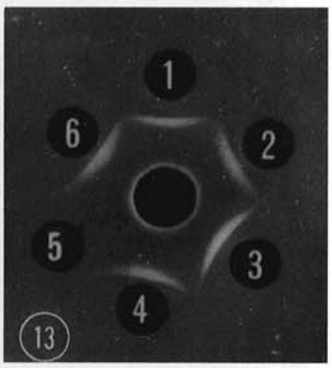
Figs. 1–10. The euonymus strain of tomato ringspot virus (TomRSV-Eu). **1,** Young leaf from a naturally infected *Euonymus kiautschovicus* exhibiting chlorotic ringspots. **2,** Persistent leaf from a naturally infected *E. fortunei* var. *vegetus* exhibiting a necrotic ringspot. **3,** Inoculated leaf of *E. fortunei* var. *vegetus* 10 days after inoculation with TomRSV-Eu. **4,** Systemically infected leaf of *E. fortunei* var. *vegetus* 4 wk after plant was inoculated with TomRSV-Eu. **5,** Leaf of *Chenopodium quinoa* 5 days after inoculation with TomRSV-Eu. **6,** Systemic tip necrosis of *C. quinoa* 10 days after inoculation with TomRSV-Eu. **7,** Inoculated leaf of *Petunia hybrida* 6 days after inoculation with TomRSV-Eu. **8,** Relative concentration of TomRSV-Eu in inoculated plants of cucumber, petunia, and fava bean. **9,** TomRSV-Eu after 2 hr of rate zonal density-gradient centrifugation in 10–40% sucrose at 52,000 *g* in an SW25.1 rotor. **10,** Electron micrograph of a purified preparation of TomRSV-Eu in 2% phosphotungstic acid. Particles of tobacco rattle virus added for calibration. (×100,000).

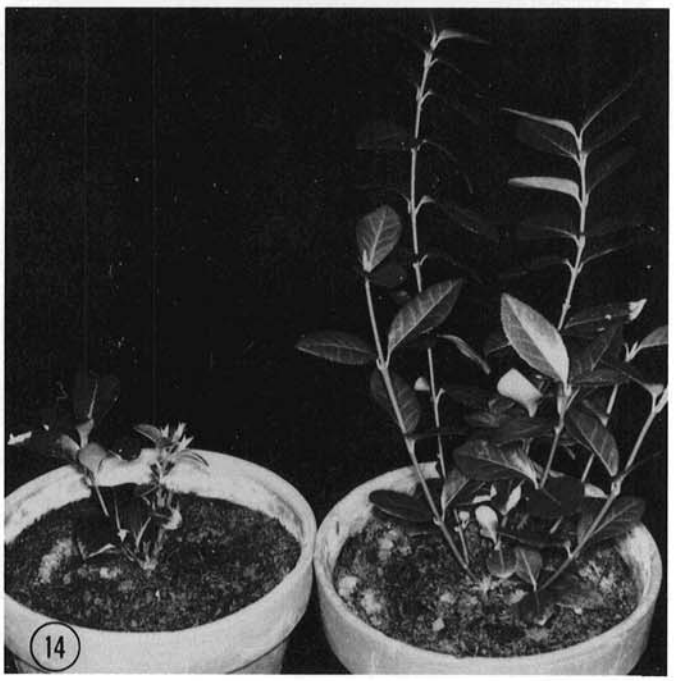
quinoa, Aster sp. Tourn., *Lactuca sativa* L., *Zinnia elegans* Jacq., *Brassica rapa* L., *Cucumis sativus* L., *Cucurbita maxima* Duch., *C. pepo* L., *Glycine max* Merr., *Phaseolus limensis* Macf., *P. vulgaris* L., *Vicia faba* L., *Vigna unguiculata* (L.) Walp., *Nicotiana clevelandii* Gray, *N. glutinosa* L., *N. rustica* L., *N. sylvestris* Speg. & Comes, *N. tabacum* L., *Nicotiana* hybrid (*N. clevelandii* ×*N. glutinosa*), *Petunia hybrida* Vilm., *Solanum melongena* L., and *S. nigrum* L.



The following plants were not susceptible to TomRSV-Eu and the virus was not recovered from the inoculated or uninoculated leaves: *Amaranthus tricolor* L., *Celosia argentea* L., *Gomphrena globosa* L., *Vinca rosea* L., *Dianthus caryophyllus* L., *Spinacia oleracea* L., *Callistephus chinensis* Nees, *Lactuca sativa* L., *Sonchus oleraceous* L., *Tagetes signata* Bartl., *Arabis alpina* L., *Brassica oleracea* L., *B. napus* L., *Matthiola incana* R. Br., *Raphanus sativus* L., *Citrullis vulgaris* Schrad., *Cucumis melo* L., *Hordeum vulgare* L., *Sorghum vulgare* Pers., *Triticum aestivum* L., *Zea mays* L., *Arachis hypogaea* L., *Cassia occidentalis* L., *C. tora* L., *Pisum sativum* L., *Trifolium repense* L., *Fagopyrum esculentum* Moench., *Portulaca grandiflora* Hook., *Torenia fournieri* Lind., *Capsicum frutescens* L., *Datura metel* L., *D. stramonium* L., *Lycopersicon esculentum* Mill., *Physalis peruvianum* L., *Solanum tuberosum* L., and *Daucus carota* L.







Figs. 11–14. The euonymus strain of tomato ringspot virus (TomRSV-Eu). **11,** Viral-like particles in plasmodesmata of TomRSV-Eu-infected leaf tissue of *Euonymus fortunei* var. *vegetus*. (×37,000). **12,** Gel double-diffusion plate 72 hr after addition of the reactants. Center well received antiserum to TomRSV (ATCC PVAS No. 15). Peripheral wells 1 and 3 received TomRSV-Eu and wells 2, 4, and 6 received TomRSV-apricot strain. Well 5 received sap from uninoculated tobacco plants. **13,** Gel double-diffusion plate 72 hr after addition of the reactants. Center well received antiserum to TomRSV-Eu. Peripheral wells 1 and 3 received TomRSV-Eu and wells 2, 4, and 6 received TomRSV-apricot strain. Well 5 received sap from uninoculated tobacco plants. **14,** Relative growth and shoot development of plants of *E. fortunei* var. *vegetus* derived from cuttings of healthy and TomRSV-Eu-infected plants. Healthy (right) and TomRSV-Eu-infected (left) plants after 16 wk of growth.

C. quinoa and tobacco cultivar Ky 35 were used as indicator or assay hosts because of the rapidity and nature of their response to TomRSV-Eu. Necrotic lesions formed on the inoculated leaves of *C. quinoa* within 3–4 days after inoculation (Fig. 5). Unfortunately, leaf distortion and premature defoliation made the lesions more difficult to count. Within 10–12 days after inoculation, tip or general necrosis of the plant had occurred (Fig. 6) even if the inoculated leaves showed only one or two lesions. The inoculated leaves of tobacco cultivar Ky 35 developed discrete necrotic rings 4–6 days after inoculation and the leaves did not become distorted or drop prematurely, which made the plant useful for assays. Systemic infection of tobacco did not occur except for systemic tip necrosis in *N. clevelandii* and Christie's *Nicotiana* hybrid.

Symptoms of TomRSV-Eu in cowpea at 4–6 days after inoculation consisted of red or purple lesions on the inoculated leaves followed by tip necrosis. The inoculated cotyledons of cucumber showed chlorotic lesions or rings within 3–5 days and a general systemic chlorosis and necrosis 14–16 days later. The inoculated leaves of petunia exhibited necrotic ringspots (Fig. 7) followed by a general systemic reaction of necrotic ringspots and line patterns.

TobRSV and TomRSV each infected tomato plants and was recovered easily from inoculated and uninoculated leaves, whereas TomRSV-Eu did not induce symptoms in four plants each of 15 tomato cultivars and the virus was not recovered from the inoculated or uninoculated leaves.

Dodder transmission. Stems of *C. campestris* transmitted TomRSV-Eu from infected cucumber and petunia source plants to healthy plants of the same species. When TomRSV-Eu-infected petunia plants were the source plants, 33% of the petunia test plants were infected 28 days after haustorial contact. When TomRSV-Eu-infected cucumber plants were the source plants, 83% of the cucumber test plants became infected. TomRSV-Eu was not recovered by mechanical transmission of extracts from transmitting dodder stems indicating that the virus did not replicate in the dodder, that the virus concentration was too low to be detected by the method used, or that inhibitors of infection in dodder prevented virus recovery.

Seeds from *C. campestris* growing on TomRSV-Eu-infected plants when germinated and established on healthy assay plants did not cause infection indicating that TomRSV-Eu was not transmitted in the seeds of *C. campestris*.

Bennett (4) reported that TomRSV and TobRSV are not transmitted by dodder, thus, the transmission of TomRSV-Eu by *C. campestris* is the first reported for a strain of tomato ringspot virus.

Nematode transmission. When healthy cucumber and petunia bait plants were planted in soil containing *Xiphinema americanum* having feeding access to either TomRSV-Eu-infected cucumber or petunia acquisition plants, 75% of the cucumber and 50% of the petunia bait plants became infected. TomRSV-Eu was isolated from roots of cucumber plants 6–8 days after exposure to *X. americanum* and symptoms appeared on leaves of cucumber bait plants within 10–12 days after exposure. However, in the case of petunia bait plants, TomRSV-Eu could not be recovered until 14–16 days after exposure to *X. americanum* and symptoms did not appear in the leaves until after 22–24 days.

Two tests each contained three plants of *E. fortunei* var. *vegetus* as bait plants. Symptoms were not observed in the leaves of euonymus and virus was not recovered from leaf extracts.

However, after 15 wk exposure, TomRSV-Eu was recovered from the roots of one plant.

Symptoms were not observed in, and TomRSV-Eu was not recovered from, any of the cucumber or petunia bait plants in the controls which consisted of soil without nematodes with acquisition and bait plants and soil with nematodes but without acquisition plants.

Properties in crude juice. TomRSV-Eu was inactivated by 10 min of exposure to 60 C but not to 55 C. Preparations diluted with water or 0.01 M sodium phosphate buffer, pH 7, were infectious at 10^{-2} but not at 10^{-3} dilution. Preparations of crude juice from TomRSV-Eu-infected cucumber or tobacco leaves, undiluted or diluted 1:5 with 0.01 M sodium phosphate buffer, pH 7, and stored at 22–25 C, were infectious after 24 hr but not after 28 hr. The addition of 2-mercaptoethanol or sodium thioglycolate to a concentration of 1% in crude sap did not prevent the rapid loss of infectivity. Crude juice from TomRSV-Eu-infected petunia leaves stored at 4 C was infectious for 26 days but not at 28 days and at −20 C retained infectivity after 128 days but not after 160 days.

Relative concentration of TomRSV-Eu in plant extracts. The maximal relative concentration, as measured by infectivity, occurred in leaves of fava bean approximately 20 days after inoculation (Fig. 8). Virus could be recovered from the inoculated cotyledons of cucumber 2 days after inoculation. The concentration in cucumber cotyledons increased until 6 days, then dropped rapidly, and virus could not be recovered after 12 days. The maximum virus concentration in uninoculated cucumber leaves occurred after 8–10 days, decreased rapidly, and was not detectable 20 days after inoculation. Rapid reduction in virus concentration in both inoculated and uninoculated leaves of cucumber was related to the development of necrosis. The concentration in infected petunia leaves reached a maximum 14–16 days after inoculation and decreased much slower than in cucumber. The highest concentration occurred in fava beans and the loss of infectivity was much slower than in cucumber or petunia. In all plants that were tested the peak concentration of TomRSV-Eu was reached before the maximal severity of symptoms was expressed. The virus concentration, especially in the case of cucumber, began to decline before the most severe symptoms were observed and continued to decrease during and after the development of necrosis.

Purification. Systemically infected leaves of fava beans, 20 days after inoculation, gave average yields of 20 mg of virus per kilogram of tissue. Of the various purification procedures that were tried, the most consistently infectious preparations of TomRSV-Eu were obtained by extracting in the presence of (w/v) 0.5M citrate buffer containing 0.1% TGA and 0.01 M Na DIECA, pH 6.2, freezing the homogenate for 24 hr or more, thawing, homogenizing it a second time in a Waring blender, and screening the mixture through cheesecloth. Clarification of plant extracts with chloroform and/or butanol, or by the use of adsorbents (hydrated calcium phosphate or activated charcoal) greatly reduced infectivity. Infectious clarified preparations were obtained by acidifying extracts to pH 4.6 with acetic acid or by the addition of 15% ammonium sulfate (w:v) and low-speed centrifugation. Clarified extracts at pH 4.6 retained infectivity for 2–3 mo at 4 C. Concentration of TomRSV-Eu with 6–10% polyethylene glycol 6000 or 40–50% ammonium sulfate gave preparations with low infectivity, whereas preparations from two or three cycles of differential centrifugation (1.5–2.0 hr at 66,000 *g* and 10–15 min at 12,000 *g*) retained infectivity for 6 mo when stored at 4 C.

Ultraviolet absorption. The UV absorption spectrum of TomRSV-Eu was similar to that of nucleoproteins with an average maximum (260 nm): minimum (240) absorption ratio of 1.4 and a 260:280 nm ratio of 1.8 which agrees with that reported for TobRSV and TomRSV (25,26). The estimated percent nucleic acid in TomRSV-Eu by the spectrophotometric method of Englander and Epstein (11), in association with dry weight determinations, was 40.

Density-gradient centrifugation. Partially purified preparations of TomRSV-Eu at a concentration of 0.4 mg/ml gave two light-scattering zones after 2 hr of centrifugation at 52,000 *g* in a 20–40% sucrose gradient (Fig. 9). In comparison to the sedimentation of SBMV and TobRSV, the top and bottom components of TomRSV-Eu had sedimentation coefficients of 52 ± 2S and 131 ± 3S, which compare to the values reported for TobRSV and TomRSV (25,26). The 52S light-scattering zone contained mainly incomplete (ghost) particles and the 131S zone, although not tested by a continuous UV-scan, most likely consisted of two nucleic acid-containing components similar to those reported by Schneider et al (24). The estimated molecular weights, based on sedimentation, of 3.3×10^6 and 5.7×10^6 daltons for top and bottom components, respectively, of TomRSV-Eu also compare with the reported values of TomRSV. The 40% nucleic acid of TomRSV-Eu, as determined by UV spectroscopy, indicates that the molecular weight of the nucleic acid in the bottom component is 2.3×10^6 daltons which compares with that reported by Schneider et al (24) and Dias and Allen (10). The values established by Reichman (19) for determining percent nucleic acid from the ratio of sedimentation coefficients of full particles (Z_2) to empty particles (Z_1) indicate that the nucleic acid content of TomRSV-Eu is 42 ± 2%, which compares to the values established by spectroscopy and reported for viruses of the nepo group (12).

Electron microscopy. Chromium shadowed leaf-dip preparations from TomRSV-Eu-infected petunia and euonymus leaves contained isometric viruslike particles that could be distinguished from cellular material. Comparable particles were not seen in preparations from healthy petunia or euonymus leaves. Electron microscopy of partially purified virus preparations that were chromium shadowed or in 2% phosphotungstic acid showed polyhedral particles that had an average diameter of 28 nm (Fig. 10). Electron microscopy of ultrathin sections from TomRSV-Eu-infected euonymus leaf tissue showed viruslike particles about 27 nm in diameter aligned in tubular membranes in the plasmodesmata (Fig. 11) similar to that reported for TobRSV (20).

Serology. In microprecipitin tests, antiserum prepared to partially purified TomRSV-Eu had an homologous titer of 1:512. Positive reactions also were obtained in microprecipitin tests with apricot strain of TomRSV. TomRSV-Eu also reacted in microprecipitin tests with antisera to the peach yellow bud strain of TomRSV and to the ATCC PVAS-15 isolate of TomRSV.

In reciprocal gel double-diffusion tests, precipitin zones of identity were obtained when TomRSV-Eu was reacted with antisera to the apricot or tobacco strains (ATCC PVAS-15) of TomRSV (Figs. 12 and 13). No precipitin zones were obtained in gel diffusion tests when purified preparations of TomRSV-Eu were placed in the center well against antisera in the peripheral wells to arabis mosaic (two isolates), carnation ringspot, cherry leaf roll, cucumber mosaic, dogwood ringspot, southern bean mosaic, strawberry latent ringspot (two isolates), tobacco necrosis, tobacco ringspot (two isolates), tomato tip necrosis, and turnip yellow mosaic viruses. TomRSV-Eu is serologically identical to the tobacco and apricot strains of TomRSV.

We also found, as reported by Koenig (13), that precipitin zone formation did not occur in homologous or heterologous reactions with TomRSV-Eu and strains of TomRSV if the agar contained 1:10 dilution of Merthiolate (Thimerosal) as a preservative.

Effect of TomRSV-Eu infection on euonymus plants. In addition to the symptoms induced on young leaves of euonymus cultivars, root development and growth of the infected cuttings were greatly reduced when compared with noninfected cuttings grown under greenhouse conditions. The infected and "healthy" cuttings developed roots but, after a 4 wk rooting period, the amount of roots on healthy cuttings was approximately three times the amount on TomRSV-Eu-infected cuttings. The roots of the healthy cuttings were longer, more diffuse and had more growing root tips than the roots on infected cuttings. After 16 wk of growth under greenhouse conditions, healthy plants had three times more shoots or branches that were three times longer than shoots or branches on infected cuttings (Fig. 14). The decreased vigor of infected plants could reflect the reduced root development, which was approximately 33% of that produced on healthy plants.

DISCUSSION

Euonymus plants exhibiting viruslike symptoms of vein clearing, vein banding, chlorosis, variegation, yellow mottle, mosaic, and light-green or white mosaic have been reported in Europe and North America (9). The agent or agents associated with some of the symptoms have been graft transmitted (23) or mechanically transmitted to herbaceous hosts (5). The euonymus mosaic reported in Czechoslovakia is an enigma. The virus (EMV) was reported to be mechanically and aphid transmitted by Bojnansky and Kosljarova (5), but Mali and Hooper (16) reported that it was transmitted by the nematode *L. euonymus*. Mali (15) later reported that EMV was soilborne, serologically related to tobacco necrosis and transmitted by the fungus *O. brassicae*.

Nepoviruses often induce chlorotic or necrotic ringspots and line patterns. Since rod-shaped viruslike particles were not detected by electron microscopy of leaf-dip preparations from infected euonymus leaves it was logical to compare the mechanically transmitted virus from euonymus with tobacco and tomato ringspot viruses that commonly occur in North America (25,26).

McLean (17) listed differential host reactions that he considered useful in distinguishing isolates of TobRSV and TomRSV. Unfortunately, they were of limited value as the host range of TomRSV-Eu was intermediate. The response of *Chenopodium amaranticolor*, *Citrullis vulgaris*, *Cucurbita maxima*, *Gypsophila elegans*, *Petunia hybrida*, *Solanum melongena*, *Portulaca grandiflora*, and *Spinacia oleracea* to TomRSV-Eu was similar to that reported for TomRSV, whereas the response of *Antirrhinum majus*, *Arachis hypogaea*, and *Phaseolus limensis* plants to TomRSV-Eu was similar to that for TobRSV. Many of the experimental test plants responded similarly to infection by all three viruses and were of limited use for virus identification. The greatest difference between TomRSV-Eu and either TomRSV or TobRSV was the inability of TomRSV-Eu to systemically infect tobacco plants or to infect plants of 14 different tomato cultivars. Also, TomRSV-Eu readily infected euonymus whereas the strains of TomRSV and TobRSV tested did not. These results were surprising but may be expected since plant response to viral infection is the most variable virus character (21).

Related viruses usually have similar methods of transmission. TomRSV-Eu was readily transmitted by mechanical inoculation, grafting, dodder (*C. campestris*) and nematode (*X. americanum*), similar to TobRSV and TomRSV transmission except for the dodder. TobRSV and TomRSV have not been transmitted by any species of dodder tested (3,25,26).

The physical properties of thermal inactivation, dilution end point, and aging in vitro of TomRSV-Eu are within the range reported for viruses of the nepo group. Also, particle size (28 nm diameter), composition (40% RNA and 60% protein), and sedimentation coefficients of TomRSV-Eu are similar to those of the nepovirus group and are of limited value for identification (12).

Serological cross reaction is the criterion most frequently used to demonstrate strain relationships among virus isolates. Precipitin zones of identity in reciprocal tests between TomRSV-Eu and the apricot strain of TomRSV show that the two isolates are serologically identical. The tobacco and peach yellow bud mosaic strains of TomRSV are serologically identical (8) and the apricot strain also forms confluent precipitin zones in gel-diffusion tests with these strains indicating similar or identical antigenic sites for these isolates of TomRSV. These positive serological tests show that the chlorotic ringspot virus of euonymus is a strain of tomato ringspot virus.

The disease in euonymus is readily recognized by chlorotic ringspots and line patterns on the spring growth but symptomatic leaves drop prematurely and by midsummer infected plants, because of premature leaf drop and recovery, may appear "healthy." Such infected plants may inadvertently be used for vegetative propagation resulting in unthrifty plants and spread of the virus. Plant material for propagation should be taken only from indexed parent plants.

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