Isolation of Tobacco Ringspot Virus from Rose

G. L. McDaniel, G. J. Buck, and R. E. Ford

Graduate Research Assistant and Associate Professor, Department of Horticulture, and Professor, Department of Botany and Plant Pathology, respectively, Iowa State University, Ames 50010.

Journal Paper No. J-6593 of the Iowa Agriculture and Home Economics Experiment Station, Ames. Project No. 1453.

The authors wish to thank the American Rose Foundation for financial assistance and R. W. Fulton and R. G. Grogan for providing antisera for serological tests.

Accepted for publication 5 August 1970.

ABSTRACT

Tobacco ringspot virus (TRSV) was isolated from petals of roses with line-pattern mosaic leaf symptoms. TRSV was purified from tobacco tissue by the chloroform-butanol differential centrifugation method. Host range, physical properties, and serological properties confirmed the identity of the virus, which has previously been called rose line-pattern mosaic virus and is listed in the rose mosaic virus (RMV) group according to symptoms. The virus was not serologically related to RMV, cherry necrotic ringspot virus, or tomato ringspot virus. Phytopathology 61:45-49.

Rose mosaic virus (RMV) has been reported throughout the USA and in other countries where roses are grown (3, 11, 16). Viruses infecting other rosaceous plants also have been found in roses, such as Prunus necrotic ringspot virus (NRSV) (9, 12) and tomato ringspot virus (TomRSV) (10). In addition, peach yellow bud mosaic virus (PYBMV) (17) and apple mosaic virus (AMV) (15) have been transmitted to rose.

RMV was isolated and has been demonstrated serologically distinct from the other viruses found in rose (7, 8). There is serological evidence suggesting that RMV, AMV, and Danish plum line-pattern virus represent two serotypes, each composed of two strains (9). In cross-absorption tests, RMV and AMV were serologically identical.

Three characteristic symptom forms for RMV have been described (6, 11): vein-banding mosaic virus, chlorotic mottle virus, and line-pattern mosaic virus (LPMV). The LPMV symptom form of RMV occurs widely in the USA and is one of the more prevalent viruses affecting nursery-grown roses (1). We isolated and purified the virus, causing LPMV symptoms in roses, to determine its relationship with other viruses found naturally in field-grown roses.

MATERIALS AND METHODS.—*Transmission.*—The virus was transmitted by bud grafts from field-grown roses to several seedling understocks and to *Rosa multiflora* Thunb. seedlings. Tobacco ringspot virus (TRSV) was transmitted to *Nicotiana tabacum* L. type Turkish, *Vigna sinensis* Endl. 'Early Ramshorn', and *Cucumis sativus* L. 'Select National Pickling' from fresh rose petals ground in 0.01 M phosphate buffer, pH 7.2, by mortar and pestle. Inoculations were made by wiping Carborundum-dusted leaves with gauze pads dipped in virus-containing extracts, and the leaves were rinsed with distilled water. All assays were made on cowpea, which produced local lesions 5 days after inoculation with TRSV. Each sample tested was assayed by the whole leaf method on a min of 5 plants.

Host range.—A host range for the virus was determined by inoculating 40 species in 13 plant families. Inoculum was taken from systemically infected Turkish tobacco inoculated 14 days earlier. All plants not expressing symptoms to infection by TRSV were tested by inoculations made from the test species to cowpea to determine whether infection resulted.

Physical properties.—Turkish tobacco plants were inoculated to increase the virus for study of thermal inactivation (TIP), dilution end point (DEP), and longevity in vitro (LIV). Systemically infected leaves were macerated without buffer.

For TIP studies, 0.2 ml of undiluted sap were placed in thin-walled test tubes (15 × 30 mm), heated 10 min, cooled immediately, and assayed on five cowpea plants. For DEP determination, 12 twofold dilutions were made by grinding known amt of tissue in a volume (g/ml) of 0.01 M phosphate buffer, pH 7.2, calculated to give the desired dilution and assayed on cowpea. For studying LIV, sap was diluted 1:2 in 0.01 M phosphate buffer and held at 25 C or −14 C. Samples held at 25 C were assayed each hr for the first 8 hr and each 8-hr period thereafter for 5 days. Samples from frozen tissue were assayed daily for 15 days, then 3, 6, and 8 weeks after freezing. Assays were made on cowpea.

Stability of the virus in desiccated tissue was tested, as described by McKinney (13). Systemically infected Turkish tobacco leaves were dried and stored at −14 C. The tissue was tested for infectivity after 10, 20, 60, 90, and 180 days by resuspension and grinding in buffer and assaying on cowpea.

Purification.—The virus was purified by the modified chloroform-butanol procedure (14). Two volumes of a 1:1 mixture of cold *n*-butanol and chloroform were added to one volume of expressed tobacco sap in 0.01 M phosphate buffer (1:1). This mixture was subjected to differential centrifugation at 106,000 *g* for 120 min in a Spinco Model L ultracentrifuge, followed by 4,080 *g* for 30 min. Two more cycles of differential centrifugation were used to further purify the virus. The resulting pellet was resuspended in distilled water to yield a 1:20 concn of the virus and was stored at −14 C.

Serology.—Antiserum was prepared by injecting a rabbit with purified virus. Normal serum was obtained by cardiac puncture before virus injections. Intravenous injections at 2-day intervals were administered in the

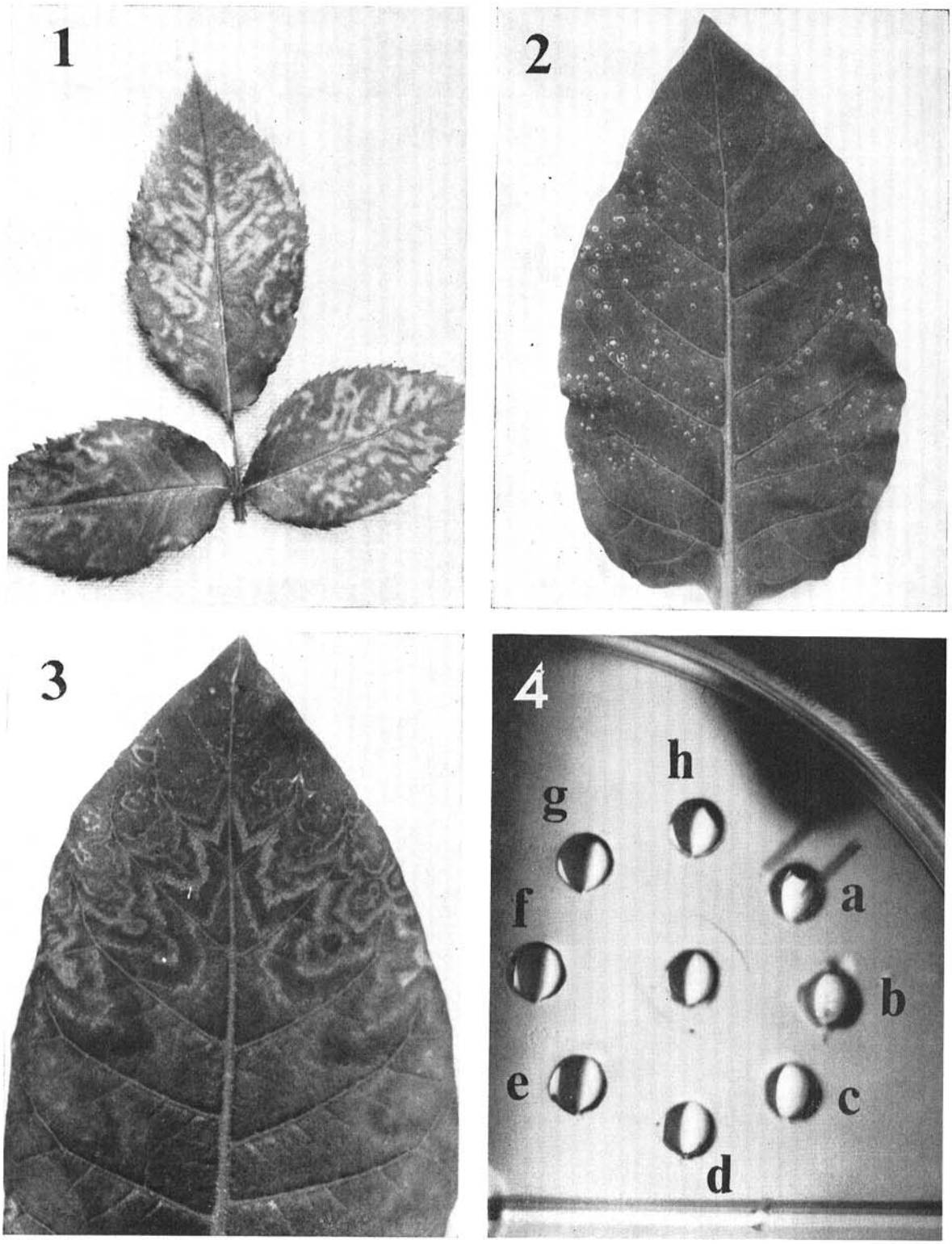

Fig. 1-4. 1) Line-pattern symptoms on *Rosa multiflora* leaves. 2) Primary ringspots on Turkish tobacco leaves inoculated with tobacco ringspot virus (TRSV) causing line-pattern symptoms on rose. 3) Systemic rings and broad bands on Turkish tobacco caused by rose TRSV infection. 4) Ouchterlony agar double-diffusion plate showing antigen-antibody precipitation zones. Wells contained antisera for the following viruses: (a) TRSV (Microbiological Associates); (b) rose mosaic virus; (c) tomato ringspot virus; (d) cherry necrotic ringspot virus; (e) TRSV (Grogan); (f) tobacco mosaic virus; (g) normal serum; (h) physiological saline. The center well contained purified TRSV from rose.

TABLE 1. Host range of tobacco ringspot virus causing line-pattern mosaic of rose

Species tested	Symptoms[a] Inoculated	Systemic
Apocynaceae		
Vinca rosea L. 'Pinkie'		VCL, St
Begoniaceae		
Begonia semperflorens Link. 'Geneva'	—	—
Chenopodiaceae		
Beta vulgaris L. 'Baby Canning'	CIR	VCl, OL
Chenopodium album L.	NLL	—
C. amaranticolor Coste & Reyn.	NLL	—
C. quinoa Willd.	NLL, Cl	Cl
Spinacia oleraceae L. 'Bloomsdale'	—	—
Compositae		
Chrysanthemum morifolium L. 'Prairie Sun'	—	—
Zinnia elegans Jacq. 'Fire Flame'	CIR	ClR, St
Cruciferae		
Brassica oleracea L. 'Early Spartan'	+	+
Cucurbitaceae		
Citrullus vulgaris Schrad. 'Dixie Queen'	+	
Cucumis melo L. 'Hale's Best'	Cl	ClR, Cl, M
C. Sativus L. 'National Pickling'	Cl	ClR, Cl, M
Cucurbita maxima Duchesne 'Butternut'	N	NR, M
Momordica balsamina L.	—	—
Geraniaceae		
Pelargonium hortorum Bailey 'Cardinal'	—	—
Labiatae		
Coleus blumei Benth. 'Candidum'	CIR	ClR, OL
Leguminosae		
Cassia occidentalis L.	NLL	N
C. tora L.	NLL	N
Cyamopsis tetragonolobus L.	NLL	E, M, D
Indigofera endecaphylla L.	NLL	—
I. hirsuta L.	NLL	—
I. subulata L.	NLL	—
I. tinctoria L.	NLL	—
Phaseolus aureus Roxbg. 'Large Oriental'	CIR	ClR, St, M
P. vulgaris L. 'Bountiful', 'Red Kidney'	NS	ClR, N, D
P. vulgaris L. 'Wade', 'Improved Tendergreen', 'White Marrowfat', 'Pea Bean', 'Resistant Cherokee', 'Valentine'	NS	ClR, N
Pisum sativum L. 'Progress No. 9'	+	
Vicia faba L. 'Long Pod Fava'	NLL	N
Vigna sinensis Endl. 'Early Ramshorn'	NLL	N, D
Malvaceae		
Abutilon theophrasti Medic.		E, D
Scrophulariaceae		
Antirrhinum majus L. 'Glacier'	NR	NR, N, D
Solanaceae		
Capsicum annuum L. 'Yolo Wonder'	—	—
Lycopersicon esculentum Mill. 'Diamond State'	—	—
Nicotiana glutinosa L.	NR	Cl, St, Rec
N. tabacum L. 'Samsun NN'	NR	Cl, St, Rec
N. tabacum L. type Turkish	NR	N, OL, St, Rec
Petunia hybrida Vilm. 'Red and White Delight'	CIR	M, St, Rec
Solanum melongena L. 'Black Beauty'		M, RS, Cl
S. tuberosum L. 'Norland'	+	

[a] Plants were inoculated with expressed tobacco sap in phosphate buffer. Abbreviations: NLL = necrotic local lesions; NR = necrotic rings; CIR = chlorotic rings; NS = necrotic spots; VCl = veinal chlorosis; Cl = general chlorosis; OL = oak leaf pattern; St = stunted, M = mottled; E = epinasty; D = eventual death of the plant; Rec = recovery from symptoms; + = symptomless Carrier; — = noninfected. The presence of the virus was confirmed by reindexing on Turkish tobacco and *Vigna sinensis*.

following volumes: three injections of 0.5 ml; one of 0.75 ml; and one of 1.00 ml of the virus. These intravenous injections were followed by subcutaneous injections of 2 ml of purified virus emulsified with 2 ml Difco incomplete Freund's adjuvant 2 weeks after the last intravenous injection. Antiserum was collected by cardiac puncture 2 weeks after the subcutaneous injections.

Antisera titers were determined by the microprecipitin method in plastic plates (2). Controls consisted of saline, normal serum, and healthy clarified tobacco sap. Serological identification of the virus was accomplished by the Ouchterlony agar double-diffusion method (20). Eight wells (4 mm in diam and spaced 6 mm apart) were prepared around a central well. Various antisera were placed in the outside wells with the virus isolate occupying the center well. Wells were covered with 0.02% NaN_3, and the plates incubated at 25 C for 24 hr.

RESULTS.—*Symptoms and transmission.*—Symptoms appeared on leaves of LPMV-infected *Rosa multiflora* as pale green, creamy-white, or yellow wavy lines, broad bands, ringspots, or blotches, which remain throughout the growing season (Fig. 1). A previously reported (5) stem necrosis occurring directly beneath the developing flower bud was not observed.

Inocula prepared from rose petals during the spring produced symptoms in inoculated herbaceous hosts. Prominent lesions formed on the primary leaves of cowpea within 5 days after inoculation. Systemic infection of leaf veins and trifoliolate leaves resulted in death of the plants within 2 weeks. Symptoms occurring on inoculated leaves of Turkish tobacco began with minute necrotic rings that were visible in 2-3 days (Fig. 2). Secondary and tertiary rings formed around primary

lesions within 10-14 days after inoculation. These rings eventually expanded to form broad bands and line formations (Fig. 3). Newly formed leaves were nearly devoid of symptoms, and assays on cowpea from these leaves demonstrated a marked reduction in lesion formation. Reciprocal inoculations from tobacco to healthy rose by sap transmission were not successful.

Host range.—The typical virus symptom for most susceptible hosts was a systemic ringspotting of newly formed primary and secondary leaflets (Table 1). Turkish tobacco consistently yielded the most inoculum of all plant species tested. No symptoms were observed on *Momordica balsamina* L., a common assay host for RMV.

Physical properties.—In 10 separate trials, the virus was inactivated after heating for 10 min at 64 C, but not after heating 10 min at 62 C. The DEP of the virus was between 1:10,000 and 1:50,000 (Fig. 5). Virus infectivity was rapidly lost upon standing at 25 C, and was completely inactive after 72 hr.

Frozen virus extracts remained infective throughout the 8-week testing (Table 2). Desiccated leaf tissue retained infectivity for 180 days, and was an adequate method for preserving the virus for short periods.

Purification.—The modified chloroform-butanol purification procedure was satisfactory for retaining most of the virus in the final pellet. Microprecipitin cross-reactions with homologous antiserum consistently gave a purified virus titer of 32 and, occasionally, a titer of 64.

Serology.—Rabbit antiserum produced against the virus isolate had a titer between 1,024 and 2,048 by the microprecipitin reactions with the virus. Immunodiffusion precipitation reactions occurred between the purified virus and its homologous antiserum and TRSV antisera (Fig. 4). All reactions between purified virus and antisera were of the same intensity and equal DEP. Extracts from a TRSV-infected Turkish tobacco leaf confirmed the identity of the virus isolate by a positive reaction with the antiserum produced against the virus isolate and both TRSV antisera. The virus isolate did not react with NRSV, RMV, or TomRSV antisera. Reciprocal tests with these viruses and antisera were not made.

DISCUSSION.—Symptomatology and general properties of TRSV from roses suggest its close relationship to the type strain of TRSV. This relationship was confirmed by serological data obtained. Therefore, we concluded that the LPMV observed in Iowa roses is caused by TRSV infection.

Use of petals as a virus source offers several advantages. Formation of quinones from naturally occurring phenolic substances by the action of the polyphenol oxidase enzyme system usually are not encountered in petal extracts (19). Therefore, the high tannin content (polyphenol substances) of rose leaf extracts may be avoided. In addition, viruses not transmissible to herbaceous hosts may be detected serologically in petal preparations.

Most rose cultivars bud-inoculated with TRSV did not show symptoms obviously different from roses infected with RMV, NRSV, or TomRSV (18). Typical RMV produced patterns of chlorotic lines and rings in rose leaves (7). Symptoms varied during the growing season and with species of *Rosa*. Chlorotic ringspots also have been reported on roses infected with Arabis mosaic virus (4). It cannot be concluded, however, that all rose viruses mentioned give similar symptoms on a given rose cultivar until this can be demonstrated by controlled inoculations.

Further studies comparing the serological and biochemical properties of rose viruses will be necessary to elucidate their relationships with one another. All viruses of rose that have been studied extensively are spherical viruses, and are evidently similar in size.

TRSV has a wide natural host range, and has been

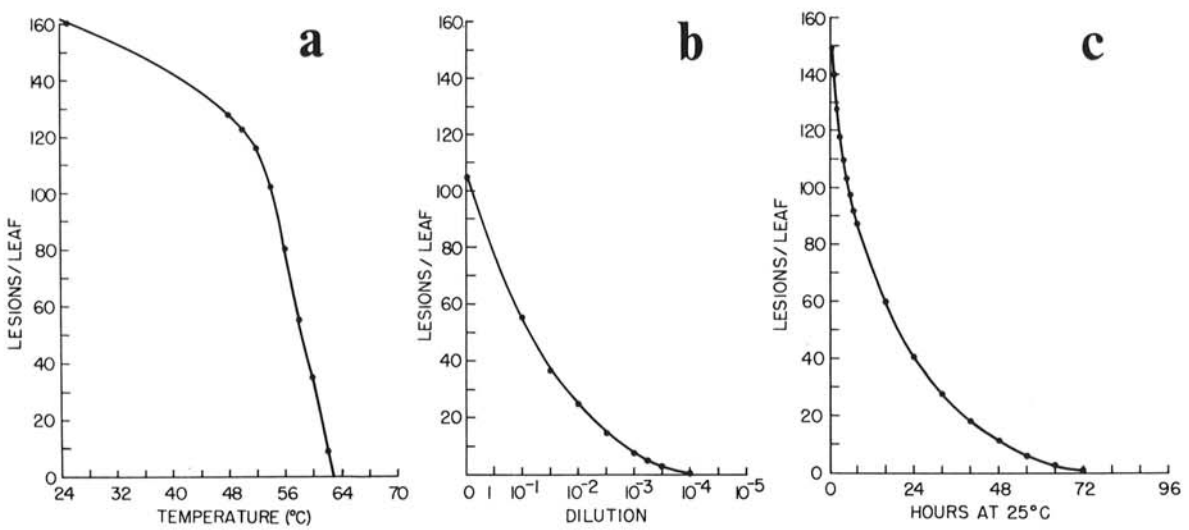

Fig. 5. Physical properties of tobacco ringspot virus causing line-pattern symptoms of rose. **a)** Thermal inactivation point; **b)** dilution end point; and **c)** Longevity in vitro. Each point represents average lesions per leaf of five cowpea plants.

TABLE 2. Longevity of tobacco ringspot virus causing line-pattern mosaic of rose in systemically infected Turkish tobacco tissue

Frozen extract[a]		Desiccated tissue[b]	
Days	Lesions[c]	Days	Lesions
1-14	200+	10	155.4
15	147.3	20	127.4
21	128.1	30	95.7
42	121.6	60	86.7
56	119.3	90	72.5
		180	47.3

[a] Expressed sap of Turkish tobacco in 0.01 M phosphate buffer, pH 7.2, was stored at —14 C.

[b] Systemically infected Turkish tobacco leaves were dried over calcium chloride crystals, cut into small strips, and stored in sealed vials over the desiccant at —14 C.

[c] Avg lesions per leaf of 10 cowpea leaves.

transmitted experimentally to many species in a large number of families of flowering plants. This first report of TRSV in roses, either naturally or by inoculation, suggests a possible susceptibility to viruses of the nematode transmitted-polyhedral virus (NEPO) group. PYBMV, a strain of TomRSV, and TomRSV have already been mentioned. These viruses are related to TRSV on the basis of their morphology and transmission by nematode vectors. Natural vectors for RMV and TRSV of rose are unknown. Comparison of RMV and this TRSV strain will be necessary to determine whether they are related.

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