Andean Potato Calico Strain of Tobacco Ringspot Virus

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ABSTRACT

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Tobacco ringspot virus (TRSV) was isolated from Peruvian potato cultivars showing yellowing symptoms that resemble potato calico disease. The virus is widespread in different potato growing areas of Peru. It produced typical symptoms of TRSV in most indicator hosts, but unusual necrotic local lesions in *Cucumis sativus* and systemic apical necrosis in *Chenopodium quinoa, C. amaranticolor,* and *Petunia violaceae*. Its dilution end point was 10^{-4}-10^{-5}, thermal inactivation point 55-60 C, and longevity in vitro 9-11 days. In immunodiffusion tests, the virus and its homologous antiserum formed spurs against TRSV strains NC-38, NC-39, NC-72, and NC-87 from tobacco and against the Eucharis mottle and Texas strains. In reciprocal tests, spurs also were produced against all these six strains. The virus can be considered as a new strain of TRSV and the name Andean potato calico strain is proposed.

Additional key words: potato viruses, serology.

RESUMEN

El virus tobacco ringspot (TRSV) fue aislado de cultivares peruanos de papa que mostraban síntomas de amarillamiento parecido a cálico. Este virus está distribuído en diferentes regiones donde se cultiva papa en el Perú. Produjo síntomas típicos de TRSV en la mayor parte de plantas indicadoras, pero síntomas poco usuales de manchas necróticas locales en *Cucumis sativus* y necrosis sistémica apical en *Chenopodium quinoa, C. amaranticolor,* y *Petunia violaceae*. Su punto final de dilución fue de 10^{-4}-10^{-5}, el punto de inactivacion termal 55-60 C, y la longevidad in vitro 9-11 días. En pruebas de inmunodifusion el virus y su antisuero homólogo formó espuelas contra los strains de TRSV NC-38, NC-39, NC-72, y NC-87 de tabaco y contra los strains Eucharis mottle y Texas. En pruebas recíprocas se formaron también espuelas contra todos estos seis strains. Este virus se puede considerar como un nuevo strain de TRSV por lo que se propone el nombre de Andean potato cálico strain.

Palabras claves adicionales: virus de papa, serología.

Calico or calico-like symptoms are produced in potato by alfalfa mosaic (3), potato aucuba mosaic (6), potato mop-top (4), tomato black ring (2, 14), and tobacco rattle viruses (16). Potato plants of the cultivar Ticahuasi (*Solanum tuberosum* subsp. *tuberosum* L. × *S. tuberosum* subsp. *andigena* Juz. & Buk.) commonly show yellowing symptoms similar to calico disease in fields in the central coastal region of Peru. Preliminary studies indicated the presence of tobacco ringspot virus (TRSV) (8, 9). This paper (i) describes an investigation of the symptomatology, host range, physical properties, and serology of the virus and (ii) shows that it is a new strain of TRSV.

MATERIALS AND METHODS

Virus source.—The virus was isolated from leaves of infected potato plants by inoculations to *Nicotiana occidentalis* Wheeler, *Vigna cylindrica* Skeels, and *Chenopodium quinoa* Willd. Five or six single-lesion transfers were made in inoculated leaves of *C. quinoa* to separate it from possible mixtures with other strains of the same virus. Final inoculations were made to *Cucumis sativus* L. and *Nicandra physaloides* Gaertn., which reacted with mosaic symptoms and were used as source of inoculum for all experiments.

Plants.—Indicator hosts were started from true seed. The seedlings were transplanted to pots containing steam sterilized muck soil. Wild *Solanum* species came initially from true seed obtained from the Potato Introduction Station, Sturgeon Bay, Wisconsin, U.S.A. They later were propagated by cuttings rooted in tap water; Peruvian cultivars were propagated by cuttings from plants previously checked as free from PVX, PVY, and PVS by inoculation to suitable indicator hosts. Three of the local cultivars used, Ccompis, Huagalina, and Imilla negra (all, *S. tuberosum* subsp. *andigena* native to Peru) came initially from true seed. For studies on host range and symptomatology in indicator plants, three rapidly growing plants were inoculated per trial and trials were

repeated at different seasons. All experiments were done under greenhouse conditions at 18-25 C. Mechanical inoculations were made by rubbing 22-μm (600-mesh) Carborundum-dusted leaves with a cotton-tipped applicator dipped in sap inoculum. Extracts were prepared by grinding infected leaves in 0.03 M phosphate buffer pH 8. For studies on physical properties, each treatment was inoculated to cotyledonal leaves of 10 cucumber plants or to five plants of *Chenopodium amaranticolor* Coste & Reyn. The lesions produced were counted.

Purification.—The virus was purified by precipitation with polyethylene glycol and ultracentrifugation. Systemically infected leaves of *N. physaloides* were homogenized (1:1, w/v) with 0.02 M Na_2SO_3 prepared in distilled water. The extract was shaken in a separatory funnel with a mixture of 4 ml of chloroform plus 4 ml of butanol per 100 ml of sap and centrifuged at 5,000 rpm for

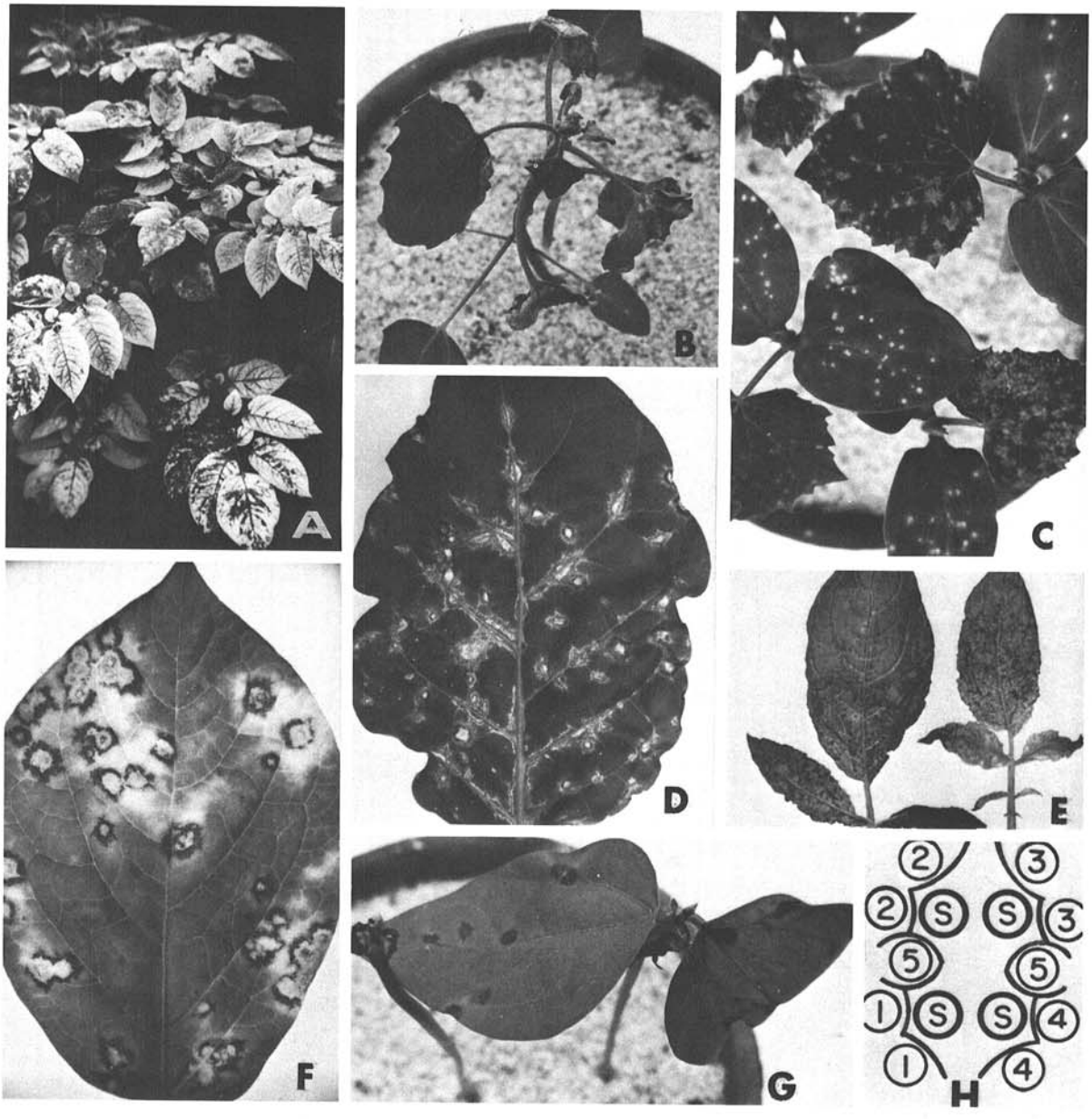

Fig. 1-(A to H). Symptoms and serological reactions of Andean potato calico strain of tobacco ringspot virus: **A)** calico symptoms in potato cultivar Ticahuasi; **B)** systemic apical necrosis in *Chenopodium quinoa;* **C)** local necrotic spots and systemic chlorotic spotting in *Cucumis sativus;* **D)** systemic ringspots in *Nicotiana tabacum;* **E)** systemic necrosis in *Solanum chanacayense;* **F)** necrotic rings in inoculated leaves of potato clone A6; **G)** local necrotic spots and systemic apical necrosis in *Vigna sinensis;* **H)** drawing representative of the serological reactions between calico antiserum (S) and TRSV strains NC = 38 (1), NC-39 (2), NC-72 (3), and NC-87 (4). Wells marked "5" were filled with calico virus.

20 minutes in the GSA rotor of a Sorvall RC-2 centrifuge. The supernatant fluid was made 0.1 M with sodium chloride, and PEG 6000 (polyethylene glycol, mol wt 6,000) was added at the rate of 8 g per 100 ml. The mixture was incubated for 2 hours and then centrifuged at 7,000 rpm for 30 minutes in the same rotor. The pellet was resuspended in 0.01 M phosphate buffer pH 7 and stored overnight at 2 C. After a low-speed centrifugation, the supernatant fluid was centrifuged at 40,000 rpm for 1 hour using rotor No. 40 in a Beckman L2-65B ultracentrifuge. Final pellets were resuspended in the same buffer.

Serology.—Antiserum was prepared by injecting a rabbit twice (1 week interval) intravenously with 1 ml of purified virus. After 2 months, four intramuscular injections were given at weekly intervals with a mixture (1:1, v/v) of purified virus and Freund's incomplete adjuvant. For titration of antibodies a modification of the microprecipitation test described by Ball (1) was used. Drops of antiserum and antigen were placed on plastic petri plates, mixed by putting the plates in a mechanical shaker for 10-15 minutes and then incubated in a moist chamber for 1 hour.

For Ouchterlony gel precipitin plates, purified Difco agar containing 1% sodium azide was used. Holes were punched with a cork borer (diameter 4 mm) at a distance of approximately 3 mm between the antisera and antigen wells. Antigens for serological tests were prepared by homogenizing infected leaves with chloroform (1:3, w/v) plus 2-mercaptoethanol to make a concentration of 0.02 M followed by low-speed centrifugation.

The other viruses used, along with their homologous antisera, were TRSV strains NC-38, NC-39, NC-72, NC-87, and the *Eucharis* mottle, and Texas strains which were kindly supplied by G. V. Gooding, North Carolina State University. All were maintained in *N. physaloides* except NC-87 and *Eucharis* mottle kept either in *Nicotiana megalosiphon* Heurck & Muell. or inoculated leaves of *N. tabacum* L.

RESULTS

Field symptoms and distribution.—The calico-like disease frequently is found in potato fields of the cultivar Ticahuasi in the irrigated desert plantings of the central coastline of Peru planted with seed tubers from the Andean highlands. It also has been found in the highland localities of Huancayo, Huasa-Huasi, and Huánuco all of which are centers for seed-potato production. Initial symptoms are yellow areas starting at the leaf margins. The yellowing then tends to progress inwards until most of the leaf is affected (Fig. 1-A). No stunting or leaf deformation is observed.

To determine if the virus could be isolated only from plants showing symptoms, 17 samples were taken from plants of the cultivar Ticahuasi with symptoms and 10

TABLE 1. Reaction of indicator host plants to mechanical inoculations with tobacco ringspot virus from Peruvian potatoes

Species	Symptoms[a]
Gomphrena globosa L.	E, M, S
Chenopodium amaranticolor Coste & Reyn.	NLL, SN
Chenopodium quinoa Willd.	NLL, SN
Cucumis sativus L.	NLL, CSL
Crotalaria juncea L.	NLL
Cyamopsis tetragonoloba (L.) Taub.	NLL
Dolichos biflorus L.	NLL
Dolichos lablab L.	NLL
Phaseolus aureus Roxb.	NLL
Phaseolus vulgaris L.	NLL
Vigna cylindrica Skeels	NLL, SN
Vigna sinensis (Torn.) Savi	NLL, SN
Datura stramonium L.	NLL, NLR, SN
Lycopersicon pimpinellifolium (Jusl.) Mill.	NLL, NSL
Nicandra physaloides Gaertn.	M, VB
Nicotiana clevelandii Gray	NLL, SN
Nicotiana debneyi Domin.	NLL, NLR, R
Nicotiana glutinosa L.	NLL, NLR, R
Nicotiana megalosiphon Heurck & Muell.	NLL, SN
Nicotiana occidentalis Wheeler	NLL, NLR, M, SN
Nicotiana rustica L.	NLL, NLR, R
Nicotiana tabacum L.	NLL, NLR, R, SLP
Petunia violaceae Lindl.	SN
Physalis floridana Rybd.	M, VN
Solanum chancayense Ochoa (P.I. 338615)	NLL, SN
Solanum demissum × *S. tuberosum* 'Aquila' (clone A6)[b]	NLR
Solanum tuberosum subsp. *andigena* 'Renacimiento'	NSL
Solanum tuberosum subsp. *andigena* × *S. tuberosum* subsp. *tuberosum* 'Ticahuasi'	C

[a]Abbreviations for symptoms: C = calico, E = epinasty, CSL = chlorotic systemic lesions, M = mosaic, NLL = necrotic local lesions, NLR = necrotic local rings, NSL = necrotic systemic lesions, R = ringspotting, S = stunting, SN = systemic necrosis, SLP = systemic line patterns, VB = veinbanding, and VN = vein necrosis.

[b]Symptoms developed on detached leaves only.

from normal-looking ones from the same field in the coastal locality of Cañete. These were inoculated to *V. cylindrica* plants. Sixteen of the 17 induced local lesions followed by a systemic necrosis typical of TRSV (18, 19) and exactly as previously observed in earlier tests, but from the other 10 samples only one produced such symptoms. Other symptomatic samples from the highlands also were tested for the presence of TRSV. Two samples of the cultivar Renacimiento (one collected in Huánuco and one in Huasa-Huasi) plus two samples of the cultivar Mariva (one from Huasa-Huasi and one from Huancayo) induced typical ringspots in *N. tabacum,* systemic necrosis in *C. quinoa,* and reacted positively in immunodiffusion tests against TRSV antiserum.

Host range and symptomatology.—All the indicator species belonging to the families Amaranthaceae, Chenopodiaceae, Cucurbitaceae, Leguminosae, and Solanaceae that were tested became infected (Table 1) except *Capsicum pendulum* L. and tomato. In general, symptoms induced were of necrotic type, but *N. physaloides, Gomphrena globosa,* and *Physalis floridana* Rybd. also reacted with mosaic. Most *Nicotiana* species reacted with local necrotic spots or ringspots followed by a systemic necrosis. The isolate induced symptoms typical of those reported for TRSV (18, 19) in *N. tabacum* (Fig. 1-D), *Phaseolus vulgaris* L., *Vigna sinensis* (Torn.) Savi (Fig. 1-G), and *C. sativus* (Fig. 1-C); however, some reactions were not typical [e.g., the necrotic spots produced in the inoculated cotyledons of *C. sativus* and the systemic apical necrosis produced in *C. quinoa* (Fig. 1-B) and *C. amaranticolor*]. Necrotic rings were produced in detached leaves of the potato clone A6 (*S. demissum* × *S. tuberosum* 'Aquila') (Fig. 1-F), an indicator which reacts with local necrotic lesions to potato virus A and potato virus Y (13) and is used to test for these viruses in seed-potato production. *Nicandra physaloides* was the best host for maintaining the virus because it developed strong mosaic and veinbanding symptoms. It also developed mosaic or necrosis followed by mosaic with TRSV strains NC-38, NC-39, NC-72, and the Texas strain. The best diagnostic hosts for detecting the virus either from indicator hosts or potato were *C. quinoa, C. amaranticolor,* and *V. sinensis.*

The wild potato species *Solanum chancayense* Ochoa (P.I. 338615) developed necrotic spots in the inoculated leaves followed by extensive necrosis and death of some leaves (Fig. 1-E). The leaves that developed later showed little necrosis. Very young plants of the cultivar Renacimiento showed systemic necrotic spots, but adult plants became symptomlessly infected. Cultivar Ticahuasi showed no typical calico symptoms when inoculated mechanically in the winter time, but the virus was recovered by back-inoculation to *C. amaranticolor.* Apparently greenhouse temperatures were too high for symptom expression. When tubers from Ticahuasi plants infected in the field were harvested and planted under greenhouse conditions during the winter season, only mild calico symptoms developed which were not as clear as those observed in the field. At warmer times of the year there were no symptoms whatsoever, but the virus still could be recovered.

Tobacco ringspot virus previously has been found to infect several species of the genus *Solanum,* both naturally and by artificial inoculation (7). These included *S. tuberosum* which was infected artificially with a strain of TRSV isolated from beans (5), that reacted with local necrotic rings and systemic invasion without symptoms. Despite this, the following when inoculated at least twice with the calico virus showed no symptoms and the virus could not be recovered from newly developed leaves: *S. berthaultii* Hawkes (P.I. 265857), *S. cardiophyllum* Lindl. (P.I. 275215), *S. curtilobum* Juz. & Buk. (P.I. 186181), *S. chacoense* Bitt. (P.I. 275136), *S. demissum* Lindl. (P.I. 230579), *S. stenotomum* Juz. & Buk. (P.I. 230512), *S. stoloniferum* Schlecht. (P.I. 230557), and *S. tuberosum* subsp. *andigena* cultivars Ccompis, Huagalina, Imilla negra, and Sipeña.

Physical properties.—The dilution end point (DEP) found was 10^{-4}-10^{-5}, the thermal inactivation point (TIP) 55-60 C and longevity in vitro (LIV) 9-11 days at 18-22 C. This LIV is the same as previously reported for TRSV at room temperature (12, 18, 19). The TIP differed from most previous reports (18, 19); however, Lister et al. (15) reported a strain from blueberry having a TIP between 55-60 C.

Purification and serology.—The purified preparations had a brown glassy color and the absorption spectrum was of a typical nucleoprotein with the peak at 254 nm. The antiserum obtained had a titer of 1:128 in microprecipitin grid titrations using purified antigen and it did not react against centrifuged healthy sap of *N. physaloides.* In immunodiffusion tests, clarified sap infected with calico virus and TRSV strains NC-38, NC-39, NC-72, NC-87, *Eucharis* mottle, and Texas produced clear precipitation lines with the calico antiserum. The titers of this serum varied between 1/32 and 1/128 (Table 2), suggesting that the relationship of potato calico virus to all the TRSV strains is close. Spurs also were readily demonstrated (Fig. 1-H). In the reciprocal tests, precipitation lines and spurs were obtained in all cases, but for spur formation with strains NC-87, *Eucharis* mottle, and Texas, the antigen preparations first had to be partially purified and concentrated about 20-fold by one cycle of differential centrifugation. These results indicate that potato calico is a strain serologically different from the previously recognized strains of TRSV.

DISCUSSION

Although there have been reports of TRSV causing calico-like symptoms in cultivated potatoes in Europe (14), the causal virus was later shown to be another nepovirus, tomato black ring (2, 11). By contrast, on the basis of its host range, symptomatology, physical properties, and serology, there can be little doubt that the virus isolated from Peruvian potatoes with calico symptoms is TRSV. Because of spur formation in reciprocal reactions, this virus can be considered serologically different from the six strains previously recognized by Gooding (10), the four from tobacco, *Eucharis* mottle (12), and Texas originally isolated from *Citrullus vulgaris.* In addition, there are differences in symptomatology between the calico and *Eucharis* mottle strain, also from Peru. The latter does not become systemic in *C. amaranticolor,* whereas the calico strain induces systemic necrosis in this host. Also, the thermal inactivation point reported for the *Eucharis* strain is 65-

TABLE 2. Homologous and heterologous serological reactions[a] between potato calico (Ca) and six other strains[b] of tobacco ringspot virus

Antiserum to strain	Antigen[b]						
	Ca	38	39	72	87	Eu	Tex
Ca	128	32	32	64	32	64	32
38	16	64					
39	32		128				
72	8			64			
87	2				32		
Eu	4					512	
Tex	8						256

[a]Reciprocal values of titers in gel diffusion tests.
[b]Ca = potato calico; 38, 39, 72, 87 = strains from tobacco; Eu = *Eucharis* mottle; and Tex = Texas.

70 C, but only 55-60 C for the calico strain of TRSV.

In the Andean highlands, wide daily fluctuations in temperatures occur and the nights are cold. Under these conditions several cultivars develop calico symptoms when infected with TRSV. In contrast, only the cultivar Ticahuasi develops symptoms on the coast during the potato-growing season, during which the daily fluctuations in temperature are less and the nights are warmer. In the greenhouse on the coast at the coldest time of year only mild symptoms were obtained in this cultivar. The failure to reproduce calico symptoms by mechanical inoculation to potato, therefore, seems to have resulted from insufficiently low greenhouse temperature at that time. Similarly, yellowing symptoms of potato mop-top virus in Peruvian cultivars require low temperatures and develop in highland- but never in coastal plantings (17).

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