Properties of Tobacco Necrosis Virus Strains Isolated From Apple

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ABSTRACT

Tobacco necrosis virus (TNV) was sporadically isolated from certain crude apple sap inocula that only occasionally incited 1-2 lesions/*Chenopodium quinoa* indicator. When these inocula were concentrated and partially purified by differential centrifugation, infectivity was often greatly increased, and isolation of TNV was more consistent. TNV was isolated from apple leaves and fruits with this technique. Several TNV isolates from apple induced a mixture of white and necrotic lesions in cowpea. Each lesion type was unstable; selections of either induced white lesions at 28 C and necrotic lesions at 15 C. These isolates were alike antigenically, and the one isolate tested selectively activated satellite virus B. Another TNV isolate from apple induced only necrotic lesions in cowpea irrespective of temperature. It differed antigenically from mixed-lesions isolates and selectively activated satellite virus C.

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We have indexed many apple cultivars during the past 7 years by inoculating *Chenopodium quinoa* Willd. with crude petal or leaf sap. Occasionally, certain of these inocula induced sparse necrotic local lesions in *C. quinoa*. The causal virus was identified as tobacco necrosis virus (TNV). Attempts to re-isolate TNV from such sources commonly failed, and these sporadic isolations were initially ascribed to chance contamination in the greenhouse. Roots of "healthy" greenhouse plants are frequently infected with TNV (2, 7).

Continued, though sporadic, isolations of TNV in apple indexing trials, coupled with the nonoccurrence of TNV in other experiments involving mechanical inoculation of *C. quinoa*, suggested that TNV was actually present in some apple inocula, however. We present evidence that TNV occurs in apple leaves and fruits, and characterize some of the properties of TNV isolated from apple.

MATERIALS AND METHODS.–*Isolation of TNV from apple leaves and fruits.*–In routine indexing for other viruses, a few TNV lesions were induced by inoculum from apple cultivars Bogo de Boskoop, Lady Carrington, and Bridgham Delicious when leaf or petal sap in phosphate-nicotine buffer (6) was assayed on *C. quinoa*. Four other apple cultivars, Allred McIntosh, Giant Lobo, Mutsu, and Spartan appeared free of TNV in similar indexing. To circumvent low infectivity of inocula and sporadic TNV recovery, crude extracts from foliage of these trees collected in early June were partially purified and concentrated before assay. Two hundred g of young leaf tissue were comminuted in 400 ml phosphate buffer (0.005 M, pH 7.6) containing 0.1% mercaptoacetic acid. Sap was strained through cheesecloth, sufficient butanol was added to bring its concentration to 8.5%, and the mixture was incubated for 12-14 hr at 3 C. After a low-speed centrifugation (8,000 *g* for 10 min), virus was sedimented with two cycles of high-speed centrifugation (69,000 *g* for 2 hr). Pellets were resuspended in cold distilled water (final volume, 1 ml/100 g leaf tissue) and the suspension was rubbed on six *C. quinoa* leaves. Lesions were counted 4-6 days after inoculation.

Essentially the same procedures were followed in concentrating sap from apple fruits. Fruits were harvested on 17 September, and thoroughly washed in running tap water to remove surface contaminants. Pulp and skins were comminuted in buffer, and the sap was incubated with butanol and concentrated with successive cycles of low-speed and high-speed centrifugation. Final volume was 1 ml for 200 g fruit tissues.

Source and preparation of satellite virus (SV).–Two TNV isolates of American Type Culture Collection, AC36TNV and AC39TNV, were increased in cowpea (*Vigna sinensis* [Torn.] Savi 'Early Ramshorn'). Each contained a different SV serotype: SV-B (AC39TNV) and SV-C (AC36TNV) (9). After initial purification of the viruses, SV was separated from TNV by two successive cycles of sucrose density gradient centrifugation (Spinco 25.1 rotor, 54,000 *g* for 3 hr). Gradients were prepared by layering 4, 7, 7, and 7 ml of 10, 20, 30, and 40% sucrose solutions, respectively, in 0.01 M phosphate buffer, pH 7.0. Preparations of SV were incubated with ribonuclease (10 ug/ml) for 0.5 hr at about 22 C (8) to eliminate final traces of TNV infectivity, freed of ribonuclease by high-speed centrifugation (86,000 *g* for 3 hr), resuspended in cold distilled water, and adjusted to OD_{260} of 0.2.

Serology.–Virus antigens and antisera were tested by double-diffusion in 0.75% Ionagar dissolved in distilled water containing 0.85% NaCl. The central antiserum depot was surrounded by eight antigen wells. Antisera were of TNV (*Chrysanthemum* strain) and SV-C (9). Plates were incubated at about 22 C for

TABLE 1. Relative infectivity of tobacco necrosis virus (TNV) in crude or partially purified apple leaf or fruit sap inocula

Apple cultivar	Crude leaf sap[a]	Purified leaf sap[b]		Fruit sap[c]
		A	B	
Bogo de Boskoop	+	226	232	9
Bridgham Delicious	+	119	3	9
Lady Carrington	+		2	
NY 44428-5	+			
Giant Lobo	–	2	0	3
Mutsu	–	5	0	74
Spartan	–	1	3	31
Allred McIntosh	–	0	0	32

[a]Assay on *Chenopodium quinoa* in mid-May; + = TNV recovery (1-2 lesions/6 inoculated leaves); – = no recovery.

[b]Leaves harvested 9 June (A) and 14 June (B), sap concentrated to a final volume of 100 g leaves/ml. Lesion numbers are totals on six inoculated *Chenopodium quinoa* leaves.

[c]Fruits collected 17 September; sap concentrated to a final volume of 200 g fruits/ml. Lesion numbers are totals on six inoculated *Chenopodium quinoa* leaves.

48 hr, and examined for precipitin lines.

RESULTS.–Tobacco necrosis virus was isolated at least once from each of the eight apple cultivars (Table 1). The virus was not recovered from concentrated leaf sap of Allred McIntosh in either of two trials, but was isolated from fruits of this cultivar. The virus was recovered 2 or more times from concentrated leaf or fruit sap inocula of cultivars Bogo de Boskoop, Bridgham Delicious, Giant Lobo, Mutsu, and Spartan. The numbers of lesions induced in *C. quinoa* by these inocula were extremely variable, ranging from 0-232 in the different trials (Table 1). When single lesions of the Bogo de Boskoop, Bridgham Delicious, and Lady Carrington isolates were transferred from *C. quinoa* to cowpea, each incited a mixture of white and necrotic lesions, with white lesions initially predominating. In spite of repeated selections from white lesions, subisolates consistently incited mixed white and necrotic lesions in cowpea at greenhouse temperatures of 28 C days and 15 C nights, but the frequency of necrotic lesions commonly increased with successive subculturing. A fourth TNV isolate, from NY 44428-5 apple, initially induced only necrotic lesions in cowpea, and this lesion type remained constant at the 28- to 15-C temperature regime.

Inoculum from a single white cowpea lesion (Bridgham Delicious isolate) was subcultured in two lots of cowpeas, one grown at 15 C and 18-hr day (2,000 ft-c), the second at 28 C with the same daylength and light intensity. At 28 C, only white lesions (Fig. 1-A) developed, and at 15 C, only necrotic lesions (Fig. 1-B). Subcultures from the necrotic lesions produced at 15 C induced only white lesions in cowpeas incubated at 28 C. Similar manipulation of the NY 44428-5 isolate induced only necrotic lesions in cowpea at either temperature.

A TNV isolate causing bean stipple streak (courtesy of R. G. Grogan, University of California, Davis) resembled the Bridgham Delicious TNV and induced white or necrotic lesions in cowpea,

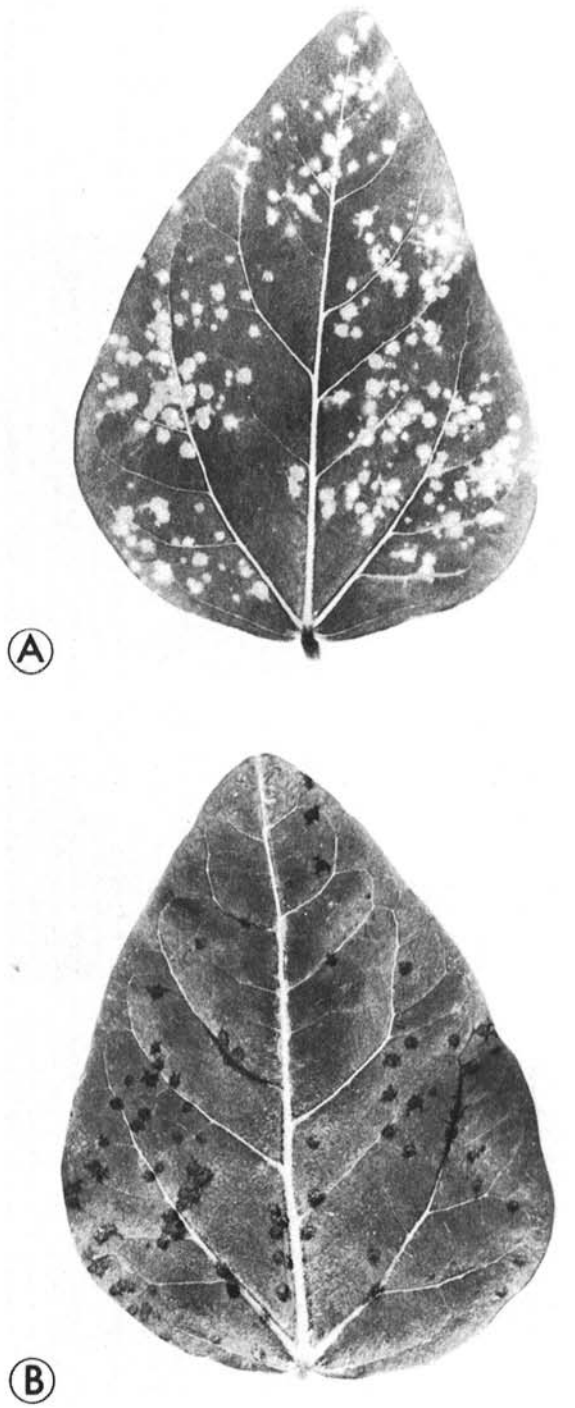

FIG. 1. Primary lesions on cowpea incited by Bridgham Delicious apple isolate of tobacco necrosis virus: **A)** White lesions induced at 28 C; **B)** necrotic lesions induced at 15 C.

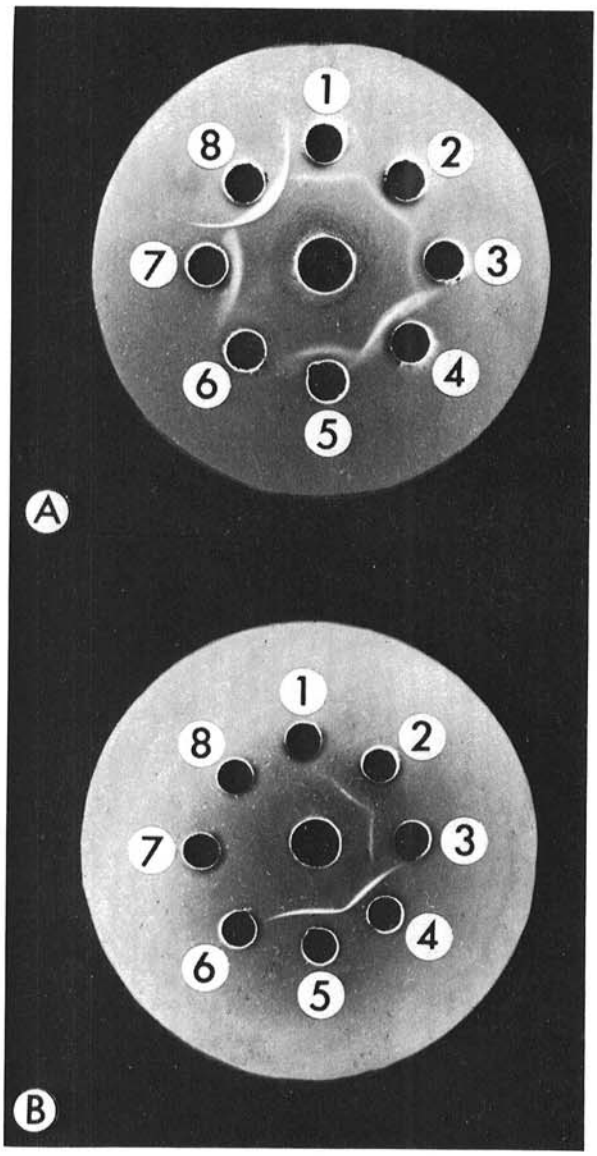

FIG. 2. Precipitin patterns in agar double-diffusion plates. A) Center well contained tobacco necrosis virus (*Chrysanthemum* strain) antiserum; peripheral wells contained partially purified preparations: 1 = Bogo de Boskoop TNV; 2 = Bridgham Delicious TNV; 3 = Lady Carrington TNV; 4 = NY 44428-5 TNV; 5 = AC36TNV; 6 = healthy cowpea sap; 7 = AC36TNV; 8 = bean stipple streak TNV. B) Center well contained satellite virus (SV)-C antiserum; peripheral wells contained purified TNV, SV, or TNV-SV mixtures: 1 = Bridgham Delicious TNV; 2 = Bridgham Delicious TNV + SV-B; 3 = SV-B; 4 = SV-C; 5 = NY 44428-5 TNV + SV-C; 6 = NY 44428-5 TNV; 7, 8 = cowpea sap from plants inoculated with SV-B and SV-C, respectively.

depending on temperature of incubation. It differed antigenically from either of the apple isolates, however (Fig. 2-A). It also induced a systemic necrosis of Black Turtle Soup bean (*Phaseolus vulgaris* L.); none of the apple isolates did so.

Serology of TNV isolates.—Concentrated, partially purified apple leaf sap did not react with TNV antiserum. Four TNV isolates from apple were propagated in cowpeas and partially purified by differential centrifugation of infected cowpea sap. Serology of these isolates demonstrated that Bogo de Boskoop, Bridgham Delicious, and Lady Carrington isolates were alike antigenically but differed from NY 44428-5 apple and AC36TNV isolates, which were antigenically similar to each other (Fig. 2-A).

Activation of satellite virus.—One ml of purified SV-B or SV-C was added to 4-ml aliquots of crude cowpea sap infected with Bridgham Delicious or NY 44428-5 TNV isolates, respectively. Twenty-four cowpea plants were inoculated with each combination. Additional groups of plants were inoculated singly with SV-B or SV-C as controls. Inoculated leaves were harvested after 5 days, and crude sap was concentrated and partially purified by differential centrifugation. Serology of these preparations demonstrated that Bridgham Delicious TNV isolate supported multiplication of SV-B but not SV-C and, conversely, that NY 44428-5 TNV isolate supported multiplication of SV-C but not SV-B (Fig. 2-B). Inoculations with SV-B alone or SV-C alone did not induce symptoms in cowpea, and neither SV isolate was detected serologically in sap from inoculated plants.

DISCUSSION.—Tobacco necrosis virus was isolated previously from foliage or petals of grapevine (1), citrus (4), and pear (5). In pears, infectivity of inocula was usually low, and isolation of TNV was sporadic (5), paralleling the data presented here. In view of the ubiquity of TNV infections in roots of "healthy" greenhouse plants (2, 7), the recovery of TNV from occasional apple inocula could be ascribed to chance contamination of *C. quinoa* leaves with water-splashed TNV particles. The considerable increases of infectivity that occurred when apple sap was concentrated, and the repeated isolation of TNV from certain apple trees, demonstrated the occurrence of TNV in apple foliage and fruits. Virus concentration in apple tissues was low, or the distribution of TNV in these tissues was erratic, perhaps restricted to small groups of cells randomly distributed.

Two biologically distinct strains of TNV were isolated from apple. One of these (NY 44428-5) induced only necrotic lesions in cowpea, irrespective of incubation temperature, and activated SV-C selectively. The second strain (Bridgham Delicious, Bogo de Boskoop, Lady Carrington) was more common. It induced lesions in cowpea that were white or necrotic depending upon temperature, and the single isolate tested activated SV-B selectively. These two TNV strains from apple were antigenically distinct from each other when tested with a *Chrysanthemum* TNV antiserum.

Fulton (3) isolated strains of TNV from tobacco roots. In cowpea, these induced white lesions in which red sectors occasionally appeared. Subcultures from these sectors were stable at 28 C; i.e., they induced red (necrotic) lesions only. Fulton concluded

that such sectors arose by mutation (3). Those TNV isolates from apple that induced mixtures of white and necrotic lesions were different. White lesion selections induced necrotic lesions on cowpea at 15 C, and when subcultures of such necrotic lesions were incubated in cowpea at 28 C, a reversion to white lesions occurred.

Tobacco necrosis virus was isolated one or more times from several other apple cultivars (including the test indicator Spy 227), from pear, and from apricot. These data suggest that TNV infections may be common in several tree fruit species. Trees infected with TNV showed no symptoms, and the possible economic importance of the virus in tree fruits is not known. Conventional indexing techniques often did not detect TNV infections in apple or pear, either because of virus concentration commonly below the detection threshold, or because of limited and erratic distribution of TNV in aerial portions of the infected trees.

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