Natural Serological Strains of Tobacco Ringspot Virus

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ABSTRACT

One hundred isolates of tobacco ringspot virus from burley and flue-cured tobacco areas of North Carolina were characterized serologically using the agar-gel double-diffusion technique. Four serological strains containing 88 isolates (strain NC-38), 7 isolates (strain NC-72), 4 isolates (strain NC-39) and 1 isolate (strain NC-87) were delineated on the basis of cross-reaction tests using antiserum for an isolate selected as the type of each strain. Each type isolate contained at least one specific antigenic site when reciprocal-absorption tests were conducted using all combinations of type isolates and their antisera. There was no correlation between isolates within a strain and the symptoms they caused in tobacco; i.e., some isolates of a strain caused severe symptoms; other isolates caused mild symptoms. No correlation was found between a strain and its geographic origin or the type of tobacco from which it was isolated. Nine isolates from outside N.C. were compared with the NC strains. Of these, seven were like NC-38; two were different. Phytopathology 60: 708-713.

Serology is one of the most effective and practical means of classifying and identifying viruses (7, 16, 18, 26). Optimal use of serology depends on knowledge of the factors which affect antigen-antibody reactions. Many of these are known; e.g., antiserum titer (5), variation in antisera from individual animals (5, 18, 24), and anomalous reactions (18). However, knowledge of antigenic variability and the biological significance of such variability is lacking for many viruses, including tobacco ringspot virus (TRSV).

Although many strains of TRSV have been reported based on symptomatology (22), only a few serological strains have been identified (13, 15, 20, 21), and the relationship among these has not been determined. Such knowledge is needed for determining the relationship between TRSV and other viruses. TRSV has a broad host range (25), and is transmitted by several arthropods and *Xiphinema americanum* Cobb (24). The identification of serological strains of this virus will serve as a basis for studies on relationships between antigenic structure and biological behavior.

MATERIALS AND METHODS.—North Carolina isolates were collected from naturally infected burley and flue-cured tobacco (*Nicotiana tabacum* L.). The 100 isolates represented each of the tobacco production areas of the state. Isolates were maintained by drying and storing the originally infected tissue over $CaSO_4$ at 4 C. The other isolates used are listed in Table 1.

Preparation of antigens and antisera.—Inject-antigen consisted of partially purified virus from tobacco cultivar Burley 21. Virus purification was accomplished by (i) homogenizing tissue in distilled H_2O (1 g/ml) containing 1% mercaptoethanol; (ii) adjusting cheesecloth-clarified juice to pH 7.2 with saturated Na_2HPO_4; (iii) adding *n*-butanol to 7%; (iv) clarification by centrifuging at 16,000 *g* (max.) for 15 min; (v) incubation of the supernatant at 25 C for 12-16 hr; (vi) another low speed clarification; and (vii) two virus sedimentations at 105,000 *g* (max.) for 90 min. Final pellets were resuspended in 0.01 M KH_2PO_4-Na_2HPO_4 at pH 7.2, clarified by centrifuging at 16,000 *g* (max.) for 10 min, and the suspension was adjusted to 1 mg/ml. Rabbits were injected intravenously four times using 1 ml/injection. After the initial injection, the times between successive injections were 2, 4, and 8 days. The first antiserum collection was made 10 days after the last injection.

Crude juice from systemically infected cucumber leaves with mosaic symptoms was used as test antigen.

Method of antigen-antibody analysis.—The agar-gel double-diffusion technique was used for determining serological relations. The medium consisted of 0.8% purified Difco-Bacto agar containing 1.0% NaN_3.

The initial criterion used to identify serological strains was spur formation in cross-reaction tests. The final criterion for establishing serological difference was the production of an antiserum against the suspected new strain and conducting reciprocal-absorption tests with the known strains.

The absorbing antigen was prepared from systemically infected National Pickling cucumber (*Cucumis sativus* L.) by homogenizing leaves in chloroform (1 g leaf/5 ml chloroform) containing 0.01 g of sodium ascorbate/g of leaves, and breaking the resulting emulsion by centrifugation at 16,000 *g* (max.) for 30 min. The supernatant constituted the absorbing antigen. The amount of absorbing antigen to use per volume of heterologous antiserum was determined by allowing $\times 2$ dilutions of antibody to react with absorbing antigen. The dilution of antiserum at which the sharpest precipitin line formed was considered near equivalence. Antisera were absorbed using the ratio of antigen to antibody indicated. For most of the antigen-antibody systems in this study, equivalence occurred at a 1/16 antibody dilution. To absorb these antisera, 16 ml of antigen were mixed with 1 ml of antiserum and incubated at 37 C for 4 hr, then incubated at 4 C for 12-16 hr. The precipitate was removed by centrifugation and the absorbed serum tested against heterologous antigen. Usually no reaction occurred, but if it did, additional absorbing antigen

TABLE 1. Source of tobacco ringspot virus isolates from outside North Carolina

Source	Designation	Geographic origin	Host origin
R. W. Fulton	Yellow TRSV	Wisconsin	*Cucumis sativus* L.
R. W. Fulton	Common TRSV	Wisconsin	*Nicotiana tabacum* L.
R. W. Fulton	Blueberry	Michigan	*Vaccinium* sp.
R. G. Grogan	Texas	Texas	*Citrullus vulgaris* Schrad.
R. G. Grogan	Bean	Unknown	Unknown
R. G. Grogan	Bean	Unknown	Unknown
W. J. Zaumeyer	AC-100	Maryland	*Phaseolus vulgaris* L.
T. T. Hebert	AC-98	Virginia	*N. tabacum* L.
R. P. Kahn	*Eucharis*	Peru	*Eucharis candida* Planch.

was used to remove unabsorbed antibodies. Absorbed sera were preserved by adding 0.005 g of NaN_3/10 ml of serum.

RESULTS.—*Identification of serological strains.*—Serological strains were identified among the 100 isolates from N.C. and the isolates from other states were compared with these strains. The 100 isolates from N.C. were used separately to inoculate Burley 21 plants, most of which developed similar symptoms. One isolate belonging to the major strain, based on symptom development, was twice local-lesioned, and an antiserum prepared against it. All isolates were screened against this isolate (NC-38) and its antiserum. Spurs formed with 12 of the isolates, so one of these (NC-39) was arbitrarily selected, local-lesioned twice, and used to prepare an antiserum. Spurs formed with eight of the 12 isolates using NC-39 and its antiserum. Using this process, two more strains were found in the remaining eight isolates. NC-72 contained seven isolates and NC-87 one isolate. Based on the procedure used, 87 of the isolates were like NC-38, three like NC-39, six like NC-72, and none like NC-87. A typical cross-reaction with spur formation is illustrated in Fig. 1.

Further serological characterization of isolates NC-38, NC-39, NC-72, and NC-87 was made using the reciprocal-absorption technique. The experiment was conducted as previously described, and repeated three times. The results of each replication were identical except in four cases (Table 2). These tests confirmed that these isolates belong to distinct serological strains. Isolate NC-72 was especially interesting because where it was used as absorbing antigen the absorbed serum was specific for its homologous antigen (Fig. 2).

Isolates from outside N.C. were tested against the four NC strains. A spur formed when NC-38 was allowed to cross-react with the Texas and *Eucharis* isolates; no spurs were formed with the other isolates. Spurs formed with all the isolates when NC-39, NC-72, and NC-87 were allowed to cross-react with them. Based on this procedure, all isolates except the Texas and *Eucharis* isolate were like NC-38. Antisera were prepared for these two isolates, and reciprocal-absorption tests were made with NC-72. Isolate NC-72, when used as absorbing antigen, also removed cross-reacting antibodies with these strains (Table 3).

Among the N.C. isolates there was no correlation between a strain and its geographic origin or the type of tobacco from which it was isolated.

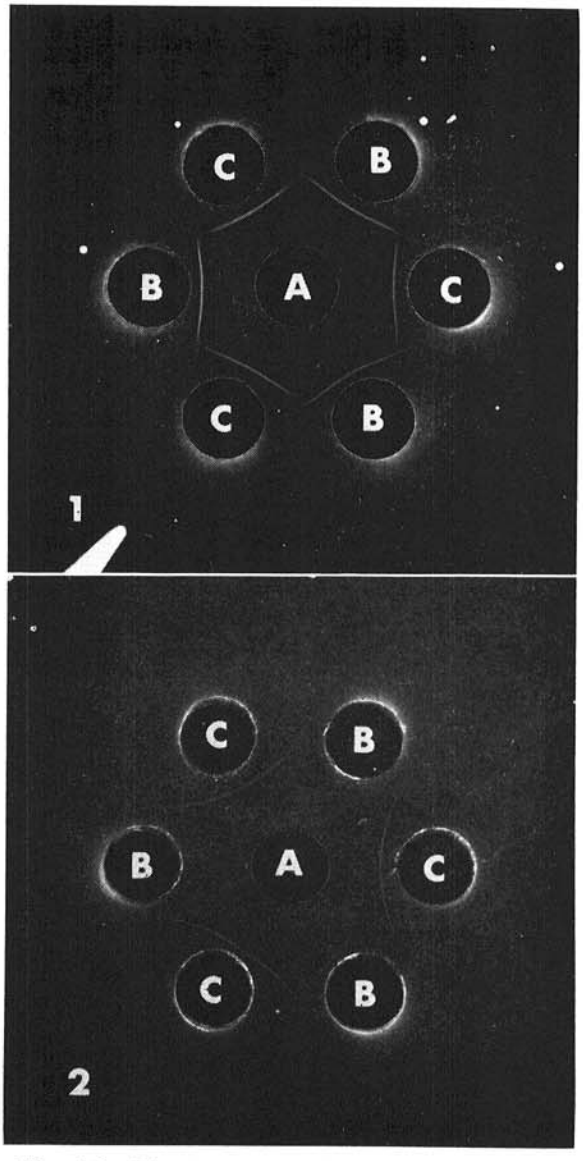

Fig. 1-2. Direct contact prints illustrating antigen-antibody reaction using the agar-gel double-diffusion technique. Tobacco ringspot virus as antigen was in crude juice from systemically infected cucumber leaves. Prints were made 3 days after incubation of plates at 24 C. **1)** Spur formation; A = NC-38 antiserum; B = NC-38 antigen; C = NC-39 antigen. **2)** Reaction using absorbed antiserum; A = NC-38 antiserum absorbed with NC-72 antigen; B = NC-38 antigen; C = NC-39 antigen.

Host range and reaction studies.—All isolates from N.C. and the Texas and *Eucharis* isolates were compared on the tobacco cultivars Burley 21 and McNair 12, cucumber, Burpee's (W. Atlee Burpee Co., Philadelphia, Pa.), Early Ramshorn blackeye cowpea (*Vigna unguiculata* [L.] Walp.), and Michigan dark red kidney bean (*Phaseolus vulgaris* L.). Inoculations to this host range, using two plants of each, were made concurrently with all isolates three times. As tests were conducted during the winter, spring, and summer, both light duration and temperature varied from test to test. The type of symptoms developing on inoculated plants also varied from test to test, but the over-all severity of reaction remained fairly constant. As an example of variation in the type symptoms, tobacco TRSV isolates NC-39 and AC-98 usually caused necrotic spots on inoculated leaves of Burley 21, whereas NC-38 and most of the other isolates caused necrotic or nonnecrotic rings. However, under some conditions (usually when the temperature was cool and light intensity low) isolate NC-38 caused necrotic lesions on Burley 21 tobacco similar to those caused by NC-39 and AC-98. Based on the severity of symptoms on Burley 21, cucumber, and bean, there was no correlation between sero-group and virulence (Table 4). All isolates killed cowpea. This is similar to the results of McLean (17), and this reaction may be useful in distinguishing between TRSV and tomato RSV, which usually causes only necrosis of the terminal shoot (9, 12, 14, 27).

Michigan dark red kidney bean, one of the better differential hosts found by Cheo & Zaumeyer (10) for TRSV strains, was the host which gave the most consistent differential reaction in these tests. Isolates consistently caused either (i) necrosis; (ii) no necrosis but a distinct mottle; or (iii) no necrosis but a faint mottle. The severity of symptoms caused by isolates

TABLE 2. Reciprocal-absorption results with tobacco ringspot virus strains from North Carolina

Antiserum	Absorbing antigen	Results when antisera tested against indicated antigen[a] 38	39	72	87
Anti-38	39	+	−	+	−
Anti-38	72	+	−	−	−
Anti-38	87	+	+[b]	+	−
Anti-38	None	+	+	+	+
Anti-39	38	−	+	+	+
Anti-39	72	−	+	−	−
Anti-39	87	+	+	+	−
Anti-39	None	+	+	+	+
Anti-72	38	−	+	+	+
Anti-72	39	+	−	+	+
Anti-72	87	+	+[b]	+	−
Anti-72	None	+	+	+	+
Anti-87	38	−	−	−	+
Anti-87	39	−[c]	−	−[c]	+
Anti-87	72	−	−	−	+
Anti-87	None	+	+	+	+

[a] Tests conducted using the agar-gel double-diffusion technique. + = precipitin line formed; − = no precipitin line.
[b] Precipitin line formed in two out of three tests.
[c] No precipitin line formed in two out of three tests.

TABLE 4. Symptoms resulting from the inoculation of tobacco, cucumber, and bean plants with certain tobacco ringspot virus isolates

TRSV isolate	Host Reactions[a] Tobacco (Burley-21)	Cucumber (National Pickling)	Bean (Michigan dark red kidney)
Sero-group-38			
NC-38	Moderate	Moderate	Moderate
NC-R-28	Severe	Moderate	Severe
NC-R-34	Severe	Mild	Severe
ATCC-98	Severe	Severe	Severe
Sero-group-39			
NC-39	Severe	Severe	Severe
NC-R-89	Moderate	Mild	Moderate
NC-R-77	Severe	Severe	Severe
Sero-group-72			
NC-72	Moderate	Moderate	Moderate
NC-R-69	Severe	Moderate	Moderate
NC-R-33	Severe	Severe	Severe
NC-87	Moderate	Moderate	Moderate
Eucharis strain	Mild	Mild	Mild
Texas strain	Moderate	Moderate	Moderate
AC-98	Severe	Severe	Severe

[a] Mild = faint mottle and/or line patterns. Moderate = distinct mottle and/or line patterns. Severe = distinct mottle and/or line patterns plus tissue necrosis.

TABLE 3. Reciprocal-absorption results with strain NC-72, the Texas strain, and the *Eucharis* strain of tobacco ringspot virus

Antiserum	Absorbing antigen	Results when antisera tested against indicated antigen[a] NC-72	Texas strain	*Eucharis* strain	NC-38	NC-39	NC-87
Anti-NC-72	Texas	+	−	−	+	−	+
	Eucharis	+	+	−	+	+	+
	None	+	+	+	+	+	+
Anti-Texas	NC-72	−	+	−	−	−	−
	Eucharis	+	+	−	+	+	+
	None	+	+	+	+	+	+
Anti-*Eucharis*	NC-72	−	−	+	−	−	+
	Texas	+	−	+	+	−	+
	None	+	+	+	+	+	+

[a] Tests conducted using the agar-gel double-diffusion technique; + = precipitin line formed; − = no precipitin line.

within a group varied, but there was generally no overlapping between groups (Fig. 3).

Cross protection tests.—Cross protection tests were conducted between NC-38 and isolates NC-39, NC-72, NC-87, AC-98, the Texas strain, and the *Eucharis* strain. All isolates were separately inoculated to Burley 21 plants. Recovered leaves were challenge-inoculated with NC-38. No symptoms developed on the challenged leaves except on plants infected with the *Eucharis* strain. Nonvirus-infected plants of the same age de-

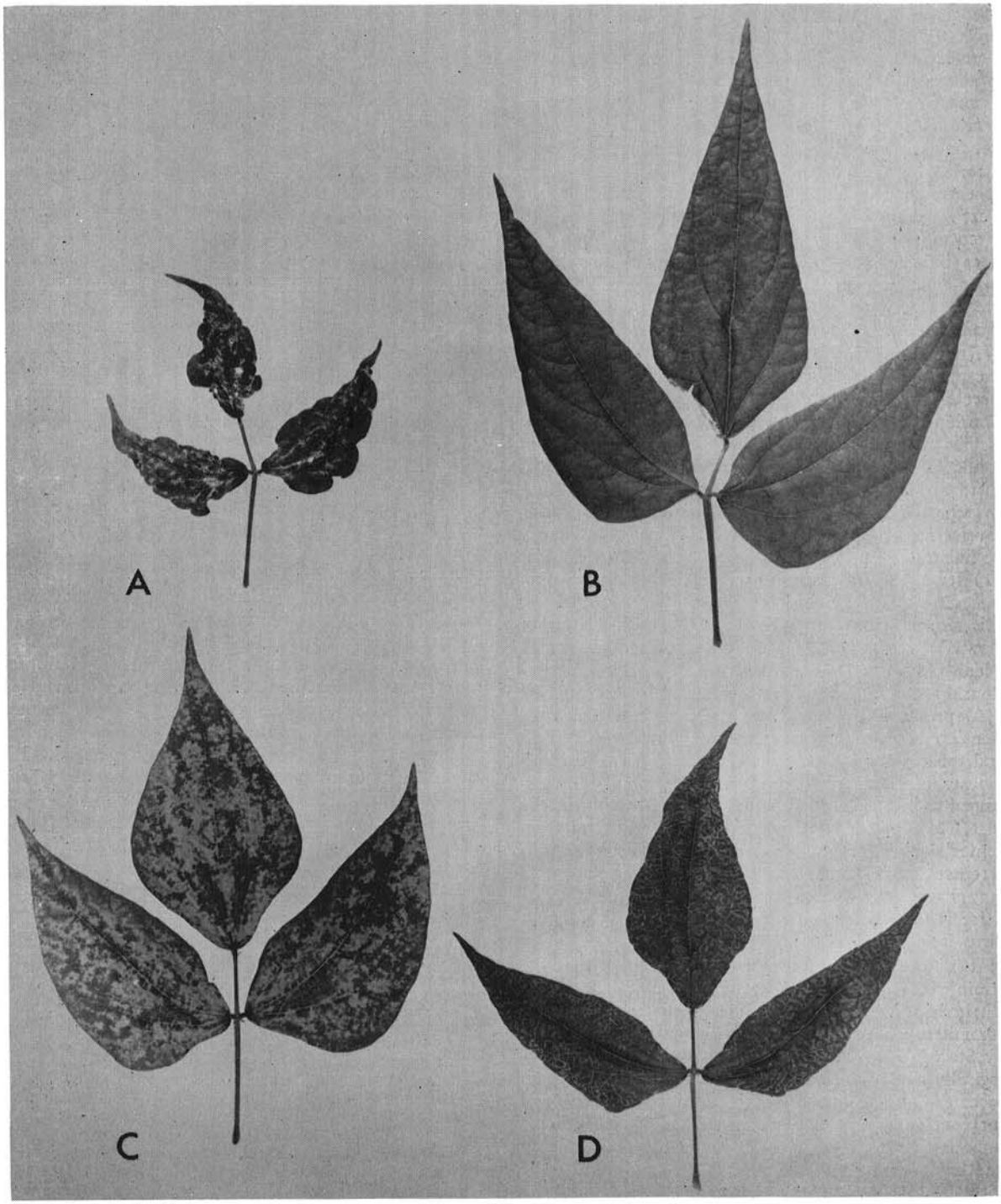

Fig. 3. Differential response of Michigan dark-red kidney bean to different isolates of tobacco ringspot virus. **A)** Necrosis; **B)** faint mottle; **C, D)** distinct mottle.

veloped from 10-20 lesions or rings/leaf. The test was repeated twice with identical results. The results with the *Eucharis* strain are similar to those of Kahn et al. (15), who found it did not protect Burley 21 against infection by several strains of TRSV.

DISCUSSION.—There are apparently many natural serological strains of TRSV. Five strains, four from tobacco in N.C. and one from watermelon from Texas, were identified in this study. The *Eucharis* strain (15) is different from these five. The strain reported by Rush et al. (20) is identical to NC-72 (Rush, *unpublished data*). The strain reported by Sauer (21) was not tested against the strains used in this study. Therefore, at least six natural strains of TRSV are known at this time; i.e., NC-38, NC-39, NC-72, NC-87, the Texas strain, and the *Eucharis* strain. Although differences were observed in the intensity of precipitin lines that formed when the six strains were allowed to interact, no attempt was made to establish the degree of relationship among the strains because no quantitative tests were made.

Knowledge of variability among individuals in a taxonomic group is basic to any classification scheme. Bercks & Gehring (6) found a distant serological relationship between potato rosette and pseudo-aucuba viruses and an isolate of TRSV. As they suggested, it would be interesting to determine the relationship of these two viruses and other serological strains of TRSV, as reported in this paper. The importance of using several strains of each virus in relationship tests may be illustrated by two recent examples. Agrawal & Maat (1) found that antiserum to one strain of cowpea mosaic virus (CowMV) would react with red clover mosaic virus (RCM), but antiserum for a second strain of CowMV would not. If only antiserum to the strain which did not cross-react had been available, no relationship would have been found. Van Regenmortel (19) presented evidence that a closer serological relationship between cucumber mosaic virus 4 (CMV-4) and tobacco mosaic virus (TMV) exists than had previously been reported. Bawden & Kassanis (4) challenged van Regenmortel's results and presented evidence that CMV-4 and TMV are, as has been generally accepted, only distantly related serologically. The differences in the results reported by these investigators may have been due to a number of factors. One of these is that their results were obtained using different virus isolates.

It is fortuitous for future investigations that the type strain of TRSV (*personal communication*, R. P. Kahn, Chairman, Amer. Type Culture Collection (ATTC) committee on plant viruses) will be the isolate used by Steere (23), because it apparently is the same serologically as NC-38, the strain which, to date, appears to be the most common, at least on tobacco. The basis for concluding that Steere's strain and NC-38 are the same is that Kahn et al. (15) found Steere's strain to be the same as AC-98, and AC-98 was found to be identical to NC-38 in this study.

Strain NC-72, when used as heterologous-absorbing antigen, left antibodies which, at the sensitivity of the test used, only reacted with the homologous system. This indicates that NC-72 has a special antigenic relationship with all the other strains tested. Regardless of the basis for this relationship, it had a practical value for the production of specific sera for most of the strains used in this study. Such specific sera can be of value for rapidly typing new isolates and in studies where a technique for identifying the different strains is needed.

The reasons for the existence of natural serological strains of TRSV are unknown. The main function of the protein coat of viruses, generally accepted as being responsible for serological activity, is considered to be a protection of the nucleic acid (3). Correlations between serological strains and vector specificity have been reported (8, 26). However, Fulton (11) reported that *X. americanum* can transmit both TRSV and TomRSV, which have not been reported serologically related but are similar in many other ways. There has been at least one report (2) of a host-induced change in serological properties. This may be the reason for the difference between the *Eucharis* and other strains. Whether or not the strains reported here resulted from host or vector selection pressure is unknown. Furthermore, they may only be mutants with no survival value. The strains of TRSV recovered from tobacco in N.C. were not correlated with geographic origin or the type of tobacco from which they were isolated. Tobacco probably serves only as a "trap crop" for TRSV in N.C. Therefore, strains found on this crop probably have their origin in indigenous hosts found in and around tobacco fields.

A preliminary report has been made of the serological strains identified from tobacco in N.C. (13). The strains were identified as "A," "B," "C," and "D." This means of identification was abandoned and the original isolate numbers were used in this paper in order to facilitate our research records in which isolate number designations have been used. Also, when more is known about strains of TRSV, a more meaningful system for strain designation may be suggested. The strain initially designated as A is now NC-38, B (NC-72), C (NC-39), and D (NC-87).

The term serological strain was used in this paper with the realization that some of the isolates may not be of frequent enough occurrence to qualify as strains in a taxonomic sense; i.e., NC-87 and the Texas isolate. However, this usage was considered justified for convenience of reference, and because it is not proposed that these be accepted as taxonomic strains at this time.

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