Studies on a Newly Recognized Disease of *Nicotiana glutinosa* of Viroid Etiology

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ABSTRACT

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This paper describes a disease of *Nicotiana glutinosa* characterized by epinasty of leaves, stunting, bunchiness, and occasional necrosis. Polyacrylamide gel electrophoresis of extracts of low-molecular-weight nucleic acid showed a disease-specific RNA migrating faster than Indian tomato bunchy top viroid (ITBTVd). This disease-specific RNA proved to be infectious on *N. glutinosa*. The causal agent differed from ITBTVd in host range and could be transferred to *N. clevelandii*, *N. debneyi*, *N. plumbaginifolia*, *Chenopodium amaranticolor*, *Cucumis sativus*, *Luffa acutangula*, *Cajanus cajan*, and *Vigna unguiculata*. The infectious RNA separated into two bands in denaturing gels, presumably linear and circular forms. It has an M_r of 1.1×10^5. These results constitute the first report of the natural occurrence of a viroid disease on *N. glutinosa*, for which I propose the name *Nicotiana glutinosa* stunt viroid (NgSVd).

Viroids are important subviral pathogens that cause several economically important plant diseases, especially in tropical and subtropical regions (9). India, with its warm climate, may be thought to provide a conducive environment for viroid diseases. However, although there have been many reports of diseases with viroidlike symptoms in India, only Indian tomato bunchy top disease has been shown to be caused by a viroid (1,7). I report the results of an investigation of a disease of *Nicotiana glutinosa* L., a wild relative of cultivated tobacco, which indicate that the causal agent is a viroid.

MATERIALS AND METHODS

N. glutinosa plants showing symptoms of epinasty, stunting, bunchiness, and necrosis were collected from the multiplication plots in the Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi. Electron microscopy of leaf-dip preparations did not show presence of any viruslike particles. The causal agent was propagated by sap inoculation, using 0.05 M phosphate buffer, pH 7.0. Young, infected leaves or aboveground portions of plants, in the case of necrotic symptoms, were harvested, chopped, and frozen at −74 C for nucleic acid analysis. Similar tissues from healthy plants of the same age served as controls.

Nucleic acid extraction. Nucleic acids were extracted at 4 C by homogenizing the plant tissues in equal amounts (1:1, w/v) of extractant containing 1.0 M glycine and 1.0 M NaCl, pH 9.5, 1% SDS, 1% mercaptoethanol, and 0.001% bentonite; water-saturated phenol (having 0.1% 8-hydroxyquinoline); and a chloroform-isoamylalcohol mixture (24:1, v/v). After centrifugation at 12,000 *g* for 20 min, the aqueous phase was reextracted with two volumes of phenol and centrifuged, and LiCl was added to the second aqueous phase to a final concentration of 2.0 M. Proteins were precipitated by dissolving 12 and 8% polyethylene glycol, respectively, in the LiCl soluble fraction, centrifuged, and low-molecular-weight (LMW) nucleic acids in the supernatant were precipitated by two volumes of ethanol at −74 C (3). Nucleic acids were collected by a 30-min centrifugation at 10,000 *g*, dissolved in 0.3–0.5 ml of sterile distilled water, and stored at −74 C for analysis in polyacrylamide gels and/or used for bioassays. Ultraviolet absorption spectrum for the LMW nucleic acid extract was measured between 220 and 280 nm using a Spectronic 2000, Bausch and Lomb spectrophotometer. The titer of LMW-RNA was calculated by multiplying its maximum absorbance at 260 nm, constant for RNA at 260 nm (40 μg/ml), and a dilution factor.

Infectivity assay. *N. glutinosa* plants at the five- to seven-leaf stage were dusted with Celite and inoculated with 5 μl of nucleic acid extracts or disease-specific RNA fractions, using a glass spatula. Leaves were rinsed with water immediately after inoculation. Generally, five plants for each treatment were used in each inoculation. The control plants were inoculated with sterile distilled water.

Polyacrylamide gel electrophoresis. Samples of 5–10 μg of nucleic acid extract were mixed with one-tenth volume of 50% glycerol solution containing 0.5% (w/v) bromophenol blue and then subjected to nondenaturing electrophoresis in 5% polyacrylamide (2.5% bis-acrylamide cross-linked) slab gel using TAE buffer (0.119 M Tris base, 0.0032 M ethylene diamine tetra acetic acid, disodium salt [sodium EDTA], and 0.036 M sodium acetate, pH 7.2) (3). After 30 min at 10 mA, the current was increased to 40 mA and electrophoresis was terminated when the dye reached near the bottom of the gel. The gels were stained with silver nitrate (4).

Determination of infectivity distribution in gels. Samples of nucleic acid extracts from infected plants were loaded in six wells of a 5% polyacrylamide slab gel. After electrophoresis, one track was separated and nucleic acids were visualized by silver staining. The other unstained tracks were cut into 1-cm slices. Each slice was homogenized in two volumes of elution buffer (0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.001 M sodium EDTA, and 0.1% SDS) and incubated overnight at 4 C. The extract was then centrifuged at 10,000 *g* for 30 min to remove gel debris. The LMW nucleic acid fractions in each supernatant were precipitated at −74 C by two volumes of ethanol. The different fractions, recovered by centrifugation for 30 min at 10,000 *g*, were dissolved in 0.3 ml of water and stored frozen at −74 C. Each of these fractions was assayed for infectivity. Ultraviolet absorption at 260 nm was measured, and the fractions containing the disease-specific RNA and 7S RNA were subjected to nondenaturing gel electrophoresis.

Nuclease sensitivity of the causal agent. The nucleic acid extracts were separately incubated with bovine pancreatic ribonuclease (10:1, w/w) at 37 C for 90 min and with deoxyribonuclease 1 (1:1, w/w) at 25 C for 20 min, and analyzed in 5% nondenaturing gel. No nuclease was added to the control. The treated samples were also assayed for infectivity.

Denaturing gel electrophoresis. The bidirectional gel system (8) was used for the detection of circular and linear forms of infectious RNA. Electrophoresis was conducted in a 7.5% nondenaturing gel, using TAE buffer (first direction), followed by 8% denaturing gel (5.2% cross-linked) containing 8.0 M urea (second direction). The second run was carried out at 60 C using TBE buffer (0.89 M Tris, 0.88 M H_3BO_4, and 0.027 M sodium EDTA, pH 8.3) at 40 mA constant current for 3 hr after the marked tracking dye had run out of the gel.

Denaturation of infectious RNA was also attempted by heating the nucleic acid extracts at 65 C for 10 min. The

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samples were immediately chilled and then subjected to 8% denaturing gel as described earlier.

RESULTS

Disease syndrome. The first symptom, epinasty of leaves, appeared 10–15 days after inoculation in all 10 of the inoculated plants, whereas the five control plants remained healthy. By 20–30 days, eight of the 10 inoculated plants became stunted with a bunchy appearance as a result of suppression of apical growth and proliferation of axillary shoots (Fig. 1A). After 2–3 mo of inoculation, secondary symptoms started as necrosis of veins and veinlets extending down to the petiole and stem (Fig. 1B). Infected plants failed to flower. The different stages of disease syndrome revealed the presence of infectious LMW-RNA in polyacrylamide gels (Table 1). The causal agent was named *Nicotiana glutinosa* stunt viroid (NgSVd).

Symptom expression and concentration of NgSVd were observed to be dependent on temperature and light. During summer months of high light intensity, long periods of sunshine (9.6 hr), and high maxiumum temperatures (40–45 C), both primary and secondary symptoms appeared more quickly (16–20 and 50–60 days, respectively) and were more severe. In winter months of low temperature (8–17 C), low light intensity, and less sunshine (7.8 hr), mild primary symptoms were expressed after 20–30 days of inoculation and none of the plants developed necrosis. The average yield of NgSVd from eight infected plants, calculated by dividing its titer (μg/ml) with the weight of leaf tissue used for extraction (g), was found to be 0.16 and 0.37 μg/g of plant tissue in winter and summer months, respectively.

Infectivity of nucleic acid extracts. *N. glutinosa* plants inoculated with nucleic acid extracts from infected plants produced all of the characteristic symptoms of the disease. Infected plants remained sterile. It was observed that gel eluates containing disease-specific RNA induced severe symptoms, including systemic necrosis leading to the death of infected plants. Similar extracts from healthy plants were never found to be infectious. The NgSVd could be transmitted to seven out of 32 plant species tested, belonging to Chenopodiaceae, Cucurbitaceae, Leguminosae, and Solanaceae.

NgSVd induced chlorotic local lesions followed by epinasty, mosaic mottling of leaves, and stunting of *Chenopodium amaranticolor* Coste & Reyn. plants; chlorotic patches and downward curling of leaf tips and margins on *Cucumis sativus* L.; mild mosaic mottling of leaves with uneven margins and downward curling of tips and margins on *Luffa acutangula* (L.) Roxb.; epinasty and chlorosis of leaves, stunting, and bunchiness of *N. clevelandii* A. Gray; epinasty and necrosis of leaves on *N. debneyi* Domin; and severe upward leaf rolling on *N. plumbaginifolia* Viv. No flowering was observed in infected *Nicotiana* species. Among the legume species tested, seven out of 10 inoculated *Cajanus cajan* (L.) Huth plants developed veinal necrosis on inoculated primary leaves and uninoculated trifoliate leaves, whereas chlorotic specks were induced on trifoliate leaves of three out of seven *Vigna unguiculata* (L.) Walp. plants, 20 and 15 days after inoculation (Fig. 2). Host susceptibility was checked by inoculating *N. glutinosa* and/or by electrophoresis. Interestingly, it did not infect *Lycopersicon esculentum* Mill., the most common host of some viroids.

Gel electrophoresis of extracts. The LMW nucleic acid extracts from healthy and infected plants revealed the presence of DNA, tRNA, 5S RNA, and 7S RNA as the most common and distinct bands in the gels (Fig. 3). However, the 7S RNA band was more prominent in tracks loaded with healthy extracts. An extra

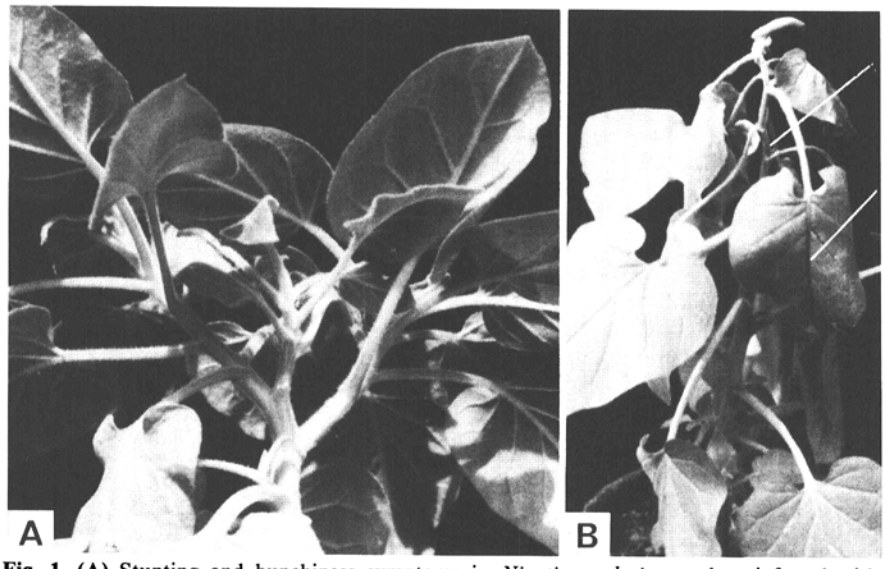

Fig. 1. (A) Stunting and bunchiness symptoms in *Nicotiana glutinosa* plant infected with *Nicotiana glutinosa* stunt viroid (NgSVd). **(B)** Systemic necrosis in *N. glutinosa* plant infected with NgSVd.

Table 1. Analysis of different stages of infection for the presence of infectious low-molecular-weight (LMW) RNA

Incubation period (days)	Symptom(s)	Presence of viruslike particles[a]	Presence of LMW-RNA	Infectivity on *Nicotiana glutinosa*[b]
10	Before symptom expression	−	+	3/4
20	Epinasty	−	+	3/3
30	Bunchiness	−	+	4/4
60	Systemic necrosis	−	+	4/4

[a] By electron microscopic examination.
[b] Number of plants infected/number of plants inoculated.

Fig. 2. Veinal necrosis on primary leaves of *Cajanus cajan* infected with *Nicotiana glutinosa* stunt viroid.

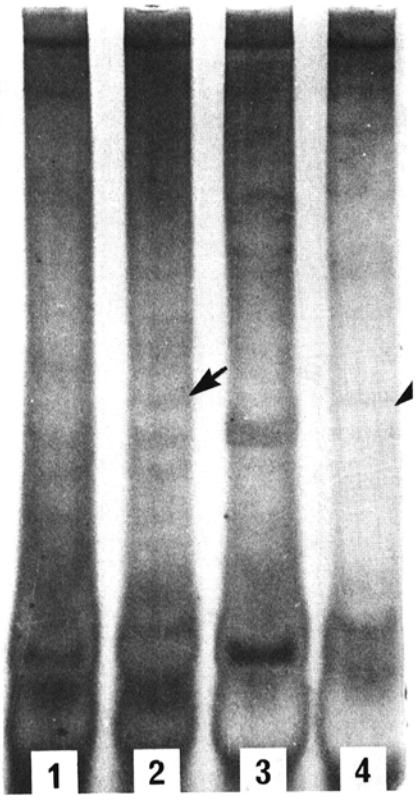

Fig. 3. Polyacrylamide gel electrophoresis of nucleic acid extracts from healthy (lanes 1 and 3) and *Nicotiana glutinosa* stunt viroid (NgSVd)-infected (lanes 2 and 4) *Nicotiana clevelandii* and *N. glutinosa* plants, respectively. The infectious low-molecular-weight RNA (viroid) is indicated in lanes 2 and 4.

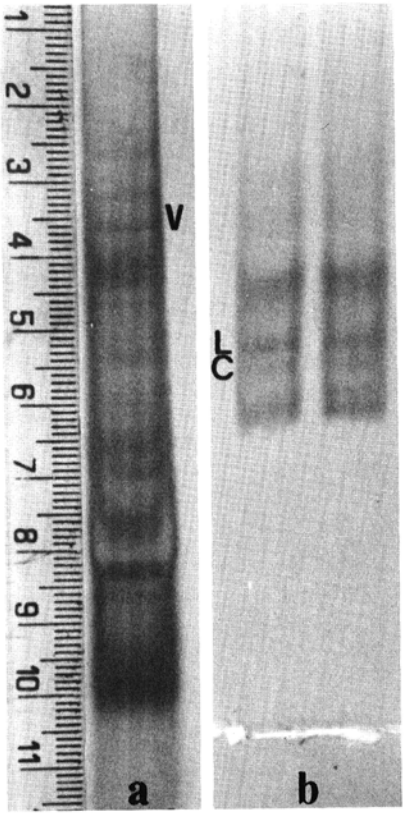

Fig. 4. Bidirectional gel electrophoresis of *Nicotiana glutinosa* stunt viroid (NgSVd). **(A)** 7.5% nondenaturing polyacrylamide gel (first direction), **(B)** 8% denaturing gel with NgSVd from *N. clevelandii* (second direction). V = viroid, L = linear form of viroid, C = circular form of viroid.

Table 2. Infectivity distribution of causal agent in polyacrylamide gel

Fraction number	Strip position from top of gel (cm)	Identity of nucleic acid species	Infectivity[a]
1	0–1	DNA	0/4
2	1–2	?[b]	0/4
3	2–3	?	0/4
4	3–4	?	0/4
5	4–5	?	0/4
6	5–6	Disease-specific RNA	9/9
7	6–7	7S RNA	0/4
8	7–8	?	0/4
9	8–9	5S RNA	0/4
10	9–10	tRNA	0/4

[a] Number of plants infected/number of plants inoculated.
[b] ? = Unknown RNA species.

RNA band was visualized only in tracks of infectious extracts.

Infectivity distribution in gels. Infectivity was detected in the gel slices containing disease-specific RNA only (Table 2), and the presence of infectious RNA in these fractions could be visualized in 5% polyacrylamide gels. No infectivity could be found in the corresponding gel slices of tracks loaded with healthy extracts.

Nuclease sensitivity of the infectious agent. Treatment of infectious nucleic acid extracts with RNase resulted in complete loss of infectivity and absence of any RNA band in the gels. Conversely, those treated with DNase could induce disease symptoms on all five *N. glutinosa* plants. Evidently, the infectious agent is composed of RNA but not DNA.

Electrophoretic mobility (R_m) of NgSVd. Coelectrophoresis of infectious nucleic acid extracts from *N. glutinosa* and standard viroid (ITBTVd) in 5% polyacrylamide gel showed that the former migrates faster than the latter. NgSVd had an M_r of 1.1×10^5, which was smaller than that of TBTVd (M_r 1.3×10^5) (7). Under denaturing conditions, the infectious RNA resolved into two bands, presumably a fast-moving linear and the slow-moving circular form (Fig. 4). Heat denaturation also produced the same results.

DISCUSSION

The results of this study (infectivity of nucleic acid extracts from diseased plants, sensitivity of the infectious agent to RNase but not to DNase, presence of an extra LMW-RNA band in polyacrylamide gel tracks with extracts from infected but not from healthy plants, and infectivity of the eluted disease-specific LMW-RNA) indicate that the causal agent of *N. glutinosa* disease is a low-molecular-weight RNA, i.e., a viroid.

To date there have been no reports of natural viroid infections of *N. glutinosa*, although it is an experimental host for potato spindle tuber viroid, PSTVd (5), and tomato apical stunt viroid, TASVd (12). The present results show that the viroid under investigation differs from the earlier reported ones in symptom expression, host reactions, and electrophoretic mobility with ITBTVd. Failure of NgSVd to infect *L. esculentum* distinguishes it from being PSTVd or TASVd, for which tomato is a diagnostic host, and also from a number of other viroids infecting tomato including citrus exocortis, columnea latent, cucumber pale fruit, hop stunt, and tomato planta macho viroids. Saraswathi (7) reported *N. glutinosa* as a nonhost of ITBTVd. Further, its infectivity on *C. cajan* and *V. unguiculata* is the first report of a viroid infecting legumes.

Viroids, often being latent or cryptic in their natural hosts, may be detected as pathogens only when transferred to other plant species on which severe symptoms develop (2,10,11). Identification of columnea latent (6) is an example of such preexisting, unrecognized viroid infections. NgSVd may have migrated by chance from an unknown symptomless host. Low titer and longer incubation period may be indicative of such transfers of these pathogens. Detailed investigations on nucleotide sequencing, however, are required to determine the relationship of NgSVd with other characterized viroids.

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